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Effects of square waveform pulsed ohmic heating on the vegetative cells and spores of *B.cereus* and *Clostridium spp* in the biogas digestate

Kare vurgu dalgalı ohmik ısıtma işleminin, biyogaz prosesinden elde edilen fermente ürün içerisindeki *B.Cereus* ve *Clostridium spp* türlerinin canlı hücre ve sporları üzerindeki etkileri

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Abstract

In this work, microbial inactivation experiments are performed on the biogas digestate samples via laboratory type ohmic heating system. The ohmic heating system ensures 2.72 V/cm voltage gradient with 50 Hz frequency square waveform pulsed alternating signals. Operations with 5 min and 15 min holding times are carried out at 70°C target temperature. Microbiological analysis performed before and after ohmic heating treatments with 5 minutes holding times revealed that, the number of Clostridium spp spores and vegetative cells suspended in the digestate with the related composition, are subjected to log reduction as 0.987 and 1.650 respectively. As a result of the 15 minutes holding time, it is determined that Clostridium spp spores and vegetative cells exhibited log reductions 0.819 and 1.725 respectively. The ohmic heating operation with 5 minutes holding time resulted in 18% decrease in the number of B.cereus spores and 55% increase in their vegetative cells in the same product. As a result of the 15 minutes holding time, the number of B.cereus spores and the vegetative cells increased by 37% and 23% respectively. Existing literature work on ohmic heating generally discusses the effects of the process parameters on the inactivation of microbial species which exist in various food matrixes. In this work, the effect of ohmic heating on the microbial inactivation of the biogas digestate which is originated from a biogas plant utilizing chicken manure as a substrate is investigated.

Keywords: Ohmic Heating, Pulsed Ohmic Heating, Square Waveform, Microbial Inactivation, Biogas Digestate.

Öz

Bu çalışmada, laboratuvar tipi ohmik ısıtma sistemi kullanılarak biyogaz santralinden alınan fermente ürün örneği üzerinde mikrobiyolojik inaktivasyon denemeleri gerçekleştirilmiştir. Ohmik ısıtma işleminde 50 Hz frekansında, 2.72 V/cm voltaj gradyanı sağlanan kare vurgu dalgalı alternatif sinyal kullanılmıştır. 70°C hedef sıcaklıkta, 5 dk ve 15 dk bekleme süreli işlemler gerçekleştirilmiştir. 5 dk bekleme süreli ohmik ısıtma işlemlerinin öncesi ve sonrasında gerçekleştirilen mikrobiyolojik analizler, ilgili bileşimdeki fermente ürün içerisindeki Clostridium spp spor ve canlı hücrelerinin sırasıyla 0.987 ve 1.650 log azalmaya tabi olduğunu göstermiştir. 15 dk bekleme süreli işlem neticesinde Clostridium spp spor ve canlı hücrelerinde sırasıyla 0.819 ve 1.725 log azalma tespit edilmiştir. 5 dk bekleme süreli ohmik ısıtma uygulaması neticesinde, aynı ürün içerisinde yer alan B.cereus spor sayısında %18 azalma, canlı hücre sayısında ise %55 artma tespit edilmiştir. 15 dk bekleme süreli işlem neticesinde B.cereus spor ve canlı hücre sayılarında sırasıyla %37 ve %23 artış meydana gelmiştir. Ohmik ısıtma kapsamındaki mevcut literatür çalışmaları genellikle, uygulanan proses parametrelerinin, çeşitli gıda ürünü matriksleri içerisinde bulunan mikrobiyal türlerin inaktivasyonu üzerine olan etkilerini tartışmaktadır. Bu çalışmada, ohmik ısıtma işleminin, proses girdisi tavuk gübresi olan bir biyogaz santralinden elde edilen fermente ürünün mikrobiyal inaktivasyonu üzerine olan etkisi araştırılmıştır.

