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THESIS

LETHAL EFFECT OF SQUARE PULSE CURRENT ON *Bacillus cereus*

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the Requirements for the Degree of
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The objective of this experiment is to study the lethal effect of *B. cereus* by electric square pulse current at non-lethal effect temperature. The apparatus was designed to suit the experiment. It is composed of three parts, i.e. DC pulse generator, cooling system and treatment chamber.

The *B. cereus* cells in logarithmic phase suspended in nutrient broth (NB) were exposed to electric square pulse current of 110 V duty cycle 50 % at variable electric frequencies (45 Hz, 50 Hz, 55 Hz and 60 Hz) under aerobic conditions. The survival fractions of *B. cereus* cells were related to exposure time. At the temperature 29 ± 3 ºC (non-lethal temperature), the surviving fractions of cells exposed to square pulse current with lower frequency were decreased faster than the cells exposed to square pulse current with higher frequency. It indicated that low frequency electric pulse gave more lethal effect on *B. cereus* than higher frequency electric pulse at the same voltage.

The present investigation shows that the electric square pulse current is an interesting technology to develop for the non-thermal disinfection of liquid foods and it may be a new technology to inactivate the contaminate harmful bacteria.

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Naruecha Kaewsanay

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LIST OF ABBREVIATIONS

M	=	Molarity. This concentration unit is defined as the number of moles of substance per liter
ppm	=	Part per million (weight/volume).
CFU	=	Colony forming unit.
N	=	Normality. This concentration unit is defined as the number of substance per liter of solution.

LETHAL EFFECT OF SQUARE PULSE CURRENT ON *Bacillus cereus*

INTRODUCTION

Consumers are increasingly aware of taste, color, flavor and nutritional value of the foods. Most of the time, fresh food preservation becomes necessary. For many years, thermal processing has been the main technology for production of safe products with long shelf-lives, although in most cases, loss of fresh flavor, vitamins, some physiochemical characteristics, some price of safety and long-term stability are inevitable.

Non-thermal methods provide as an option because during non-thermal processing the temperature of the food is hold below the temperature normally used in the thermal processing (60 to 100 $^{\circ}$C) for a few minutes. Therefore, the quality degradation expected from high temperature is minimal. Idea of non-thermal method for microbial control in foods is applied by using electrical treatment. The easiest method is using low-voltage alternating current (AC), since it is possible to control temperature below lethal temperature during processing. Kheamrutai (2004) indicated that the survival fractions of *Bacillus cereus* were related to current density through the suspension.

Several types of food samples analyzed by Food Microbiology at The Institute of Nutrition, Mahidol University (INMU) during 1995-2002; four food born pathogens, i.e. *Bacillus cereus*, *Salmonella*, C*lostridium perfringens* and *Staphylococcus aureus* were identified (Jiraporn, 2003). It was found that *B. cereus* was the most common organism in 20 % of the 213 seasoning samples. *B. cereus* is a Gram positive rod-shaped bacterium. It forms spore that can survive for a long time under unfavorable conditions. Its spore is difficult to kill due to the inactivation kinetics of spore. *B. cereus* has been suspected for many years as a food born pathogen since the 1950s (Reginald *et al*., 1993). *B. cereus* can produce toxin during

log phase of growth (Rawiwan, 2002).

The passage of low-voltage AC through the cell suspension at non-lethal temperature has been known to be primarily due to toxic substances formed in the suspension by electrolysis. By using Ag-metal electrodes, Fritz believed that silver salts and free chlorine generated by electrolytic action of AC were responsible for killing effect of yeast cells (Shimada and Shimahara, 1981). Tracy (1932) proposed that the formation of temporary toxic substance like free chlorine might cause the killing effect. Rosenberg *et al.,* (1965), by using certain group of the metal complexes, e.g. Pt (IV) complexes or irons like Ni^{2+} produce in the medium at the level of about 1-10 ppm by electrolysis, it caused inhibition of *E. coli* cell division or resulted in bacterial death. Using stainless steel electrodes in suspension containing chlorine, Pareilleux and Sicard (1970) reported that the toxicity was due mostly to labile compounds whose effect on *E. coli* k-12 cells could be reduced by the addition of cysteine or albumin to the suspension. Shimada and Shimahara (1981) used high quality carbon electrodes. They concluded that the toxicity was due to hydrogen peroxide rather than free radicals. Some minor factors such as AC itself are affecting all lethality during AC-exposure. In order to get rid of minimal electrolysis by-product production, carbon electrodes were employed in this research because carbon electrode is a gas-ion electrode. It does not have any effect because of chemical inertness (Rieger, 1987).

In this research *B. cereus* was suspended in nutrient broth (NB) and exposed to the square pulse current for 0-6 hrs. The result was standardized by comparing with the controlled *B. cereus* which was kept in the same conditions but without exposure to the pulse current.

The benefit of this research is to reveal the bactericidal actions by low-voltage square pulse current at non-lethal temperature. It is valuable information to generate new technology for preservation of liquid foods. The knowledge might be applied to eliminate pollution of fresh water or used as fundamental information for further study. Therefore, the objectives of this study are:

1. To study non-thermal effect of square pulse current on *B. cereus*,

2. To investigate the effect of low-voltage square pulse current (110 V, duty cycle 50%) at non-lethal temperature (29±3 ºC during the treatment) on the viability of *B. cereus* at different electric frequencies (45Hz, 50 Hz, 55Hz, and 60 Hz).

LITERATURE REVIEW

Technologies of Food Preservation by Non-thermal Method

Low-Voltage Alternating Current

Low voltage AC was early electrical method to inactivate the food-born pathogens in liquid food. From available information, Tracy (1932) was the first one who investigated in this field. He inoculated grape juice with yeast cells and subjected the suspension to 120 V, 60 Hz AC in a chamber with carbon electrodes. The surface areas of electrodes used were 0.95, 1.17 and 4.9 cm^2, and the electrode gap was 5 to 7 mm. A rapid stream of cold water externally cooled the chamber. The highest temperatures attained were recorded by a thermocouple and potentiometer. A set of lethal temperature and time combinations to obtain complete sterilization was determined and the minimum lethal temperature reported was 46 °C. The initial concentration of yeast cells used was 1.5×10^6 CFU/ml, and the temperature of solution varied from 30-48 °C with an average of 44 °C.

A killing effect was obtained at all used current levels, and this was reported as being due to the passage of AC through the sample. An increased killed percentage was reported with increased current and increased exposure time. The killing effect at different current levels was compared without keeping the diameter of the chamber constant; thus the current density was not constant for all studies. The authors suggested that the microbiocidal action of AC depended upon a definite quantity of electricity applied at or above a certain minimum current density. The maximum reduction in yeast cells was only two to three log cycles and this was explained by the consideration that there might have been an increase in resistance to killing action of AC when the cells decreased in number. Chemical analysis showed no toxic gases to have evolved after treatment but it was speculated that the formation of temporary toxic substances like free chlorine might be the reason for the death of yeast cells. Since the temperature in these studies were not controlled, so even a few seconds at the lethal temperatures (>46 °C) might have caused microbial death.

Pareilleux and Scard (1970) attempted to use low-voltage AC (50 Hz) ranging from 10 to 200 mA to kill the cells of *E. coli K-12*. *E. coli K-12* was grown in nutrient broth medium. The log-phase culture was used. Stainless steel electrodes were used and the temperature measured after the treatment was below 40 °C at the end of the treatment in all experiments. The total time of exposure never exceeded 10 seconds.

They reported that the minimum current required to kill bacteria was 25 mA. There was no decrease in the number of viable cells immediately after the treatment but the number decreased with the holding time. However the bacteria could be completely repaired after treatment if they were plated immediately on complete medium. Therefore it seemed likely that there was no direct lethal effect but an indirect effect. When untreated cells were added to a medium, which the current had just passed, the loss of viability is not as high as the loss observed after treatment of the cells in suspension. After 30 minutes, the treated medium lost its toxic activity completely. The toxicity might be due to labile compound whose effect could be inhibited by the addition of cysteine or protein in the medium. Most of the toxicity was developed with unalterable electrodes in the presence of chlorides in the medium. These results seemed to indicate that the mechanisms influencing bacterial death were extremely complex, involving a number of interactions between the organisms, medium and electrode material. They also concluded that the bactericidal effect depended on the current passing through the suspension, the presence of chloride containing compounds and the time during which the cells were left stand in the medium after the treatment.

Rowley (1972) measured of growth rate change produced by electrical current undertaken under conditions of minimal electrolysis byproduct production. *E. coli B* cells were used in this experiment and log phase cells were used to initiate the experiments. Both low level alternating and direct currents were used. The alternating currents were at 15 and 30 mA at 1, 10, 30, and 60 Hz. The direct currents were at 0.2, 1, 14, and 140 mA. Platinum with 10% iridium was used for the electrodes. Hence no metallic byproducts that might affect cell growth would be produced. The

electrochemical reaction produced hydrogen gas and hydroxide ions at the cathode and oxygen gas and hydrogen ions at the anode. Hence the cathode area became alkaline and anode area acidic. In the case of direct currents, this condition would continue to build up unless the hydrogen and hydroxide ions migrated together and reacted to form water. The same process would occur using AC, however, since each electrode, changed from a cathode to an anode periodically, the byproducts would combine into water. Therefore, an alkaline or acid condition would not persist.

In order to provide the microorganism with a proper environment, the growth chamber was placed in a constant water bath. The temperature of the water bath was adjusted to maintain the growth medium at a temperature of 37 °C. Rowley (1972) reported that AC had little or no effect on growth rate of *E. coli B* and direct current increased its generation time. When filters that inserted between electrode section and the center chamber, were removed; the effect was greater than with filters. This would seem to indicate that the agents causing the decrease in bacterial growth rate were byproducts of the electrochemical reaction. Byproducts would be more concentrated near the electrodes and without filters the microorganisms would be more exposed.

Shimada and Shimahara (1985a) presented results showing higher concentration of UV- absorbing materials in the supernatant fractions of cell suspension exposed to a current density of 600 ± 60 mA/cm^2 and temperature of 34 ± 3 °C. Based on an increased absorbance of 260 nm and increased exposure time, they suggested that the intracellular materials (including DNA) were released from the cells during AC exposure. Electron micrographs of thin sections showed more disorganized materials in the central areas within exposed (5 hrs) cells than in unexposed (only shaken for 5 hrs) or fresh (untreated) cells. They concluded that AC exposure enhanced the aggregation of DNA related materials that exist within cells and following he leakage of cellular contents from the cells into the suspension during AC-exposure. They also reported that another effect in bactericidal was hydrogen peroxide, which chemical compound formed in suspension when exposed to the current.

In 2004, Kheamrutai used low voltage AC to kill *B. cereus*. The bacteria were suspended in 0.2 M phosphate buffer solution (pH 7.0) and the temperature was maintained at 29 ± 3 ºC. The result of this research was reported that when the current densities and exposure time were increased the surviving fractions of cells would definitely decrease.

High Hydrostatic Pressure

High pressure technology was originally used in the production of ceramics, steels, and super alloys. In the past decade, high pressure technology expanded to food industry. High pressure usage to inactivate microorganisms has been recognized since the beginning of the twentieth century, but only in the past decade researchers began to study the potential commercialization of high pressure technology in food industry. At a pressure of 4000 – 9000 atm, enzymes and bacteria are inactivated, but taste and flavor remain unaffected.

Fioretto et al. (2005) showed that result of inactivation two food-borne pathogens, *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 were inactivated by the pressure of 150 to 550 Mpa for 15 minutes at room temperature.

Pulse Electric Field

Pulse Electric Field (EMF) is a method used to preserve liquid foods, e.g. milk, apple juice (Gustavo *et al*., 1999). This method, the high intensity pulse electric filed in the form of short pulse in a few microseconds or milliseconds is applied while the temperature is maintained at the nonlethal effect level. Therefore this process is a non-thermal method. Microbial inactivation increases with the increases of the electric field, number of pulses, pulse duration, pulse shape, temperature of medium, maturity of the bacteria, and ionic strength of the medium.

Magnetic Filed

Magnetic fields, in general, influence the direction of migration and alter the growth and reproduction of microorganisms. Magnetic fields increase DNA synthesis, change orientation of biolecules and biomembranes to a direction parallel or perpendicular to the applied magnetic field, and change ionic drift across plasma that is reduced when placed in oscillation magnetic fields which is a method to facilitate the treatment of cancer. The energy of magnetic fields is also transfer to the metabolic activities involving the ions (Na^+, Ca^{2+}, K^-). Transferring of energy to the ions results in increase of the ionic drift velocity and therefore it is an increase in the net transport of ions such as Ca^{2+} across membrane. An increase induces in metabolic activities which occur over a range of frequencies.

The sites that interact with the magnetic field are the cell tissues affected the most by the magnetic filed. The effect of magnetic fields to tissues and organs is from the ions that transmitted from the interaction site to the other. For example, Figure 1 suggests that the cell membrane is the interaction site and the effect of the magnetic fields is transmitted to organelles, unclear membranes, chromosomes, and protein molecules. Therefore, the intensity of response to the magnetic field is diffused and delayed in the other tissues besides the interaction sites.

Bacillus cereus

Bacillus cereus (pronounce "bah-sill-us" "serious") is a spore forming bacteria, Gram positive, aerobic or facultative anaerobic. This microorganism commonly found in soil, dust, natural environment and variety of foods such as milk, vegetables, spices, rice and sauces (Chorin *et al*., 1997). Its ability to sporulate makes it quite resistant to heat treatments. Consumption of foods that contain the bacteria higher than 10^5 CFU/g may result in food poisoning (Johnson, 1984). Two types of illness have been attributed to the consumption of foods contaminated with *B. cereus*. Rhodehamel and Harmon (1998) concluded that the first and better know type is characterized by abdominal pain and diarrhea. It has an incubation period of 6-15 hrs

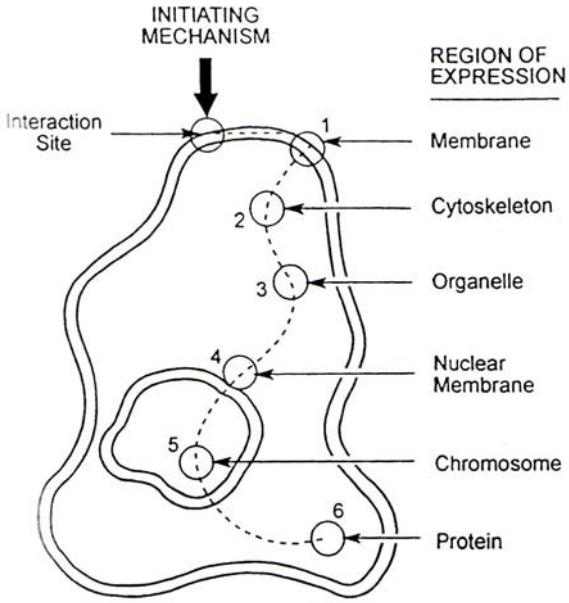


Figure 1 Cascade of responses in a biological cell exposed to magnetic field.
Source: Liboff (1985)

and symptoms that last for 12-24 hrs. The second type is characterized by and acute attack of nausea and vomiting occurs within 0.5-6 hrs. after consumption of contaminated food. Diarrhea is not a common in this type of illness. Symptoms generally are within 6-24 hrs.

Morphology of *Bacillus cereus*

Bacillus cereus is Gram positive rod, square ended, vegetative cell size 1.0 μm × 3.0-5.0 μm. Vegetative cells belong to the large group of the *Bacillus spp*. (Goepfert, 1976). Frequently, *B. cereus* cells arrange themselves in long chains. The spores are oval or cylinder shapes, center position, do not swell the sporangium (Johnson, 1984). It is active motile with peritrichous flagella. Colony feature of *B. cereus* is similar to *B. anthracis*; i.e. rough, cloudy white opaque glass, border not smooth, and its magnitude is 4 to 7 mm (Rawiwan, 2002).

An important components of a biological cell is its membrane because it (a) acts as semi-permeable barrier, (b) extrudes extracellular enzymes and cell wall

materials, (c) is the site of many complex activities, including RNA, protein and cell wall synthesis, electron transport, and oxidative phosphorylation, and (d) plays important role in the control of DNA synthesis (Rogers *et al*., 1980). In a cell subjected to no stress, the membrane acts as semi-permeable barrier. Any damage to the membrane affects its functions and may lead to the inhibition of cell reproduction.

Cell membrane or plasma membrane is beneath cell wall. It is a thin structure that completely surrounds the cytoplasm both in cell that contain and those that lack a cell wall. If the cell membrane is broken, the integrity of the cell is destroyed, the internal contents leak into the environment and the cell dies.

Plasma membrane contains approximately 60 % protein and 40% lipid, primarily in the form of phospholipids bilayer (Nester *et al*., 1995). Lipids aggregate in an aqueous solution, they tend to form lipid bilayer structures spontaneously – the fatty acids point inward each other in a hydrophobic environment, while the glycerol molecules are remain expose to the aqueous external environment (Figure 2). Different protein molecules are embedded in the phospholypid bilayer, which account for some of the different functions that membrane performs (Figure 3).

Growth Factors of *Bacillus cereus*

Johnson (1984) described that at optimum temperature (35 to 40 °C), generation time ranged from 18 to 24 minutes for four stains of *B. cereus*. Some stains have the ability to growth at low temperature and can be regarded as psychrotrophs with minimal growth temperature of 5 °C, the maximum at 50 °C (FSHN420 Food Microbiology, 1994). The range of pH is 4.5-9.3 (FSHN420 Food Microbiology, 1994) and the minimum water activity of *B. cereus* growth was report to be 0.95 (Scott, 1957). *B. cereus* was sporulated and germinated simply, they have short germination time about 20 to 30 minutes (Jiraporn, 2003). Vegetative cells were damaged at 47 °C (Rapport and Goepfert, 1978).

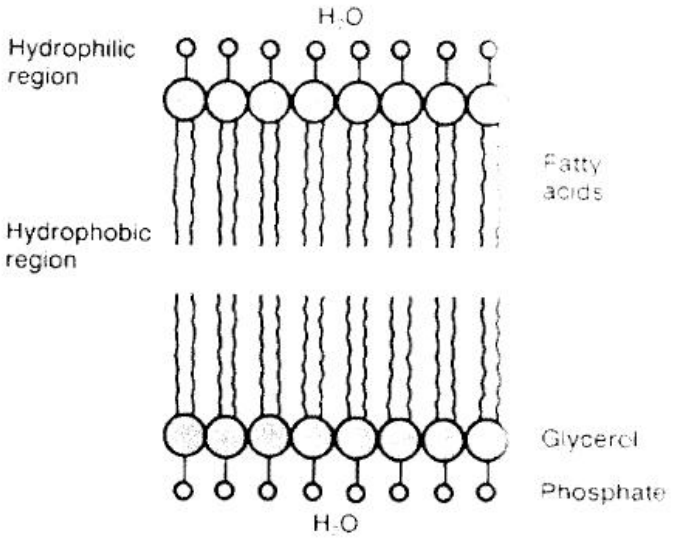


Figure 2 Fundamental structure of phospholipids bilayer.

Source: Block and Madiga (1988)

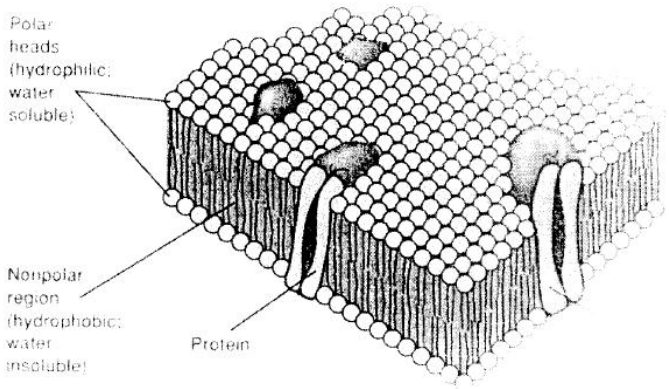


Figure 3 Diagram of membrane showing the lipid bilayer with proteins embedded in the membrane.

Source: Nester *et al*. (1955)

Outbreaks of *Bacillus cereus* Gastroenteritis

B. cereus is food-borne pathogen which causes two types of illness. The example of food-borne illness outbreaks and clinical manifestations caused by *B. cereus* are show in Table 1.

Table 1 Food-borne illness outbreaks and clinical manifestations caused by *Bacillus cereus.*

Type	Food	Symptoms[a]	Incubation period (hrs)	Country
Emetic	Packed lunches	V	0.5-3	Japan
	Boiled rice	V	0.5-4	Finland
	Cooked rice	V	0.5-4	Japan
	Macanoni and chess	N, C, V	1-3	?
	Fried rice	N, V, (D)	1-6	U.K.
	Fried or boiled rice	V	1.5-4.5	U.K.
	Fried rice	V	2	Netherlands
	*Cooked rice	V	2	Canada
	*Feta chess	V	4	Canada
	Skim milk powder	N, V	6	Canada
	Vanilla slice	V	8	U.K.
	Fried rice	V	?	U.K.
	Risotto, beef curry	V	?	U.K.
	With rice, lamb with rice			
Intermediate	*Chinese food	N, V, D	2	Canada
or not well	*Instant breakfast	N, V, D	2-3	Canada
defined	*Fried rice	N, V, D	3-5	Canada
	*Malted milk powder	N, V, D	6	Canada
	*Canned tuna	N, V, D	6	Canada
	*Curried chicken	N, V, D	6-12	Canada
	*Baked fish	N, V, D	9-13	Canada

Table 1 (continued)

Type	Food	Symptoms[a]	Incubation period (hrs)	Country
Diarrheal	Cooked rice	C, D	2-12	Japan
	Mashed potatoes	C, D (V)	3-10.5	U.S.A.
	Chicken ala king	C, D (V)	5-10	Canada
	Potatoes, stuffing, beans	C, D (V)	5-14	U. K.
	Meat, gravy	C, D (V)	5-14	U. K.
	Vegetable sprouts	C, D	6-15	U.S.A.
	Green bean salad	C, D	7-15	Canada
	Meat loaf	N, C, D (V)	10	U.S.A.
	Vanilla sauce	N, V, D	12-13	Norway
	Chicken pot pie	N, D	14	U.S.A.
	Barbeque chicken	N, C, D	15	Canada
	Turkey loaf	C, D	?	U.S.A.
	Barbeque chicken	N, C, D	15	Canada
	Turkey loaf	C, D	?	U.S.A.

* *Bacillus cereus*-like organism isolated, but not enumerated.

[a] V = vomiting, N = nausea, C = cramps, D = diarrheal, ( ) = mild or few responses.

Source: Johnson (1984)

Diarrheal Type

It is caused by enterotoxins, produced during vegetative cells of *B. cereus* exponential growth (Goepfert *et al*., 1972). Toxin production occurs within a pH range form 6 to 8.5, with optimum of 7 to 7.5. Toxicity lost after the exponential phase of growth is complete (Spira and Silverman, 1979). Toxin can produced form 18 to 43 °C (Spira and Goepfert, 1972). It is activated by heating at the temperature 56 °C for 5 minutes. It is stable at 45 °C for 30 minutes (Turnbull, 1976). Cell lysis is not required for toxin production (Terranova and Blake, 1978). Johnson (1984)

reported that *B. cereus* diarrheal type outbreaks have symptom parallel to those of *Closidium perfringens*. Onset of watery diarrhea and abdominal cramps and occur 6 to 15 h following consumption of contaminate foods. Nausea may accompany the diarrhea.

Emetic Type

It is caused by emetic toxin that present in the cell-free filtrate, stable to 126 °C for 90 minutes, 4 °C for 2 months and a range from 2 to 11. The first report outbreaks were in 1971 (Public Heath Laboratory Service, 1972). Johnson (1984) explained that emetic outbreaks are characterized by nausea and vomiting within 0.5 to 6 h after consumption of contaminated foods. Occasionally, there are abdominal cramps and/or diarrhea.

Since this research concerns the lethal effect of square pulse current of *B. cereus*, the elements of square pulse current are presented below.

Microbial mechanisms by low – voltage alternating current

In previous sections, the microbial inactivation was discussed. The main microbiocidal mechanism was the result of hydrogen peroxide formation (Shimada and Shimahara, 1981, 1982 and 1983). Thus the mechanisms of killing microorganisms via hydrogen peroxide will be elaborated in the following section.

Hydrogen Peroxide

Hydrogen peroxide (H_20_2) was first discovered by the French chemist Louis Jacques Thenard, in 1818 (Ascenzi, 1996). Pure H_20_2 is a colorless, syrupy liquid with a sharp and penetrating odor (Branen *et al*., 1990). It is a disinfectant rather a preservative since it rapidly kills microorganisms if it is applied at a sufficient concentration (Luck and Jager, 1997). The antimicrobial effect of H_20_2 is based essentially on its oxidation action. This causes all characteristics of irreversible

changes in the microorganism cell. Enzymes, membrane constituents and lipids are possibly oxidized and thereby inactivated (Luck and Jager, 1997). The bactericidal effectiveness of H_20_2 varies with the pH concentration, temperature, time, and type and numbers of microorganisms present (Davidson and Branen, 1993). H_20_2 has no long-lasting action since it easily decomposes to oxygen and water by heat or the enzymes catalase and peroxidase (Block, 1991). At room temperature it slowly decomposes. Changes in environmental conditions such as pH and temperature influence the rate of H_20_2 decomposition (Branen *et al*., 1990).

Antimicrobial Action. Many literatures report that the mode of action of H_20_2 is not due to the H_20_2 molecule itself but to the production of a most powerful oxidant, the hydroxyl free radical (Peloux *et al*., 1962; Miller, 1969; Cohen and Heikkila, 1974; Frolander and Carlsson, 1977; Symonyan and Nalbandyan, 1979). Hydroxyl can enter into reaction with organic materials. It can attack membrane lipids, DNA, and other essential cell components and alter molecule structure, and then these contribute to lethal and sub-lethal changes in living cells (Block, 1991). Conversion of H_20_2 to a biological poison can be divided to 3 cases, as follows:

The first mechanism which H_20_2 participates in the destruction of bacteria involves the reaction of the superoxide ion radical with H_20_2 to produce the hydroxyl radical.

$$O_2^- \bullet + H_2O_2 \longrightarrow OH \bullet + OH^- + O_2 \qquad (1)$$

In the presence of iron salt such as iron in microorganisms (Repine *et al*., 1981), this leads to the production of more the highly toxic hydroxyl radical by the well-known Fenton reaction (Equation 2) (Block, 1991). Repine *et al*. (1981) observed that scavengers of $OH\bullet$, such as dimethyl sulfoxide (DMSG), react and inactivate $OH\bullet$ and protect bacteria from being killed by OH•. It was observed that the killing effect of H_2O_2 was proportional to the intrinsic iron content of the

microorganism and that increasing concentrations of OH• scavengers increasingly inhibited killing of microbial by H_2O_2.

$$H_2O_2 + Fe^{2+} \longrightarrow OH\bullet + OH^- + Fe^{3+} \quad (2)$$

In the presence of myeloperoxidase enzyme, chloride in the bacteria may be oxidized by H_2O_2 to hypolchlorite (Klebanoff, 1968).

$$Cl^- + H_2O_2 \xrightarrow{\text{myeloperoxidase}} OCl^- + H_2O \quad (3)$$

Hypochlorite is well-known oxidant and germicide. Transition metals are believed to catalyze the formation of the hydroxyl radical (Dittmar *et al*., 1930).

Catalases from cells may be the most important factor in protection against high H_2O_2 concentrations because catalases generally have high rates (Juven and Pierson, 1996). Catalase can decompose H_2O_2 into oxygen and water. Thus OH• that is toxicity to cell is not produced.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (4)$$

Although catalases produced by respiration cells may be adequate to protect the cell from damage caused by steady-state levels of metabolically produced H_2O_2, this defense is overwhelmed by concentration (3% and greater) used for practical disinfections.

Food Industry Application. H_20_2 is considered so safe that it has been approved to use in foods in many countries (Schumb *et al*., 1955). It has been suggested to use as an antimicrobial agent in water (Yoshpe and Eylan, 1968) and dairy products (Fox and Kosikowski, 1962; Naguib and Hussein, 1972). It is an approved bactericide in the USA in the processing of milk used in certain varieties of

cheese (Juven and Pierson, 1996). Food grade H_2O_2 is a lightly stabilized preparation formulated to meet Food Chemicals Codex specifications; it is being recommended to use as an antimicrobial agent in milk intended to use in cheese making, in whey intended to use as modified whey, in corn starch and dried eggs (Juven and Pierson, 1996). Certain preparations in which H_2O_2 is the active compound are also being marketed as disinfectants for fruits and vegetables (Falik *et al.*, 1994). Residual Peroxide must be removed by an appropriate means, typically by addition of catalase.

H_20_2 was formed in suspension when exposure to AC or DC current (Patermarakis and Fountoukidis, 1990; Matsunaga *et al*., 1992, and Zhai *et al*., 1998). Kheamrutai (2004) concluded that hydrogen peroxide concentration was increased with increased current density at definite time. H_20_2 concentration affected the viability of *B. cereus*.

An objective of this research is to measure the lethal and the survival percentage of the bacteria in the square pulse current. However, the bactericidal affecting factors are found to be both H_20_2 and AC. Thus the justified calculation methods to identify the relevant lethal factors have to be found.