Anahtar kelimeler: Ohmik Isıtma, Vurgulu Ohmik Isıtma, Kare Dalga, Mikrobiyal İnaktivasyon, Biyogaz Digestat

1 Introduction

Heat treatment processes are widely used to protect food from microbiological and biochemical changes as well as to obtain shelf life extension. Appropriate heat treatment methods can destroy both vegetative and spore forms of microorganisms so that the food to be ensured as microbiologically safe. However, most of the conventional heat treatment applications can cause decrease in the nutritional value and sensory quality of the food as well as formation of toxic components, depending on the process temperature [1].

Ohmic heating is among the alternative thermal processes to be generally applied to the food products in order to obtain microbial inactivation. The food to be processed via ohmic heating acts as a conductive medium, thereby allowing passage of the alternating current within it. Ohmic heating has advantages over conventional heating process in terms of rapid and homogenous heating as well as achieving higher nutrient retention and less energy consumption. Bacterial spores with a multiple layered resistant structure, are able endure stress conditions such as; heat, pressure, UV and chemical agents which could normally inactivate vegetative cells. Regarding thermal inactivation methods, bacterial spores require much higher target temperatures to obtain sufficient inactivation which would lead to excessive energy consumption, nutrient loss and degradation. Therefore, emerging technologies which

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enable inactivation of both vegetative cells and spores with optimum energy requirement and highest nutrient retention have already been an outstanding research subject.

In literature, there are studies investigating the bacterial inactivation mechanisms of the ohmic heating. A hypothesis supports that unlethal temperatures can trigger the activation of the spores and transition to the vegetative form. Losing their protective structure to an extent, activated spores are more likely to be inactivated at lower temperatures than their dormant state forms[2]. A research results declare that, activation of the spores causes changes in the tertiary structure of the some cystine rich spore coat proteins which are responsible for the dormancy of the spores[3]. A study on the ohmic heating attributes the inactivation success of the bacterial spores mainly to unlethal thermal effect but also points out the synergistic effect of the electric field[4]. Another study indicates that ohmic heating induces heat activation of the spores, thereby allowing the release and oscillation of some ionic molecules such as Ca-DPA as well as parts of denatured proteins[5]. The ionic components which are released from the spore increases the electrical conductivity of the spore, thus the spore becomes vulnerable to the electric current passing through the medium[6]. Another hypothesis can also be derived from the germination mechanisms of the spores. Due to combined effects of unlethal heat and electric fields, germination related mechanisms may be triggered within the spore, resulting in losing the protective structure against stress conditions. For instance; some studies observe calcium dipicolinate leakage from the spore being exposed to ohmic heating related heat and electric field effects. At this point, calcium dipicolinate can act as a germination trigger agent, possibly inducing cortex hydrolysis. In addition, change in the tertiary structure of the spore coat which occur as a result of heat activation may also trigger cortex-lytic enzymes that are assumed to be found on the spore coat [7]. Considering all of these possible germination related mechanisms, the protective structure of the spore can be deformed, leading to be more vulnerable to stress conditions.

There are internal and external factors which have effect on the microbial inactivation success of the ohmic heating. Internal factors include product related parameters such as; physicochemical properties, pH, water activity, electrical conductivity, matrix, composition, electrolytes, fat and oil content, viscosity, microorganisms type, growth phases, genetics, sporulation medium etc. External factors generally cover the process parameters which are applied on the product during ohmic heating process including voltage gradient, average current values, frequency, heating rate and period, maximum temperature and holding time [8],[9],[10]. Internal and external factors have synergistic effect on microbial inactivation of the ohmic heating process.