Natural Mortality of *B. cereus*

The responses of the test subjects are assumed to be entirely due to the effects of the applied stimuli such as chemical reagent and physical method, and no allowance has been made for any responses, which might have occurred without these stimuli. The observed percentage of test subjects must be corrected in order to obtain the true percentage of subjects killed by the stimuli alone.

In toxicity test, any parameters can be divided into two sets; testing set and controlled set. T_1 and T_2 is test population before and after treatment, respectively and C_1 and C_2 is control population before and after treatment, respectively.
The true mortality ratio is P'.

$$P' = \frac{\frac{T_1 - T_2}{T_1} - \frac{C_1 - C_2}{C_1}}{1 - \frac{C_1 - C_2}{C_1}} \tag{5}$$

When available data are in form of live individuals and uniform population; $T_1 = C_1$ thus

$$P' = \frac{\left(\frac{T_1C_1 - T_2C_1 - C_1T_1 + C_2T_1}{T_1C_1}\right)}{\frac{C_1 - C_1 + C_2}{C_1}}$$

$$P' = \frac{C_2T_1 - T_2C_1}{T_1C_2}$$

$$= \frac{C_2 - T_2}{C_2}$$

$$= 1 - \frac{T_2}{C_2},$$

or

$$\text{corrected mortality percentage} = \left(1 - \frac{\mathbf{T_2}}{\mathbf{C_2}}\right) \times 100. \tag{6}$$

This is commonly known as Abbott's formula, on account of its use in paper by Abbott (1925) (Finney, 1964). In contrary, if available data are in form of live individuals and non-uniform population, $T_1 \neq C_1$ (Bakr, 2004), Handerson-Tilton's formula is used. Handerson (1955) modifies Abbott's formula for calculation corrected % mortality.

$$P' = \frac{\frac{T_1 - T_2}{T_1} - \frac{C_1 - C_2}{C_1}}{1 - \frac{C_2 - C_1}{C_2}}$$

$$= \frac{\left(\frac{T_1C_1 - T_2C_1 - C_1T_1 + C_2T_1}{T_1C_1} \right)}{\frac{C_1 - C_1 + C_2}{C_1}}$$

$$= \frac{C_2T_1 - T_2C_1}{T_1C_2}$$

$$= 1 - \frac{T_2C_1}{T_1C_2},$$

or

$$\text{corrected mortality percentage} = \left(1 - \frac{T_2C_1}{T_1C_2}\right) \times 100. \quad (7)$$

If available data are shown in percentage, Schneider-Orelli's formula will be used for the calculation of corrected % mortality. Schneider-Orelli's formula is demonstrated by:

$$\text{Corrected \% mortality} = \left(\frac{\text{Mortality\%in treated} - \text{Mortality\%in control}}{100 - \text{Mortality\%in control}} \right) \times 100 \quad (8)$$

Square Pulse Current

Characteristics of square pulse waveform

Normally, a square pulse waveform is consisted of various sinusoidal waveform frequency that is n-fold increase (n = 1, 2, 3,…) from fundamental frequency.

Basically, a pulse can be described as a very rapid transition from one voltage or current level (baseline) to another level, and then, after an interval of time, a very rapid transition back to the original baseline level. The transitions in level are called *steps.* An ideal pulse consists of two opposite-going steps of equal amplitude. When

the leading or trailing edge is positive-going, it is called a rising edge. When the leading or trailing edge is negative-going, it is called a falling edge.

Figure 4 (a) shows an ideal positive-going pulse consisting of two equal but opposite instantaneous steps separated by an interval of time called the pulse width. Figure 4 (b) shows an ideal negative-going pulse. The height of the pulse measured from the baseline is its voltage (or current amplitude).

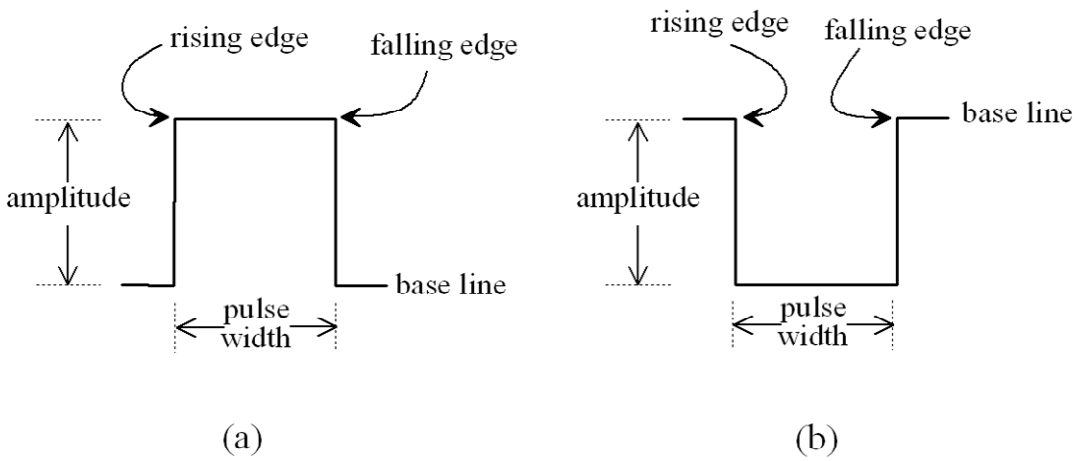


Figure 4 The characteristics of ideal pulse electric waveform.

In many applications, analysis is simplified by treating all pulses as ideal (composed of instantaneous steps and perfectly rectangular in shape). Actual pulses, however, are never ideal. All pulses possess certain characteristics that cause them to be different from the ideal. Figure 5 (a) represents an interval of time during which the pulse is rising from its lowest value to highest. This interval is called the rise time, t_R. Rise time is the time required for the pulse to go from 10% of its amplitude to 90% of its amplitude. The interval of time during which the pulse is falling from its higher value to its lower value is called the fall time, t_F. Fall time is the time required for the pulse to go from 90 % of its amplitude to 10% of its amplitude. Pulse width is the time between the point on the leading edge where the value is 50% of the amplitude and the point on the trailing edge where the value is 50% of the amplitude which shown in Figure 5 (b).

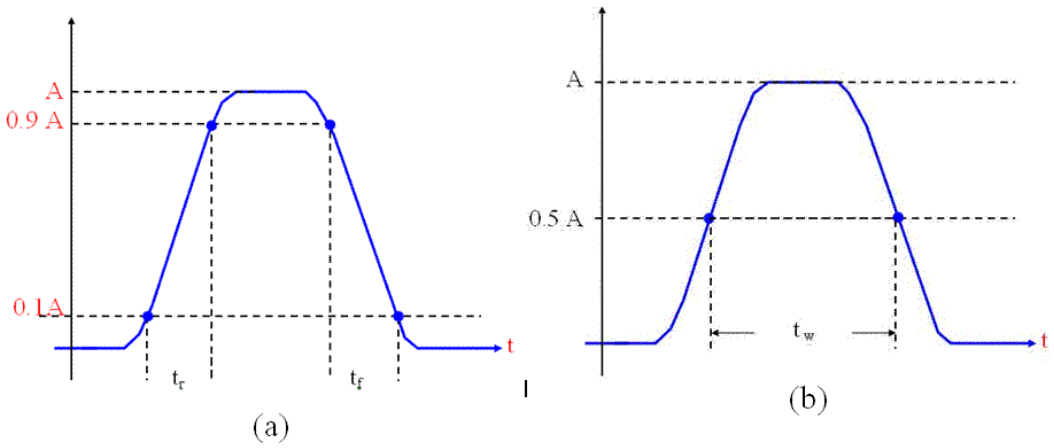


Figure 5 The characteristics of non-ideal pulse electric waveform pulse.

Any waveform was repeated itself at fixed intervals is periodic. Figure 6 shows some examples of periodic pulse waveforms. Notice that in each case, the pulses repeat at regular intervals. The rate at which the pulses repeat is the pulse repetition frequency, which is the fundamental frequency of the waveform. The frequency can be expressed in hertz or in pulses per second. The time from one pulse to the corresponding point on the next pulse is the period (T). The relationship between frequency and period is the same as with the sine wave, $f = 1/T$.

An important characteristics of periodic pulse waveforms is the duty cycle. The duty cycle is the ratio of the pulse width (t_W) to the period (T) and is usually expressed as a percentage.

$$\text{Percent duty cycle} = \frac{t_W}{T} \times 100\% \qquad (9)$$

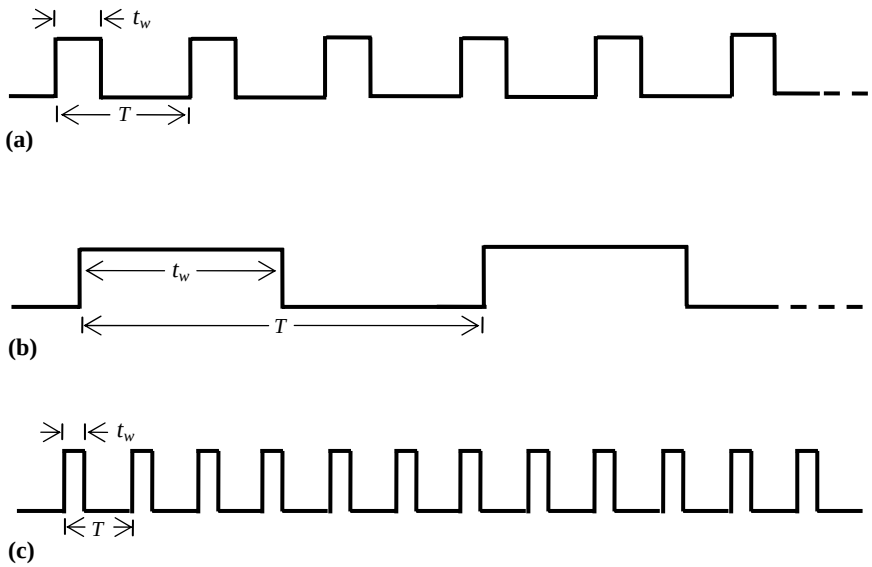


Figure 6 Repetitive pulse waveforms

For a square wave which is a pulse waveform with 50 percentage of duty cycle. Thus, the pulse width is equal to one-half of the period. A square wave is shown in Figure 7.

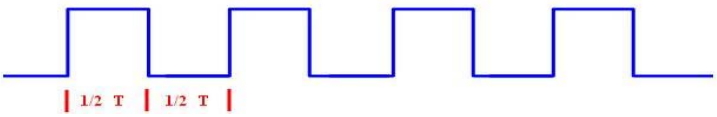


Figure 7 Characteristic of square wave with 50 percentage of duty cycle.

The average value (V_{avg}) of a pulse waveform is equal to its baseline value plus the product of its duty cycle and its amplitude. The lower level of a positive waveform or the upper level of a negative waveform is taken as the baseline. The formula is as follows

$$V_{avg} = \text{baseline} + (\text{duty cycle})\ (\text{amplitude}). \qquad (10)$$

MATERIALS AND METHODS

The Research Apparatus Design

This designed equipment is composed of three parts. They are the pulse current generator which transforms AC to pulse signal, temperature controller and treatment chamber as shown in Figure 8. The treatment chamber is prepared into two sets. The first one is connected to pulse current generator and another one without current while both samples are in the same water bath at the same temperature.

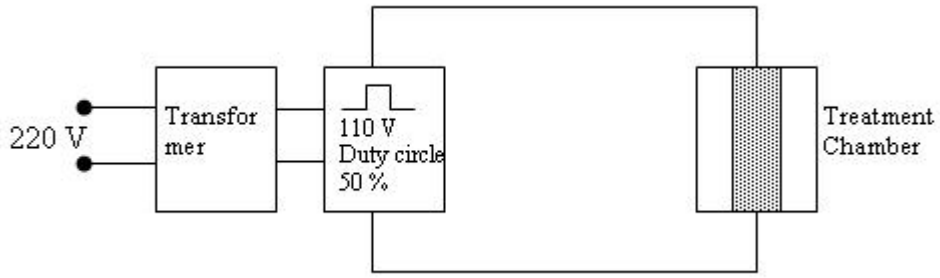


Figure 8 Fundamental components of the pulse current method.

Pulse current generator

Square pulse direct current was transformed from AC by the circuit that placed in box as shown in Figure 9. A variable electric frequency controlled by outer button that was at the side of the box.

Treatment Chamber Design

Treatment chamber is designed to hold cell suspension during current application and to house the electrodes. Static chamber is mainly suitable for laboratory use. Materials selected to construct a treatment chamber need to be washable and able to use with autoclave at 121 ˚C for 15 minutes. Additionally the materials should efficiently transfer heat because the purpose experiment is to

investigate the effect of low-voltage direct pulse current to the cell suspension at non-lethal temperature. Consequently, glass is chosen material to use because it can endure high temperature condition and transfer heat rather well. Figure 10 shows a glass chamber for this experiment. The glass chamber is constructed to form a vertical pipe with four holes connecting with four vertical arms. The chamber is 14 cm long and has volume of 25 ml.

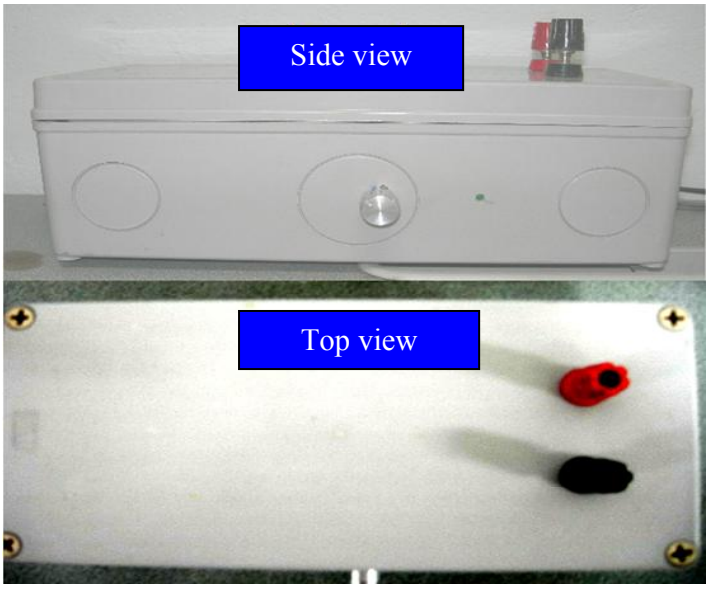


Figure 9 The box that contain the pulse sign transformed circuit.

The chamber cross sectional area is 1.77 cm^2. The inner diameter of all arms is 1.5 cm. The first and forth arms are used to accommodate the electrodes. The second arm is provided to support a mercury thermometer for temperature measurement during the treatment of cell suspension with pulse current. The third arm serves as a passage to introduce and remove a sample (Figure 14).

The carbon rods are used as electrodes in the present work. They are made of commercial carbon (Figure 11), fine graphite and nontoxic type. They are purchased from Thai Carbon and Graphite Co., Ltd. Electrode specific resistance was 11 μΩm. Each carbon rod is 15 cm in length by 1 cm in diameter. Carbon electrodes are inserted into the both end side arms. The length of the electrodes is 12.5 cm. Carbon

rod is connected to wire, which had copper plate as contact agent at the terminal. Thermo tolerant tube (of diameter 14 mm) is used as water shield and

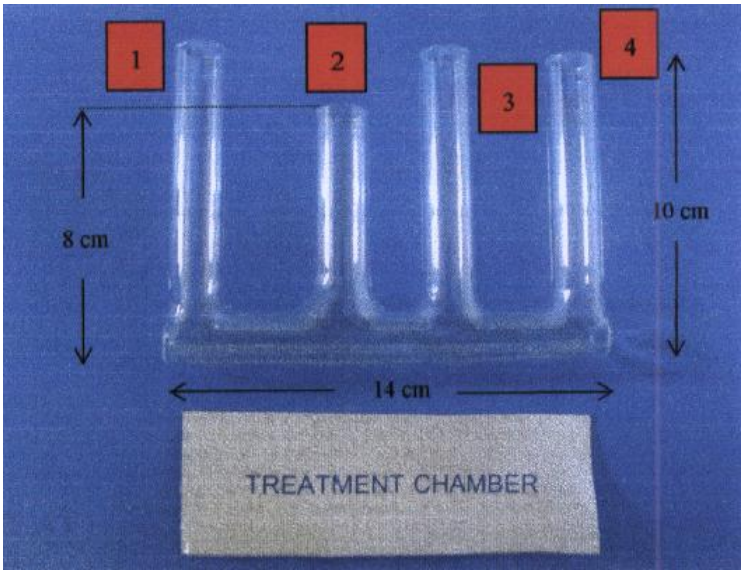


Figure 10 A glass chamber for pulse current exposure.

wire-electrode sheathe, (Figure 12) since chamber must be placed in water in order to obtain equilibrium temperature. Electrodes and thermometer are held in their position by insulating material suck as rubber plug. Treatment chamber is fixed on a wood-iron stand (Figure 13). This part is immersed in water bath. Assembled treatment chamber is shown in Figure 14.

Cooling System

The cooling system is used to maintain a constant temperature because the aim of this research is to investigate the effect of low-voltage pulse current (duty cycles 50%) on the viability of *B. cereus* at non-lethal temperature.

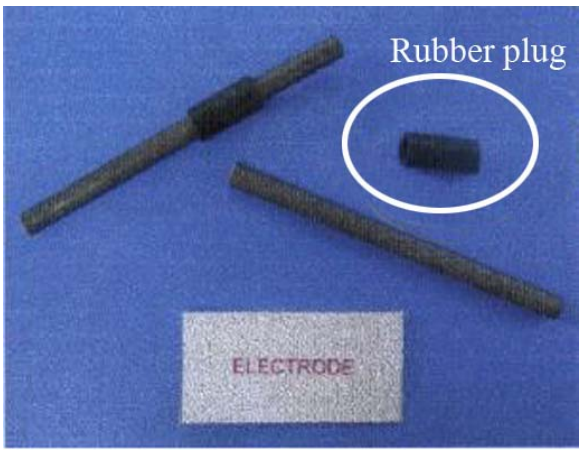
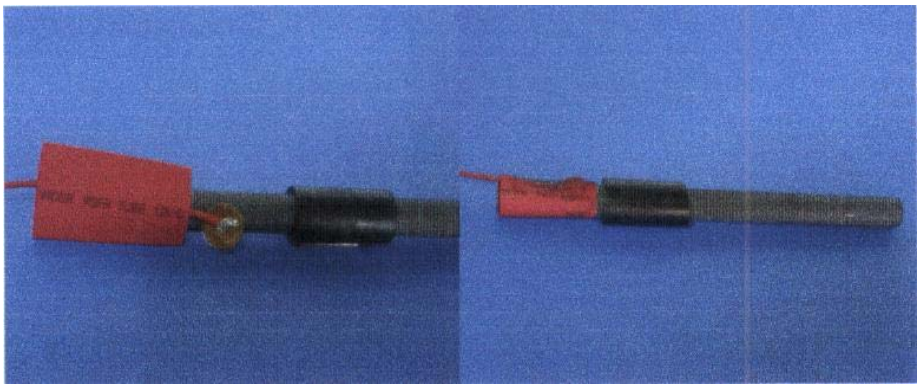


Figure 11 Carbon electrodes and rubber plug. Rubber plug is hollow cylinder



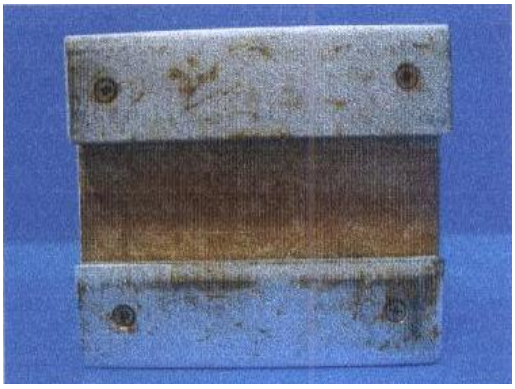
(a) Unassembled (b) Assembled

Figure 12 Connection between wire and rod

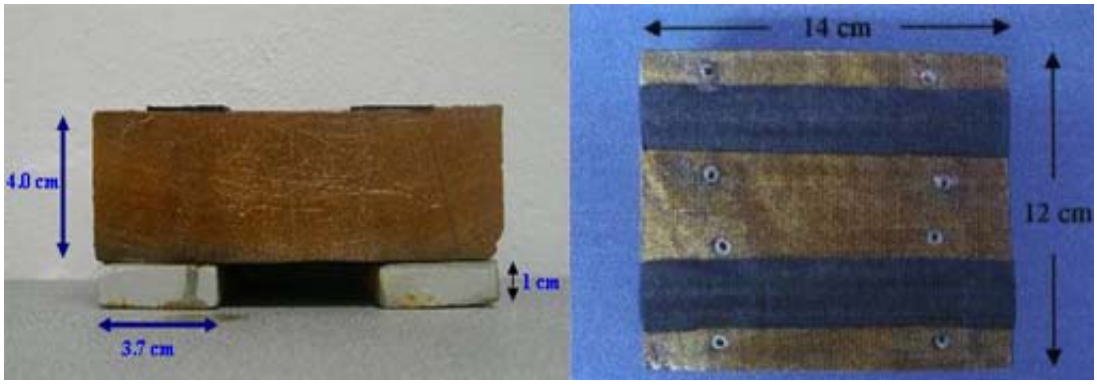
Preparation of Bacterial Culture

B. cereus was used throughout the experiment. The culture was inoculated into 250 ml flask containing 100 ml nutrient broth (NB, Appendix A), and incubated at 37 °C with shaking at 216 strokes/minute for 18 hrs in incubator. After 18 hrs of incubation, one loop of cell suspension was streaked on nutrient agar (NA, Appendix A) plate for bacterial isolation in order to have pure culture. The plate with bacteria was incubated at 37 °C for 18 hrs. Then, single colony was chosen and maintained on NA slant at 37 °C for 18 hrs. Thereafter it was kept in a refrigerator at 4 °C ready for

utilization. The culture was subcultured every month on the same medium. It was isolated for pure culture by streak plate method and refreshed in the same way as above process every time before use in the experiments.



(c) Bottom view



(a) Side view (b) Top view

Figure 13 Stand is made of wood that fastened with iron. Rubber plates (Aeroflex, 1/8″ in thick) are slapped gently on the top plane. A platform can support two glass chambers.

Effect of Pulse Current on Survival of *B. cereus*

Vegetative cells in logarithmic phase were prepared (Appendix A). They were suspended in NB. The initial cell concentrations used were approximately 10^6 CFU/ml. 23 ml of cell suspension (approximately 23×10^6 CFU) were taken to treatment chamber. Square pulse current was used at 110 V at 50% of duty circle. The temperature of cell suspension was maintained at 29 ± 3 °C during pulse current exposure by cooling system. For untreated cell suspension, it must be kept under the same condition as treated cell suspension. Thus it was introduced in the same glass chamber and placed in the water bath at temperature 29 ± 3 °C.

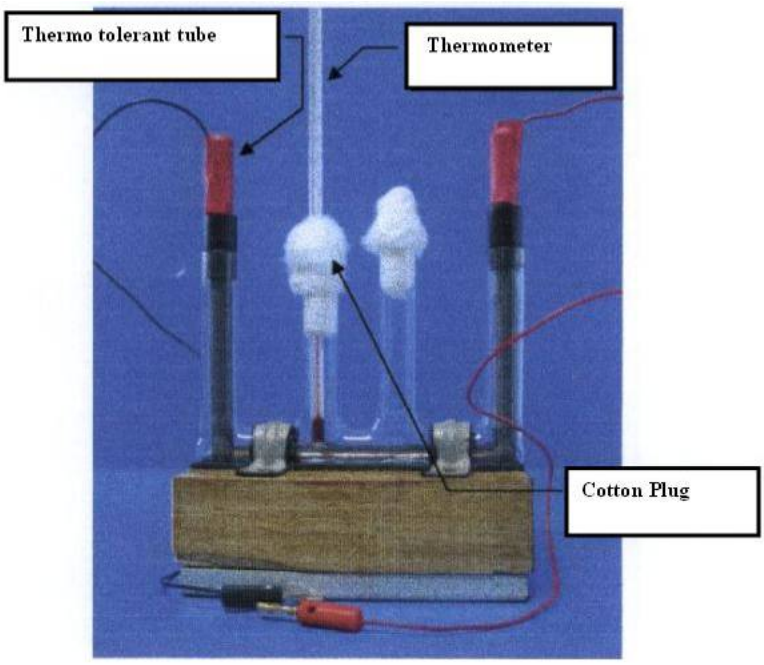


Figure 14 Assembled treatment chamber.

Treated cells were exposed to square pulse current. At the start of the exposure and at 2, 4 and 6 hrs thereafter, 0.1 ml of cell suspension was taken out from the chamber to determined viable count instantaneously by spread plate count technique. Viable counts of untreated cell were served as control.

Quantitative Assay of Hydrogen Peroxide

Cells were collected and exposed in the same way as described in the above section. Square pulse current was used at 110 volt at 50% of duty circle. The temperature of cell suspension was maintained at 29 ± 3 °C during square pulse current exposure. Cell suspension was measured the content of H_2O_2 every hour for 6 hrs by using peroxide test strips (Merckoquant). Quantitative assay of H_20_2 was described as follows:

1. Dipping the test strip into the cell suspension to be testing for 1 second, such that the reaction zone was completely wetted.

2. Moving, the test strip slightly for 3-30 seconds until the suspension had evaporated from the reaction zone, then dipping it into distilled water for 1 second and shook off the excess water.

3. After 15 seconds, comparing the reaction zone with the color scale (Figure 15).

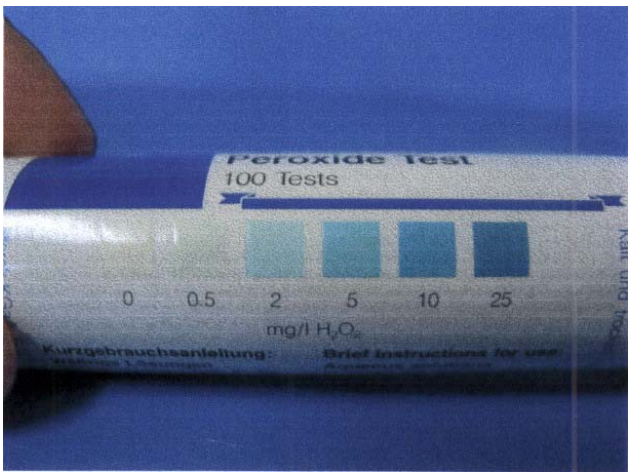


Figure 15 Color scale for determination hydrogen peroxide concentration.

The appearance of any blue coloration within 3 minutes can be interpreted as a positive reaction. The results of H_20_2 concentration in this research were confirmed by investigation in duplicate experiments.

Cell Number Counting Method

The cell number was determined by the spread plate count technique. It is a viable cell counting method. A viable cell is defined as the one that is able to divide and form offspring. The usual way to perform a viable count is to determine the number of cells in the sample capable to form colonies on a suitable agar medium. Because of this reason, the viable count is often called the dilution plate count or colony count. The assumption made in this type of counting procedure is that each viable cell can yield one colony. Procedures of spread plate method are described as follows. Sample is diluted by sterilized distilled water. Since one rarely knows the approximate viable count ahead of time, it is usually necessary to make more than one dilution. Ten fold serially dilution of the sample is frequently used. A 0.1 ml of the last three appropriate diluted samples was spread over the surface of NA plate using sterilized glass spreader. Each dilution was spread on triplicate NA plates. Subsequently plates were incubated for 24 hrs at 37 °C. After incubation, the colonies in countable plates were counted. The typical practice, which was the most valid statistically, was to count colonies only on plates that contained between 30 to 300 colonies. The viable counts of sample that were plate counts multiple by dilution factor were averaged and reported as CFU/ml.

In parallel experiment, cell growth was measured by Jenway spectrophotometer, Model 6305, at 650 nm.

Statistical Analysis

The results shown in this research were taken from the average of observation in duplicate experiments. Statistical analysis of the data from all experiments was performed using the software program Sigma Stat by SPSS Inc.

Place and Duration

Place: Biophysics Laboratory at Department of Physics and Microbiology Laboratory, Faculty of Science, Kasetsart University Duration: April, 2005 - February, 2008.

RESULT AND DISCUSSION

Lethal Effect of Square Pulse Current on *Bacillus cereus*

The lethal effect of square pulse current on *B. cereus* is studied by the pulse current with duty circle 50 % at different frequencies (45Hz, 50 Hz, 55Hz, and 60Hz) in duration time of 6 hrs. In this study, the result of treated cells compared with untreated cells reported in terms of number of viable cells. Effect of square pulse current on *B. cereus* at different electric frequency is shown in Figures 16 to 19.