A literature work comparing the microbial inactivation via exponential decay and square wave form pulsed electric fields states that inactivation success also depends on the wave form. Results of the pulsed electric field (PEF) applications on *Saccharomyces cerevisiae* suspended in the apple juice indicate that the square wave form pulses enable higher inactivation success as well as notable energy savings compared to the exponential decay wave form pulses. The authors also revealed that under identical peak electric field and energy input parameters, oscillatory decay pulses lead to considerably lower inactivation success with respect to exponential decay wave form[11]. Another paper focusing on the PEF application for the microbial inactivation of the food products also compared the effect of the wave forms. Regarding studies performed on the orange juice samples, three different pulse forms such as; square wave, exponential decay, under damped RLC has been used. The authors suggests that in contrary to the square wave form pulses, both exponential decay and under damped RLC pulses are not able to sustain target electric field densities during the application period. As a result, square wave form pulses are found to be the most effective for obtaining required level of pasteurization [12]. During PEF applications, bacterial cells which are suspended in the treated matrixes may be exposed to reversible or irreversible electroporation. In theory, electroporation occurs once the applied electric field strength exceeds the critical threshold to increase the trans membrane potential of the cell to an extent which results in the permeability of the membrane with consequent pores. Hence, the cell loses its homeostasis while allowing membrane impermeable molecules to flow into the cell as well as outflow of the cell structures[13]. Literature review suggests that electroporation occurs as a result of the electrical pulses. Besides, permeabilization strength is stated to be strongly correlated with the shape of the applied electrical pulses[14]. A PEF study performed on the vegetative cells, germinated and dormant spores of *Bacillus atrophaeus* demonstrates that the vegetative cells to be destroyed whereas germinated spores to be more resistant than vegetative cells under identical application procedures. On the other side, under all application parameters, no significant reduction of the dormant spores to be obtained [15]. Some research results indicate that spores are resistant to heat and PEF applications [16],[17],[18],[19]. Strong heat resistance of the spore is attributed to the vegetative cell losing significant portion of its water content during sporulation which results in the core to have a considerably low water content [20],[21]. On the other side, the mechanism which enable the spore to be resistant against PEF has not been clarified yet. PEF applications are mainly considered to inactivate vegetative cells by electroporation and electrical breakdown mechanisms which are not found to be effective for the destruction of the spores. Some paper on this topic demonstrate that even though PEF application may cause cracks on the spore surface, this will not be sufficient for spore inactivation [22],[23]. Some authors claim that as a result of the complex multiple layered and significantly dehydrated protective structure of the dormant spore, the interaction between extracellular material and the spore core is not maintained, thereby preventing the potential destructive effect of the PEF application on the spore [24]. Another approach is that the spores having smaller size with respect to their vegetative cells tend to be less sensitive to electroporation related damages [25]. Numerous papers on the inactivation of the vegetative cells and the germinated spores via PEF treatment declare that electric field strength and number of pulses to be the primary effective factors. Besides there are other parameters which affect the inactivation efficiency of the PEF such as; wave form of the pulses, frequency, width and treatment temperature [26],[27],[28],[29]. In brief, literature review regarding PEF applications generally indicate that considerable inactivation success on vegetative cells are obtained. Despite the fact that germinated spores are also inactivated, those still tend to be significantly more resistant to PEF with respect to vegetative cells. The concept of the germinated spores to be a laboratory scale condition because the environmental conditions to be either compelling enough to keep them to be dormant or

sufficiently nutritious to ensure germination followed by outgrowth. Evaluated papers suggest that PEF is not effective on the destruction of the dormant spores. Last but not least, wave form of the pulses has been stated among the factors that influence PEF inactivation success on the vegetative cells.

Various literature work on the ohmic heating draws attention to considerable inactivation of vegetative cells as well as spores of some species due to the synergistic effect of unlethal temperature and electric fields.

In this work; a simple laboratory scale ohmic heating system utilizing square waveform pulsed, low frequency alternating signals has been prepared to investigate the microbial inactivation success on *Bacillus cereus* and *Clostridium spp* vegetative cells and spores. In contrary to conventional ohmic heating processes which use sinusoidal waveform alternating current coming from the public utility, this study aims to observe the effect of square waveform pulses on the microbial inactivation. On the other side, a literature study suggests that during ohmic heating of food products, the use of square wave form pulses can prevent electron build up and some undesirable electrochemical reactions to be occurred at the interfaces between the electrodes and the food samples[30]. This approach also promoted to perform the microbial inactivation experiments via ohmic heating with square wave form pulses.