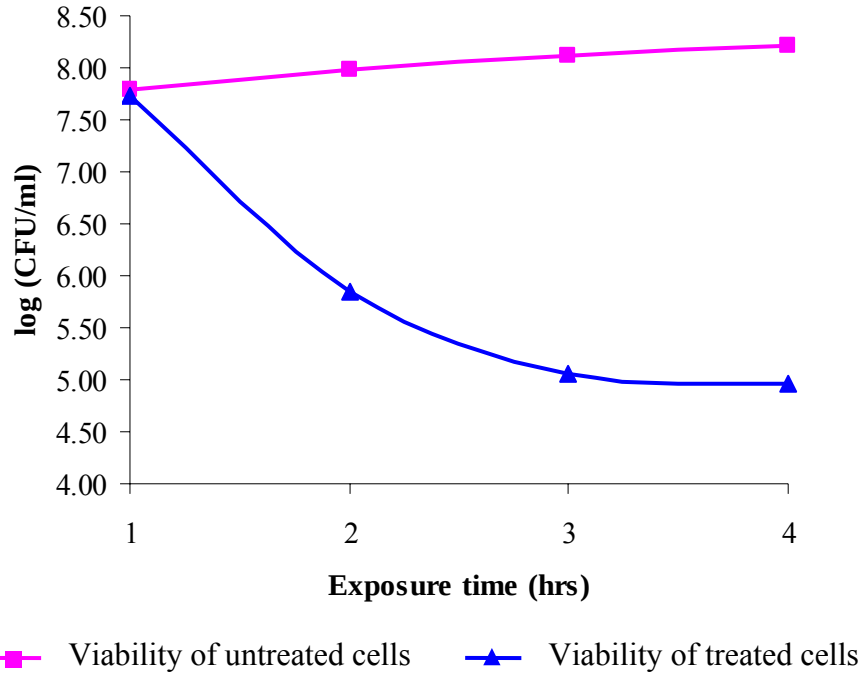


Figure 16 Logarithmic plot of *B. cereus* growth curves of treated and untreated cells exposed to square pulse current with frequency 45 Hz.

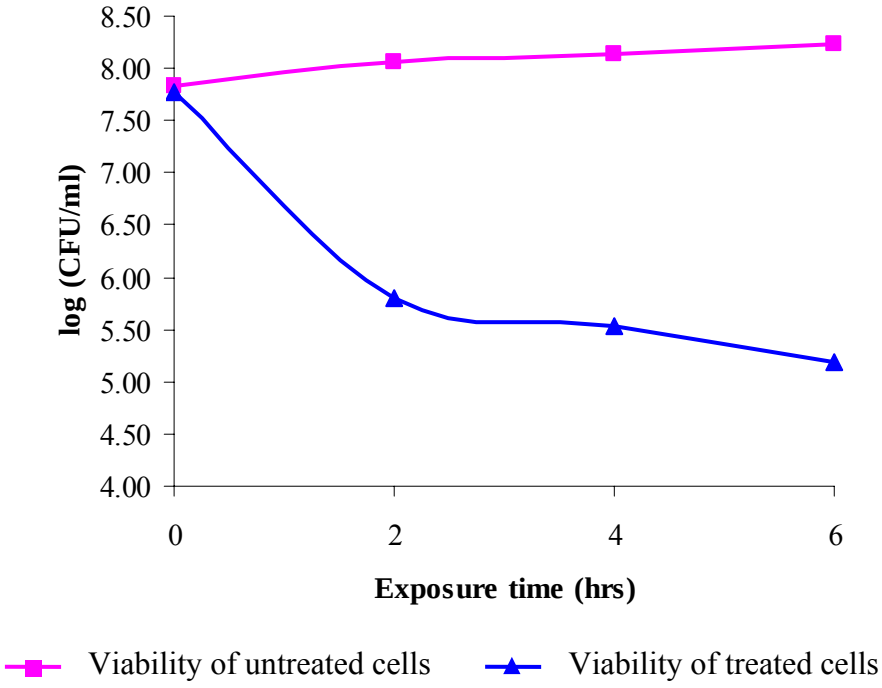


Figure 17 Logarithmic plot of *B. cereus* growth curves of treated and untreated cells exposed to square pulse current with frequency 50 Hz.

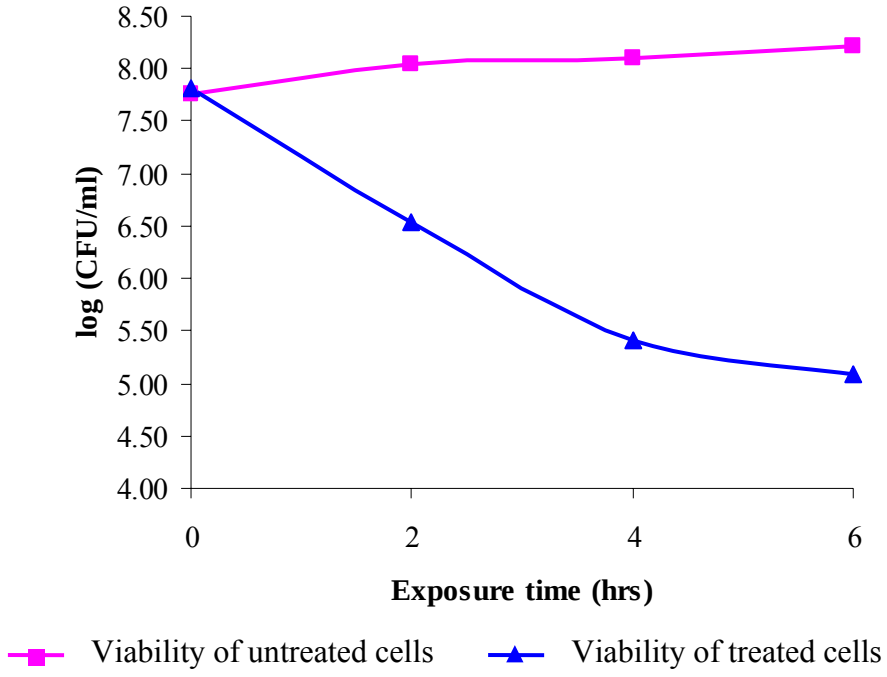


Figure 18 Logarithmic plot of *B. cereus* growth curves of treated and untreated cells exposed to square pulse current with frequency 55 Hz.

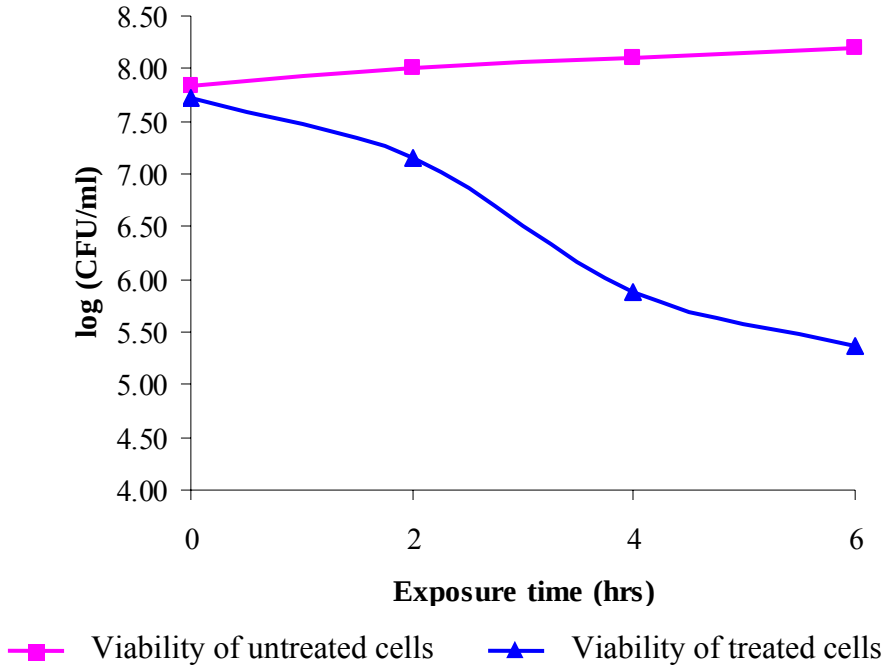


Figure 19 Logarithmic plot of *B. cereus* growth curves of treated and untreated cells exposed to square pulse current with frequency 60 Hz.

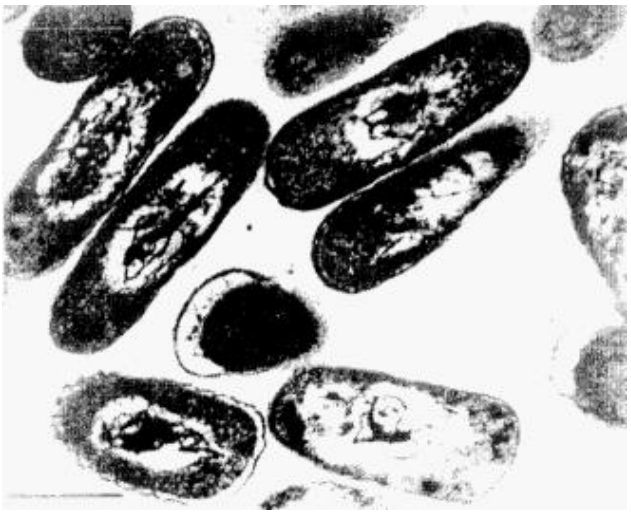
Figures 16 to 19 show the difference of viable number cells between treated and untreated samples by semi logarithm (log N) format versus exposure times. The plotting data were taken from four experiments with different electric frequencies. All experiments showed that the similar results that the numbers of viable of untreated cells were increased while treated samples were decreased. Moreover, Figures 16 to 19 show the same form of curves. The initial times used in all experiments were when the sample reached the stationary phase. Thus, the growth rates of untreated cells were little increased. However lethal effect of square pulse current on *B. cereus* in this experiment was quite obvious. The growth rates of the treated cells decreased with time.

The lethal effects of AC to microbial cells directly have been reported that the cell died because it was found that surface charges and physiological properties of cells, e.g. respiratory rate and stainability with crystal violate, varied when the cells

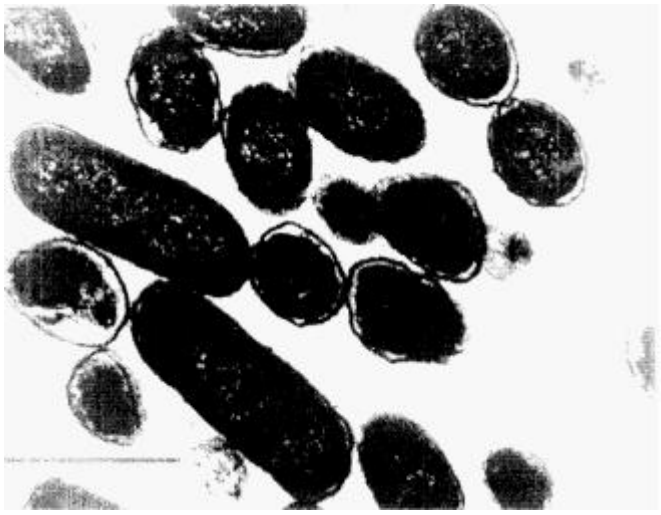
exposed to AC and it was inferred that permeability of the cell membrane is modified by AC-exposure. AC causes the release of the intracellular content of cells together with changes in the electron micrographic appearance of cellular materials located in the nucleus region within cells (Shimada and Shimahara, 1985 (a); Shimada and Shimahara, 1985 (b) and Lui *et al.*, 1997).

Shimada and Shimahara (1985a) found that electron microscopic observation revealed some interesting differences between cells treated and untreated with AC as shown in Figure 20. Electron micrograph of thin section shows that *E. coli* cells exposure to AC for 5 hrs in a phosphate buffer (20a) possessed more organized material in the central areas than unexposed cells (Figure 20b). In the unexposed cells, the cell nuclei have less electron density than the surrounding cytoplasm and membranes. These areas are presumably densely packaged DNA. When cell are exposed to AC, the arrangement of the materials in the nucleus areas varies from diffuse granular inclusions to irregularly dense aggregates. The electron transparent portions occur within the areas as shown in Figure 20a. Bacterial chromatin aggregates into compact masses under a variety of circumstances such as on exposure to a high salt concentration, low temperature, UV irradiation, metabolic inhibitors or starvation. These suggest that AC-exposure enhances the aggregation of DNA relating materials within cells following the leakage of cellular contents from cells. The present experiment was to determine the lethal effect of square pulse current on *B. cereus* at non-lethal temperature in nutrient broth (NB).

In similar experiment, Chutima (2005) showed that the generation time of *B. cereus* in nutrient broth (NB) that treated with microwave were longer than untreated cells. The results indicated that the viability of *B. cereus* was decreased during exposure to microwave.



(a) Cells with AC-exposure of 600 mA/cm^2 for 5 hrs.



(b) Cells untreated with AC.

Figure 20 Thin sections of *E. coli* cells treated and untreated with AC.
Source: Shimada and Shimahara (1985b)

In research of *B. cereus* with different medium, Kheamrutai (2004) put *B. cereus* in phosphate buffer, which was not appropriate condition for cell activities such as cell metabolism, then treated with AC. At non-lethal temperature, the

experimental results showed the lethal effect of AC to *B. cereus* cells. In Kheamrutai's experiment, H_20_2 produced by electrodes was found quite a few. As a matter of fact, H_20_2 is one of main cause of bacterial death.

Quantity Assay of Hydrogen Peroxide

In this experiment H_20_2 concentration was undetectable by using H_2O_2 test strip. In general, electrode causes electrolysis. For direct current, electrolysis is quite strong. For AC, it is expected that H_20_2 should be produce by the electrode and this is the case of Kheamrutai's experiment. Khemrutai (2004) reported that H_20_2 concentration was related to current. The present experiment, DC square pulse current was used to treat the *B. cereus* cells. However no evidence of H_20_2 was found in this experiment. The undetectable of H_20_2 might be from two reasons. The first one is that the used current is too low to produce H_20_2. The second reason, it was expected that the small amount of H_20_2 did exist, but it was decomposed by the catalase enzyme that the *B. cereus* produced.

Effect of electric frequency on *B. cereus*

Figure 16 to 19 performance comparison of viable cells between treated and untreated suspension. From the ratio of viable cell in Figure 26, the number of viable cells was increased with increasing electric frequency. Concerning the rate of change, the British economist Thomas Malthus (1966 - 1934) was the first who observed that many biological populations increase or decrease at a rate proportional to the population (Crack, 1990). Let $y(t)$ be the population of the given species at time t. The simplest hypothesis concerning the variation of population is that the rate of change of y is proportional to the current value of y that

$$\text{is } \frac{dy}{dt} = ay . \qquad (11)$$

Where the constant of proportionality a is called the rate of growth or decline, depending on whether it is positive or negative. The mathematical problem is the same as radioactive decay if $a < 0$. Solving Equation (11) subject to the initial condition

$$y(0) = y_0 \tag{12}$$

$$\text{give } y(t) = y_0 e^{at} . \tag{13}$$

Thus the cell number at any instantaneous time can be determined if the rate of growth and the initial quantity of all are know.

This hypothesis was applied to generate survival mathematical model of cells that were exposed to square pulse of electric frequency 45 Hz, 50 Hz, 55 Hz and 60 Hz with duty cycle 50 %. The best fitting-line of survival curves in Figure 20 was generated from least square method by using Microsoft Excel. The reliability of the fitting was indicated by R^2. R was called correlation coefficient. Perfect fit occurs when $R^2 \rightarrow 1$. Figure 20 shows fitting-line of survival curves and equations in exponential function. Where y was surviving fraction and t was exposure time and the initial of value was 1. It found that decline rate of surviving fraction at electric frequency 45 Hz, 50 Hz, 55 Hz and 60 Hz were 0.1224, 0.1109, 0.1037 and 0.0837 respectively. These indicated that decline rate of surviving fraction of cell decreased with increased stimulus level.