For the laboratory scale application, a simple rectangular-trough design is preferred. A laboratory scale design with electrical circuit components and their functions will be described in detail in Part 2. Microbial analysis are performed both on the treated and untreated samples in triplicate. Obtained results are interpreted in terms of the reduction percentages by the use of average counts values of vegetative cells and spores suspended in the treated and untreated biogas digestate samples. According to the obtained results, square wave form pulsed ohmic heating to have significant inactivation effect on both vegetative cells and spores of *Clostridium spp*. Application at 2.72 V/cm voltage gradient and 50 Hz frequency accompanied by 70°C target product temperature with 5 minutes and 15 minutes holding times lead to approximately same level of decrease in number of *Clostridium spp* spores and vegetative cells which are suspended in the digestate. Nevertheless, ohmic heating utilizing the same application parameters will not be an appropriate method for the inactivation of *B.cereus* spores and vegetative cells which are suspended in the digestate with the related composition and properties.

Literature review on ohmic heating suggests that existing work generally focuses on the effects of the voltage gradient, frequency and temperature parameters on the inactivation of microbial species which exist in various food matrixes. In our study, ohmic heating is applied to the biogas digestate which is originated from the chicken manure.

During anaerobic digestion of organic matter which is found in biomass, biogas which is mainly composed of CH_4 and CO_2 is produced to generate electricity and heat. The resulting slurry of this process is called as biogas digestate. Since the carbon and hydrogen content of the organic matter feedstock of the process is obtained as biogas, the digestate comprises the residual nutrients of the feedstock. Many applications demonstrate that digestate can be utilized as fertilizer in agriculture and its support effect on crop yields has been proven to be equivalent to many mineral fertilizers[31]. As the number of biogas plants increases,the quantity of the digestate used on agricultural land increases as well. Therefore, hygiene condition of the digested products eventually becomes a crucial subject. According to most of the studies regarding hygienization in biogas plants, treatment through biogas process can ensure reduction of some pathogen species. Nonetheless, due to their ability to form highly resistant structures under stress conditions, some of the persistent species manage to stay unaffected through anaerobic fermentation process. Rules and best practice recommendations regarding the use of digestate on agricultural area indicate that it shouldn't impose risk for humans,animals and plants as well [32]. During the sanitization process conventionally applied in biogas plants, the biomass is heated to the target temperature before being fed into the anaerobic digester and conserved at this temperature for a specified period of time. There are also applications in which the sanitization process is performed on the digestate. Many countries have set standards for the hygienization process. According to EU Regulation No. 142/2011; prior to anaerobic fermentation, the biomass which has maximum particle size of 12 mm, must be kept at 70°C for 60 minutes without interruption. EU countries also approve the application of alternative methods that are proven to have equivalent effectiveness to the standard pasteurization application of 70 °C-60 minutes. Pasteurization inactivates the vegetative cells of a significant quantity of the bacteria population in the biomass. However, pasteurization is not sufficient to destroy bacteria which are resistant to heat treatment at such temperature, particulary spore-forming species and their spores. This conventional pasteurization treatment is expected to destroy gram-negative bacteria ,while the heat resistant enzymes of gram-positive bacteria remain viable. Advanced heat treatments such as HTST and UHT are required to eliminate gram-positive bacteria. Our study presented in this paper suggests an approach to a hygienization application utilising ohmic heating which aims to inactivate both vegetative cells and spores, providing an alternative to conventional pasteurization for use in biogas plants.

Conventional ohmic heating processes generally utilize sinusoidal waveform alternating current coming from the public utility. In our study, the effect of square waveform pulsed ohmic heating on *B.cereus* and *Clostridium spp* vegetative cells and spores suspended in the biogas digestate is investigated.

2 Material method

2.1 Basic principles of the ohmic heating and the laboratory scale set up design

Ohmic heating set up basically consists of a product with conductive property which acts an electrical resistor and the electrodes which are in contact with the product. Determined voltage is applied between the metal electrodes via power supply unit (PSU) which is connected to the grid. Ohmic heating fundamentally depends on the Ohm's Law which is given in Equation (1), determines the relationship between voltage, current and resistance in a circuit.

$$\mathrm{I} = \frac{V}{R} \tag{1}$$

I: Current (A)

V: Voltage (V)

R: Resistance (Ohm)

The heat generated by this method is defined by Joule's Law which is given in Equation (2). This law states that when an electric current passes through a material having an electrical resistance, electrical energy is converted into heat energy, thereby increasing the temperature of the material.

$$Q = I^2RT \quad (2)$$

Q: The amount of heat (Joule)

I: Electric current (A)

R: Amount of electrical resistance in the conductor (Ohm)

T: Time (s)

Schematic of the laboratory scale set up design is given in Figure 1.