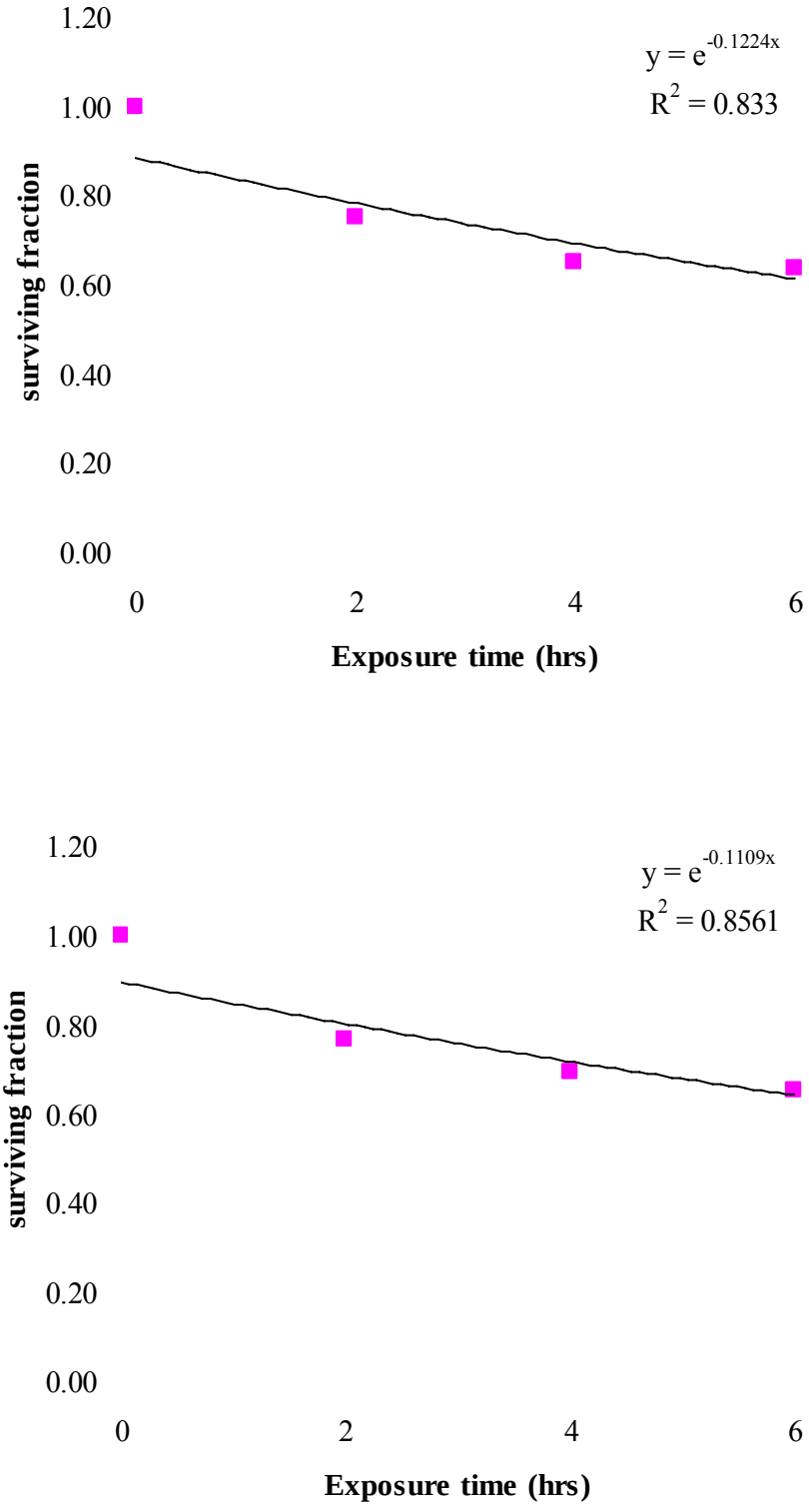
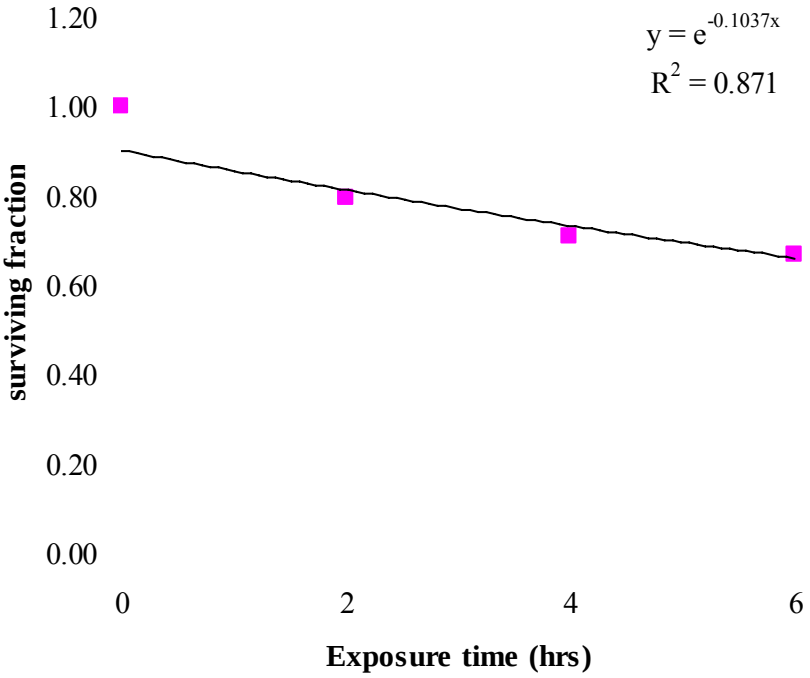


Figure 21 Fitting survival curves of cells exposed to square pulse current with electric frequencies 45 Hz, 50 Hz, 55Hz and 60 Hz.



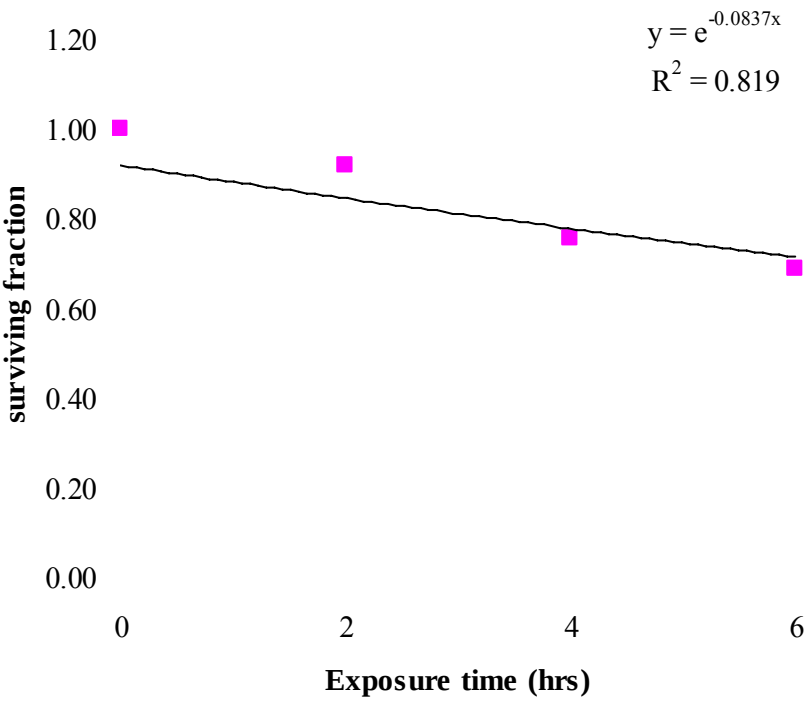


Figure 21 (continued)

Design of Processing Equipment

The studying in lethal effect of square pulse current on *B. cereus* involves the application of pulse wave generator and cooling system.

The pulse wave generator

The square wave generator circuit consists of IC 555, capacitors (C) and resistors (R_a and R_b). This circuit can change electric frequency by varying capacitance or resistance. Variable capacitor forms pulse repetition 40 to 68 Hz. The circuit of pulse generator is shown in Figure 22.

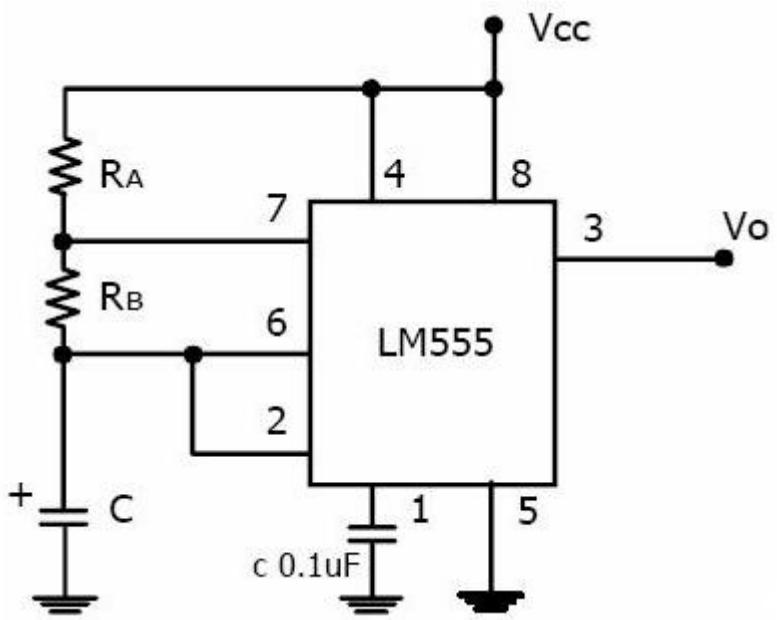


Figure 22 Assembly of the circuit of a pulse generator.

The circuit is connected to the step down voltage transformers (220 V to 110 V) manufactured by Intertek Testing Services (Thailand) Ltd. It can make output direct square pulse current at 110 voltages duty cycle 50 %. The pulse shape was checked by Oscilloscope (IWATSU, Model SS-7825). The assembly shape of output signal was shown in Figure 7.

In 2004 Kheamrutai used AC passing through suspension. The current densities were kept at stable level in each experiment. Current density was varied by using variable resistors which connect in series with the treatment chamber. The current was measured by true-rms multimeters (Fluke, Model 179), which connected in series to a variable resister. During experiment, the circuit generated heat and the cooling fan had to be used for ventilation to reduce the temperature of the circuit. Comparing with the present experiment, the circuit used to generate DC square pulse did not produce any detectable heat.

The cooling system

In order to investigate the direct effect of the current to the microorganism, the temperature has to be controlled. The previous cooling systems were designed. Usually, each experiment has it own suitable cooling system. However, the basic requirement of cooling system is more or less the same that is to maintain the temperatures during samples were exposed to current. This experiment needs the cooling system that facilitated more than previous research. Kheamrutai (2004) designed the cooling system to maintain temperature of samples at 29 ± 3ºC.

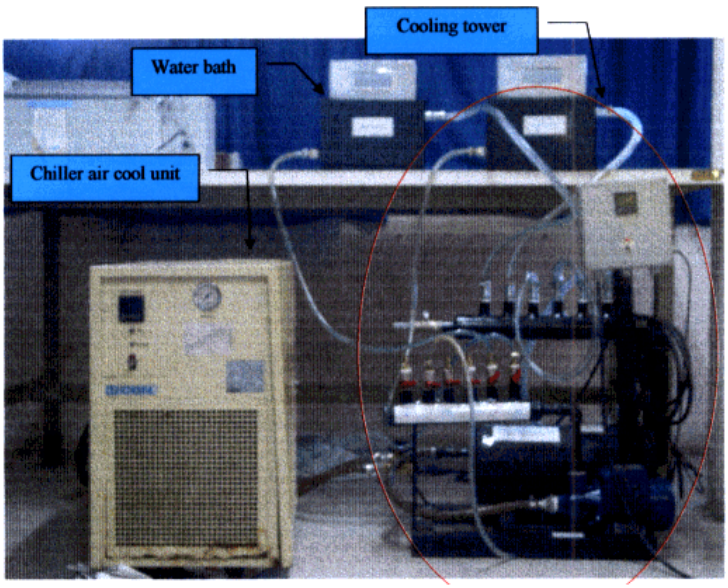


Figure 23 The cooling system used in Kheamrutai's experiment.

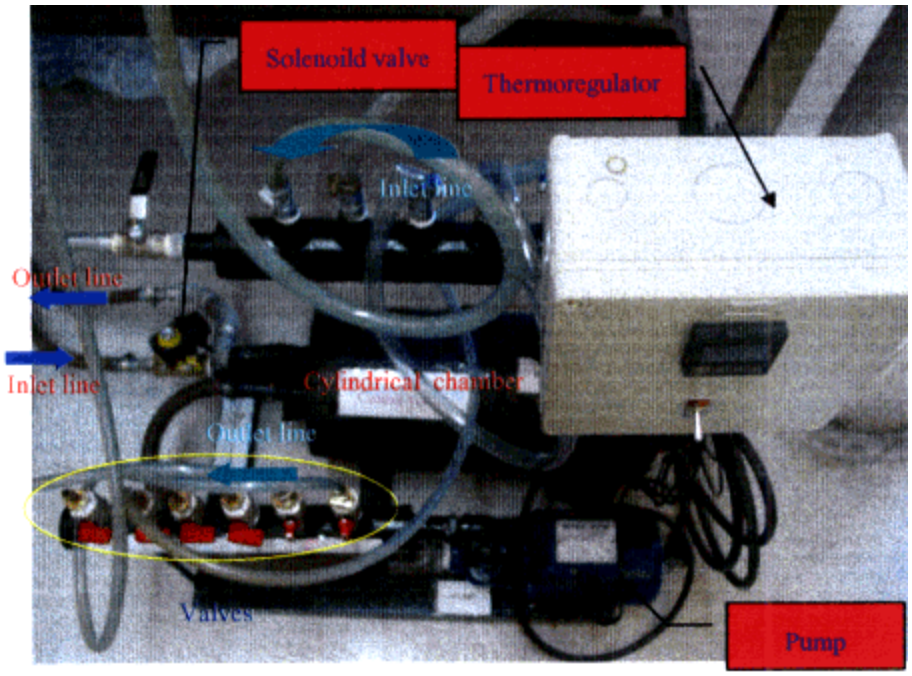


Figure 24 The major elements of cooling tower in Kheamrutai's experiment.

The cooling by water bath was chosen, the cooling system is composed of water bath, chiller air cooled unit (Model AC1P75) and cooling tower as showed in Figure 23. The cooling system is divided into two parts. The first part, cooled water from chiller flows through inlet line (blue arrow) into cooling coil and flowed backward to chiller through outlet line (blue arrow) cyclically (Figure 24). Cooling coil is inside cylindrical chamber wrapped in rubber envelope to prevent heat from environment. When instantaneous temperature of water in the water bath is equal to the set temperature, the solenoid controlled valve is closed. In contrary, the solenoid controlled valve is opened when instantaneous temperature varies higher than the set temperature. The past cooling systems that showed in Figure 23 and 24 were efficient to control the temperature. The variation of cooling temperature of this cooling system was shown in Appendix C.

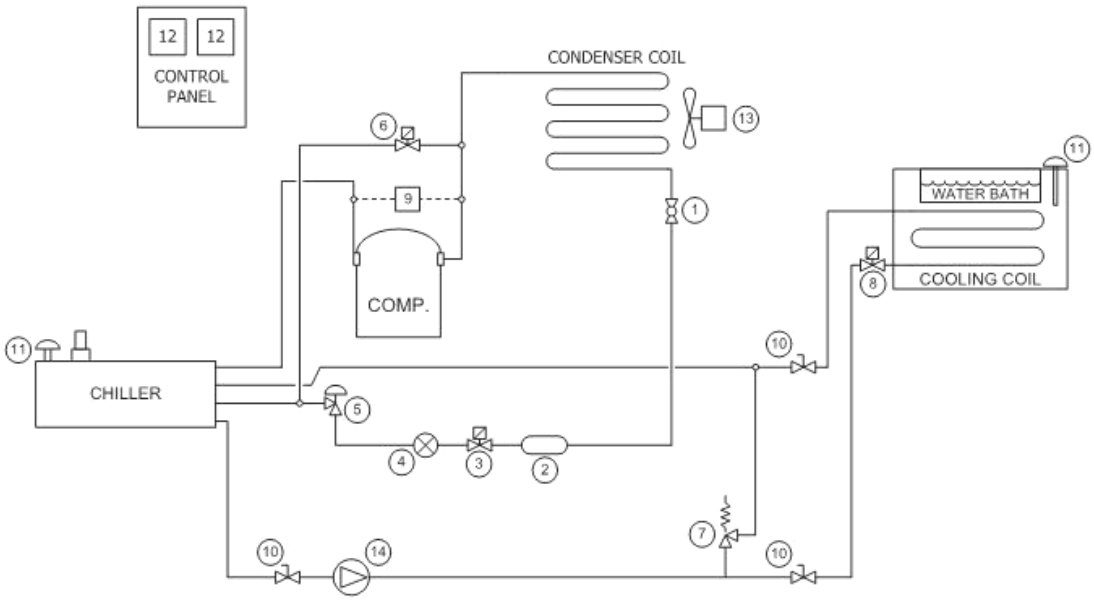


Figure 25 The diagram of cooling system circuit. The reference numbers of components were descript in Table 2.

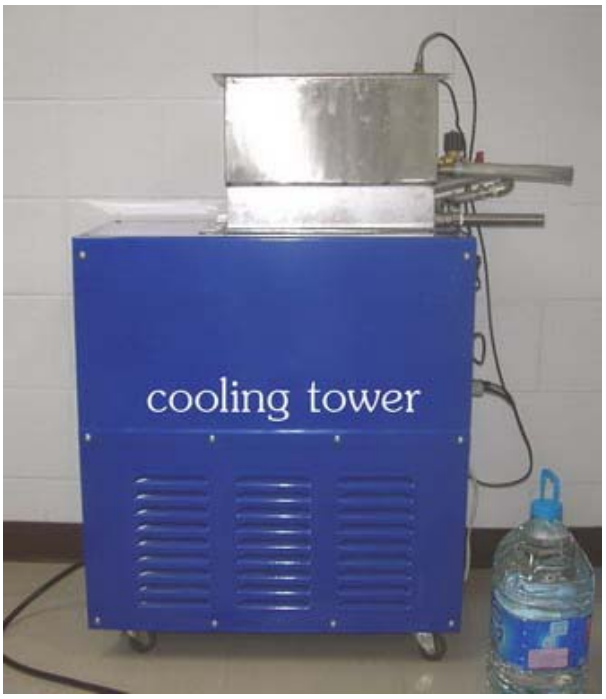
Table 2 The components of cooling circuit

Reference number	Particulars name
1	Liquid valve
2	Drier
3	Solenoid valve R22
4	Sight glass
5	Expansion valve
6	Solenoid by pass
7	Safety valve
8	Solenoid valve (Water)
9	High-Low pressure control

Table 2 (continued)

Reference number	Particulars name
10	Ball value
11	Thermocouple PT 100 Ω
12	Temperature controller with digital setting and display
13	Condenser fan
14	Chiller pump

However, from the arrangement of the system is difficult to move from one place to another. In the present experiment, the cooling system that used the same principle as the past system had been designed and developed. Figure 26 demonstrated the developed cooling system. The designed cooling tower had dimensions of 130 cm height, 50 cm width and 75 cm length. The system was the same as previous cooling system but developed the temperature measurement part. The diagram and the components of cooling system were shown in Figures 25 and Table 2. Figure 26 to 28 shows the cooling tower.



Figiure 26 The cooling – tower to maintain temperature.

Figure 27 Control board

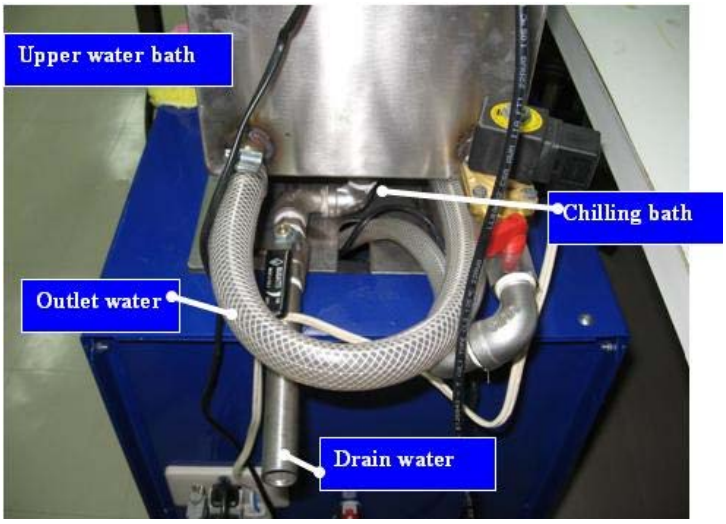


Figure 28 Water system of cooling tower

CONCLUSION

Square pulse current affected to the viability of *B. cereus* cells. Surviving fractions of cells decreased with increased exposure time. The low frequency electric pulse (45 Hz) gave more lethal effect on *B. cereus* than higher frequency electric pulse (60 Hz) at the same voltage. Therefore the decrease in surviving fractions was related to a quantity of electricity and duration and the frequency of applied square pulse current. Comparing with the past designs of apparatus, the present apparatus design is found to be more efficient and suitable for the bacterial experiment.

These results inferred that the square pulse current with 50% duty cycle is able to be utilized as a non-thermal processing method for preservation of food. It can inactivate *B. cereus*, which is food-born pathogen, or other microorganisms.

LITERATURE CITED

Ascenzi, J.M. 1996. **Handbook of Disinfectants and Antiseptics.** Marcel Dekker, Inc., New York.

Block, S.S. 1991. **Disinfection, Sterilization, and Preservation.** 4th ed. Lea and Febiger, Philadelphia.

Block, T.D. and M.T. Madiga. 1988. **Biology of Microorganisms.** 5th ed. Prentice Hall, New Jersey.

Bakr, E. LdP Line. Available Source: http://www.ehabsoft.com/ldpline/index.html, January 6, 2004.

Branen, A.L., P.M. Davidson, S. Salminen. 1990. **Food Additives.** Marcel Dekker, Inc., New York.

Chorin, E., D. Thuault, J.J. Cléret and C.M. Bourgeois. 1977. Modeling *Bacillus cereus* Growth. **Int. J. Food Microbiol.** 38: 229-234

Chutima, O. 2005. **Effect of weak microwave radiation on bacillus cereus growth**. M.S. Thesis Kasetsart University.

Clark, C.W. 1990. **Mathematical Bioeconomics.** 2nd ed. Wiley-Interscience, New York.

Cohen, G. and R.E. Heikkila. 1974. The generation of hydrogen peroxide, superoxide radical and hydroxyl radical by 6-hydroxyl dopamine, dialuric acid, and relate cytotoxic Agents. **J. Biol. Chem.** 249: 2447-2452.

Davidson, M.P. and A.L. Branen. 1993. **Antimicrobial in Foods.** 2nd ed. Marcel Dekker, Inc., New York.

Dittmar, H.R., I.L. Baldwin and S.P. Miller. 1930. The influence of certain inorganic salts on the germicidal activity of hydrogen peroxide**. J. Bacteriol**. 17: 203 – 211.

Falik, E., Y. Aharoni, S. Grinberg, A. Copal and J.D. Kline. 1994. Postharvest hydrogen peroxide treatment inhibits decay in eggplant and sweet red pepper**. Crop Prot**. 13: 451-454.

Finney, D.J. 1964. **Probit Analysis**. 2nd ed. The Syndics of The Cambridge University Press, New York.

Fioretto, F., C. Cruz, A. Largetan, T.A. Sarli, G. Demazeau and A. E. Moueffak. 2005. Interactivation of *Sataphylococuss aureus* and *Samonella entilistidis* in tryptic soy broth and caviar samples by high pressure processing. **Braz J Med Biol Res**. 38(08): 1259-1265

Fox, P.E. and F.V. Kosikowski. 1962. Heat-treated and hydrogen peroxide treated milk for cheddar Cheese. **J. Dairy Sci**. 45: 648.

Frolander F. and J. Carlson. 1977. Bactericidal effect of and anaerobic broth exposed to atmospheric oxygen tested on *Peptostrepto coccus anaerobius*. **J. clin Microbiol**. 6: 117-123.

FSHN 420 Food Microbiology. 1994. ***Bacillus cereus***. Available Source: http://www.ag.iastate.edu/departments/foodsci/classes/fshn420/bcereus.htm, September 6, 2003.

Gutavo, V.B., M. Marcela, U.R. Pothakamury and B.G. Swanson. 1999. **Preservation of Foods with Pulsed Electric Fields.** Academic Press, San Diego.

Goepfert, J.M. 1976. **Compendium of Methods for the Microbiological Examination of Foods**. M.L. Speck ed. Amer. Publ. Hlth. Assoc., Washington, D.C.

Goepfert, J.M., W.M. Spira and H.U. Kim. 1972. *Bacillus cereus*: food poisonings organism. A Review. **J. Milk Food Technol.** 35: 213-227.

Handerson, C.F. 1995. Tests with acaricides against the brown wheat mite. **J. Econ. Entomol.** 48: 157-160.

Jiraporn, S. 2003. **Effect of Instant Noodles Preparation and Identification of the Proper Critical Control Points for Controlling *Bacillus cereus* Contamination in Instant Noodle Seasoning.** M.S. thesis, Mahidol University.

Johnson, K.M. 1984. *Bacillus cereus* foodborn Illness an Update. **J Food Prot.** 47: 145-153.

Juven, B.J. and M.D. Pierson. 1996. Antibacterial effects of hydrogen peroxide and methods for its detection and quantitation. **J. Food Prot.** 59: 1233-1241.

Kheamrutai, T. 2004. **Lethal Effect of Low Voltage Alternating Current on *Bacillus cereus***. M.S. Thesis Kasetsart University.

Klebanoff, S. 1968 Myeloperoxidase-halide-hydrogen peroxide antibacterial system. **J Bacteriol**. 95: 21-31.

Liboff. A. R. 1985. Cyclotron resonance in membrane transport, interactions between electromagnetic fields and cells series A: **Light science**. 97: 281- 296.

Luck, E. and M. Jager. 1997. **Antimicrobial Food Activities: Characteristics, Uses Effects**. 2nd ed. Springer – Verlag. Germany.

Lui, W.K., M.R.W. Brown and T.S.J. Elliott. 1997. Mechanisms of the bactericidal activity of low amperage electric current (DC). **J. Antimicrob**. **Chemother**. 39: 687-695.

Matsunaga, T., S. Nakasono, T. Takamuku, J.G. Bergess, N. Nakamura and K. Sode.1992. Disinfection of drinking water by using a novel electrochemical reactor employing carbon-cloth electrodes. **Applied and Environmental Microbiology.** 58: 686-989.

Miller ,T.E. 1969. Killing and lysis of Gram - negative bacteria through the synergistic effect of hydrogen peroxide, ascorbic acid and lysosome. **J. Bacteriol.** 98: 949-950.

Naguib, K. and L. Hussein. 1972. The effect of hydrogen peroxide on the biological quality and nutritive value of milk. **Milchwessenschaft**. 27: 758-762.

Nester, E.W., C.E. Roberts and M.T. Nester. 1995**. Microbiology**: a Human Perspective. Dubuque, Iowa.

Pareilleux, A. and N. Sicard. 1970. Lethal effects of electric current on *Escherichia coli*. **Appl. Microbiol.** 19: 421-424.

Patermarakis, G. and E. Fountoukidis. 1990. Disinfection of water by electrochemical treatment. **Water Research**. 24: 1491-1496.

Peloux, Y., C. Nofre, A. Cier and L. Colobert. 1962. Poliovirus in activation by means of chemical systems production of free hydroxyl radicals: mechanisms of the virucidal activity of hydrogen peroxide and of ascorbic acid. **Ann. Inst. Pasteur.** 102: 6 - 23

Public Heath Laboratory Service. 1972. Food poisoning associated with *Bacillus cereus*. **Brit. Med. J.** 1972 (i): 189.

Rapport, H. and J.M. Goepfert. 1978. Thermal injury and recovery of *Bacillus cereus.* **J. Food Prot.** 41: 533-537.

Rawiwan, P. 2002. **Factor Affecting Toxin Production of *Bacillus cereus* in Thai Chili Pastes.** M.S. thesis, King Mongkut's University of Technology Thonburi.

Reginald, W.B., M. Gpala, K. Lawrence, C. Samuel and M.H. Standly. 1993. Biological characterization and serological identification of *Bacillus cereus* diarrhoeal factor. **Neth. Milk Dairy J.** 47: 105-120

Repine, J.E., R.B. Fox and E.M. Begger. 1981. Hydroxyl peroxide kills *stephylococcus aureus* by reacting with staphylococcal iron to from hydroxyl radical. **J. Biol. Chem.** 256: 7094-7096.

Rhodehamel, E.J. and S.M. Harmon. 1998. **Food and Drug Administration Bacteriological Analytical Manual**. 8th ed. AOAC International, Gaithersburg.

Riger, P.H. 1987. **Electrochemistry.** Prentice-Hall, Inc., New Jersey.

Roger, H.J., H.R. Perkins and J.B. Ward. 1980. **Microbial Cell Walls and Membranes.** Chapman & Hall, London.

Rosenberg, B., L.V. Camp and T. Krigas. 1965. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. **Nature.** 205: 698-699.

Rowley, B.A. 1972. Electrical current effects on *E.coli* growth rates. **Proc. Soc. Exp. Biol. Med.** 139: 929-934.

Schumb, W.C., C.N. Satterfield and R.L. Wentworth. 1955. **Hydrogen Peroxide.** Van Nostrand Rheinhold, New York.

Scott, W.J. 1975. Water relations of spoilage microorganisms. **Avian. Food Res.** 7: 81-124.

Shimada, K. and Shimahara. 1977. Effect of alternating current on growth lag in *Escherichia coli B.* J. Gen. **Appl. Microbiol.** 23: 127-136.

_____,_____. 1981. Factor affecting the surviving fractions of resting *Escherichia coli* B and K-12 cell exposed to alternating current. **Agric. Biol. Chem.** 45: 1589-1595.

_____,_____. 1982. Responsibility of hydrogen peroxide for the lethality of resting *Escherichia coli B* cells exposed to alternating current in phosphate buffer solution. **Agric. Biol. Chem.** 46: 1327-1337.

_____,_____. 1983. Sublethal injury to resting *Escherichia coli B* cells aerobically exposed to alternating current. **Agric. Biol. Chem.** 47: 129-137.

_____,_____. 1985a. Change in surface charge, respiratory rate and stainability with crystal violate of resting *Escherichia coli B* cells aerobically exposed to alternating current. **Agric. Biol. Chem.** 49: 405-411.

_____,_____. 1985b. Leakage of cellular contents and morphological changes in resting *Escherichia coli B* cells exposed to alternating current. **Agric. Biol. Chem.** 49: 3605-3607.

Spira, W.M. and G.J. Silverman. 1979. Effect of glucose, pH, and dissolved. Oxygen tension on *Bacillus cereus* growth and permeability factor production in batch culture. **Appi. Environ. Microbiol.** 37: 109-116.

_____ and J.M. Goepfert. 1972. *Bacillus cereus*-induced fluid accumulation in rabbit ileal loops. **Appl. Microbiol.** 24: 341-348.

Symonyan, M.A. and R.M. Nalbandyan. 1979. Generation of superoxide radicals in alkaline solution of hydrogen peroxide and the effect of superoxide dismutase on this system. **Biochim. Biopys. Acta.** 583: 279-286.

Terranova, W. and P.A. Blake. 1978. Current concept: *Bacillus cereus* food poisoning. **New Engl. J Med.** 298: 143-144.

Tracy. R.L. 1932. Lethal effect of alternating current on yeast cells. **J. Bacteriol.** 24: 423-438.

Trunbull, P.C.B. 1976. Studies on the production of enterotoxins by *Bacillus cereus*. **J. Clin. Pathol**. 29: 941-948.

Yoshape, P.Y. and E. Eylan. 1968. Disin faction of water by hydrogen peroxide. **Health Lab. Sci.** 5: 233-238.

Young, H.D. 1992. **University Physics**. 8th ed. Addison-Wesley Publishing Company, Massachuserrs.

Zhai, Z.H., Y. Sakagami and T. Opaka. 1998. Toxicity of hydrogen peroxide produce by electroplated coating to pathogenic bacteria. **Can. J. Microbiol.** 44:441-447.

APPENDICES

APPENDIX A

Culture Medium and Analytical Methods

Nutrient Broth (NB)

Beef extract	3.0	g
Peptone	5.0	g
Distilled water	1.0	l

Nutrient Agar (NA)

Beef extract	3.0	g
Peptone	5.0	g
Agar	15.0	g
Distilled water	1.0	l

The pH of culture medium adjusted to be 7.0 ± 0.5 by adding up 1 N NaOH and 1 N H_3PO_4. Mixed ingredients were sterilized by autoclave at 121 ºC for 15 minutes.

The pure and fresh culture was prepared. It was inoculated into NB 50 ml (in flask 250 ml) and incubated at 37 °C with shaking at 216 strokes/minute for 18 h: Afterwards, starter was adjusted at 0.5, two percentages of volumes of OD adjusted starter were transferred to NB 100 ml (in flask 250 ml). It was maintained at 37 °C, 216 strokes/minute for 4 hrs. After 4 hrs, several l0-fold dilutions of sample by sterilized distilled water were used. Each dilution was taken to measure OD at wavelength 650 nm and determined viable counts by spread plate count technique. Relationship between OD and viable counts in logarithmic units (Appendix Table A1) were plotted as shown in Appendix Figure A1.

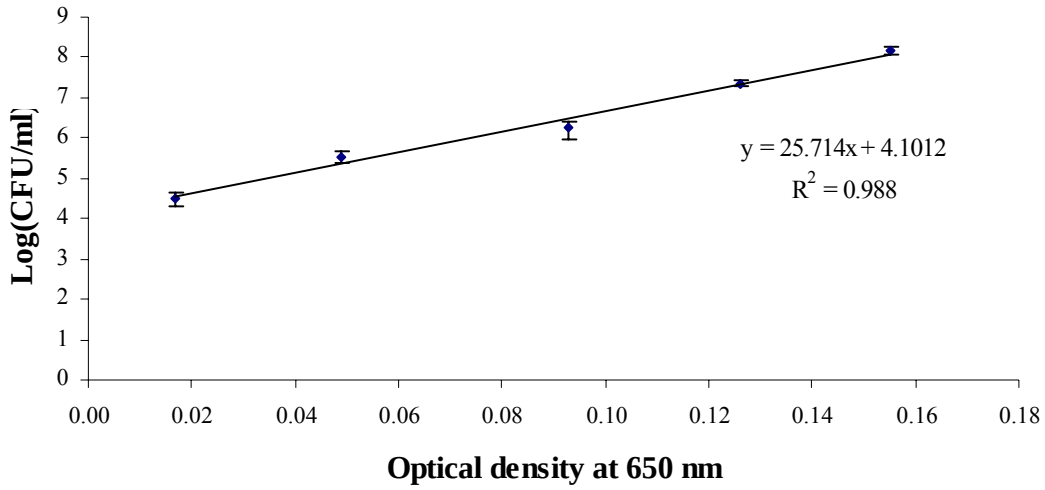
Linear curve fitting method provided linear equation for calculation OD when viable count was 10^6 CFU/ml. From linear equation

$$y = 25.714x + 4.1012;$$

if viable counts were 10^6 CFU/ml, y will be 6. Consequently OD (x value) was defined to be 0.07.

Appendix Table A1 Relation between OD at 650 nm and viable cell counts in logarithmic units.

Optical density	CFU/ml	log(CFU/ml)
0.155	$1.51 \times 10^{8} \pm 3.73 \times 10^{7}$	8.18 ± 0.10
0.126	$2.21 \times 10^{7} \pm 3.72 \times 10^{6}$	7.34 ± 0.07
0.093	$1.76 \times 10^{6} \pm 8.25 \times 10^{5}$	6.25 ± 0.17
0.049	$3.44 \times 10^{5} \pm 1.03 \times 10^{5}$	5.54 ± 0.11
0.017	$3.27 \times 10^{4} \pm 1.27 \times 10^{4}$	4.51 ± 0.14



Appendix Figure A1 Standard curve of cell concentrations in logarithmic phase

APPENDIX B

Vegetative cells in Exponential Phase Preparation and Standard Curve of Cell Concentration

Vegetative Cells in Exponential Phase Preparation

The bacteria *B. cereus* in logarithmic phase was used. In order to obtain the culture in this phase, growth curve had to be determined. The fresh and pure culture was prepared. Then it was transferred from NA slant to NB 50 ml (in flask 250 ml) and incubated at 37 °C, with shaking at 216 strokes/minute for 18 hrs. Afterwards the optical density (OD) of starter was measured by spectrophotometer. Wavelength was set up as 650 nm. An OD of starter was adjusted as 0.5 (cell concentration about 10^7 CFU/ml) by addition of NB. Two percentages of net volumes in OD adjusted starter were transferred to NB 100 ml (in flask 250 ml). Cell suspension was incubated at 37 °C, with shaking at 216 strokes/minute. One ml of sample was taken to measure the survival counts every 2 hrs for 48 hrs. Viable *B. cereus* counts were determined by spread plate count technique. The counts were averaged and reported as CFU/ml (Appendix Table B1). Finally, growth curve that showed relationship between viable counts in logarithmic units and time was generated (Appendix Figure B1).

Appendix Table B1 Variable count of *B. cereus* (37 °C, 216 strokes/minute) at different incubation times.

Time (hrs)	Viable counts	log(CFU/ml)
0.00	$3.70 \times 10^5 \pm 1.53 \times 10^5$	5.57 ± 0.02
1.76	$4.04 \times 10^5 \pm 8.37 \times 10^4$	5.61 ± 0.10
4.00	$1.69 \times 10^7 \pm 4.08 \times 10^6$	7.23 ± 0.12
6.86	$5.06 \times 10^7 \pm 6.69 \times 10^6$	7.70 ± 0.06
8.00	$3.93 \times 10^7 \pm 6.89 \times 10^6$	7.59 ± 0.08
10.00	$5.60 \times 10^7 \pm 1.29 \times 10^7$	7.75 ± 0.12
12.00	$5.37 \times 10^7 \pm 3.05 \times 10^7$	7.73 ± 0.36
14.35	$6.60 \times 10^7 \pm 2.74 \times 10^7$	7.82 ± 0.23
16.36	$9.63 \times 10^7 \pm 2.09 \times 10^7$	7.98 ± 0.09
18.42	$1.10 \times 10^8 \pm 2.92 \times 10^7$	8.04 ± 0.13
20.20	$1.25 \times 10^8 \pm 2.23 \times 10^7$	8.10 ± 0.09
22.30	$1.52 \times 10^8 \pm 3.89 \times 10^7$	8.18 ± 0.13

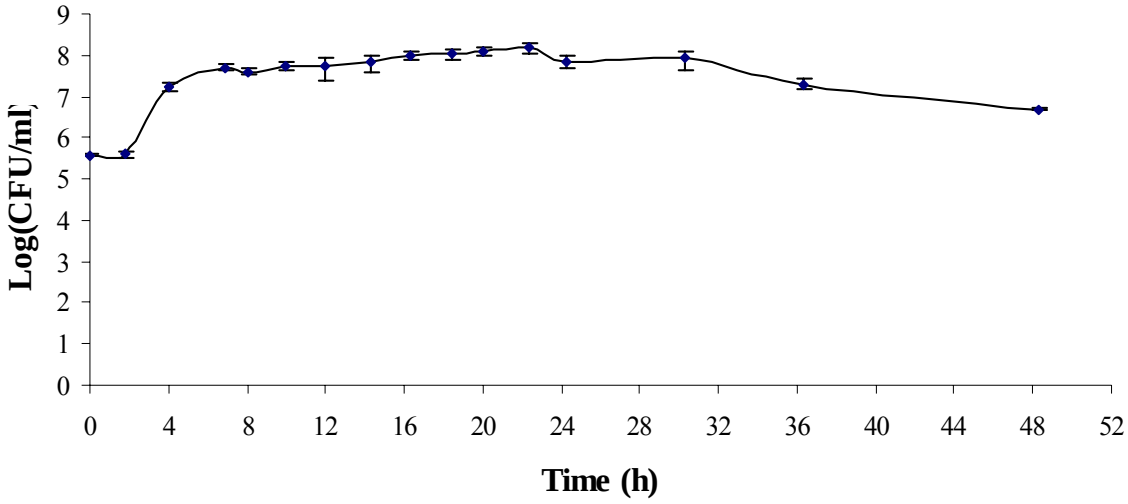
Appendix Table B1 (continued)

Time (hrs)	Viable counts	log(CFU/ml)
24.30	$6.97 \times 10^7 \pm 2.33 \times 10^7$	7.84 ± 0.17
30.30	$8.50 \times 10^7 \pm 4.15 \times 10^7$	7.93 ± 0.29
36.30	$2.01 \times 10^7 \pm 5.61 \times 10^6$	7.30 ± 0.14
48.30	$5.00 \times 10^6 \pm 4.04 \times 10^5$	6.70 ± 0.04

As shown in Appendix Figure B1, the incubation time to attain exponential phase was proximately 2-6 hrs. The averaged incubation time of 4 hrs was employed in all experiments.

Standard Curve of Cell Concentration

Initial cell concentrations before pulse current exposure in this research were fixed as 10^6 CFU/ml. Therefore standard curve that demonstrate association optical density versus viable counts should be elucidated.



Appendix Figure B1 Growth curve of *B. cereus* at 37 ºC, 216 strokes /minute

APPENDIX C

The Setting Temperature of Cooling System

Appendix Table C1 The setting temperature during exposure current

Exposure Times (hrs)	0	1	2	3	4	5	6
Water bath Temperature (ºC)	27	28	27	26	26	25	27
Chilling Temperature (ºC)	18	18	17	19	17	19	18

APPENDIX D

Number of Viable Cells Exposed to Square Pulse Current

Appendix Table D1 Number of viable cells exposed to square pulse current with electric frequency 45 Hz in logarithmic scale.

Exposure time (hrs)	Viability of untreated cells log (CFU/ml)	Viability of treated cells log (CFU/ml)
0	7.79	7.73
2	7.97	5.86
4	8.12	5.07
6	8.21	4.95

Appendix Table D2 Number of viable cells exposed to square pulse current with electric frequency 50 Hz in logarithmic scale.

Exposure time (hrs)	Viability of untreated cells log (CFU/ml)	Viability of treated cells log (CFU/ml)
0	7.84	7.77
2	8.07	5.79
4	8.13	5.54
6	8.23	5.19

Appendix Table D3 Number of viable cells exposed to square pulse current with electric frequency 55 Hz in logarithmic scale.

Exposure time (hrs)	Viability of untreated cells log (CFU/ml)	Viability of treated cells log (CFU/ml)
0	7.76	7.81
2	8.04	6.54
4	8.10	5.42
6	8.21	5.08

Appendix Table D4 Number of viable cells exposed to square pulse current with electric frequency 60 Hz in logarithmic scale.

Exposure time (hrs)	Viability of untreated cells log (CFU/ml)	Viability of treated cells log (CFU/ml)
0	7.83	7.72
2	8.00	7.15
4	8.11	5.88
6	8.20	5.36

CURRICULUM VITAE

NAME	: Miss Naruecha Kaewsanay
DATE OF BIRTH	: June 28, 1975
BIRTH PLACE	: Kalasin, Thailand
WORK PLACE	: Kangnachan Pittayakhom School, Kalasin