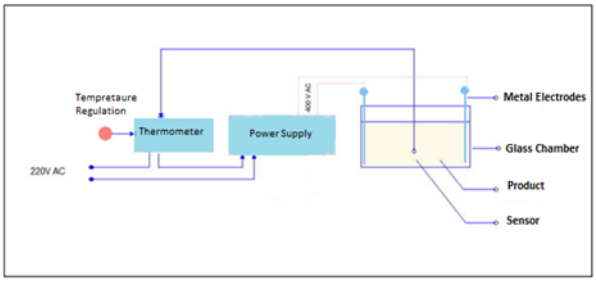

Figure 1. Schematic of the laboratory scale set up.

Ohmic heating chamber and electrodes allocation can have various types of design. Chamber and electrodes geometry can be adjusted to obtain target voltage gradient across the product. For instance, a literature study utilizes zig-zag shaped sequential elbow type electrodes design to enhance continuous ohmic heating efficiency[33]. In this study, biogas digestate which will be processed via ohmic heating is placed in a rectangular-trough glass chamber. Stainless steel electrodes that are shaped in accordance with the chamber geometry to be mounted at the ends of the rectangular trough heating chamber. Voltage will be applied to the electrodes that are connected to the power supply unit. Electrodes to be immersed in the product and be in contact with it. Temperature sensor which is thermocouple will be mounted in the middle of the two opposing electrodes, submerged in the product with its tip towards the bottom of the chamber. Minimum and maximum product temperature settings will be made at the thermometer device. Thermometer device which is connected in series with the circuit has relay contact allowing either open or close the circuit in accordance with the signals received from the thermocouple. When the product reaches the maximum target temperature, the relay will be opened and the power supply unit to be de energized. The oscilloscope is connected to the power supply output to monitor the actual wave form. Ohmic heating systems can be operated in a continuous or batch mode. In this work, experiments are performed under batch type operation. The glass chamber is filled with 500 mL of the biogas digestate for each trial and discharged once the operation is completed. Heating chamber is insulated with styrofoam to minimize heat and evaporation related losses. Heating chamber used for ohmic heating of the biogas digestate is given in Figure 2.

Figure 2. Heating chamber.

Mixing can be applied in order to prevent the formation of hot and cold spots within the product. In this respect, appropriate mixing can enhance microbial inactivation success by improving thermal distribution within the product. Biogas digestate has a heterogenous structure. On the other side, bacteria can be sensitive to mechanical stress and develop unanticipated behavior towards mixing which can mask the sole effect of ohmic heating on the microorganism. Thus, digestate will not be mixed before or during the ohmic heating trials.

2.2 Electrical system and electrical circuit design

Prior to the first trials, electrical circuit design is simulated on computer. The circuit is introduced to the computer by list of texts and the program processes the data and provides the results. Obtained signals are captured by the digital oscilloscope so that the wave forms are visualized.

2.2.1 Square wave pulse signal

Ohmic heating applications are performed using square wave pulses at 2.72 V/cm voltage gradient, 50 Hz frequency and 70°C target product temperature with 5 minutes and 15 minutes holding times. Square wave pulse signal is given in Figure 3.

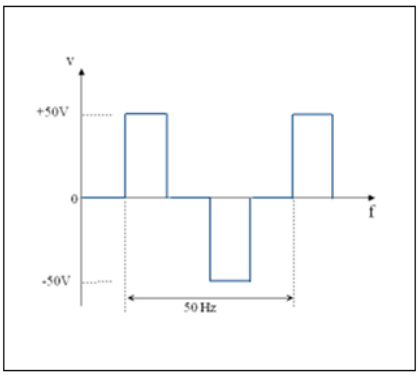

Figure 3. Square wave pulse signal.

The peak value of the pulses applied between the two electrodes from the PSU is 50 V AC. $V_{effective}$ value which is obtained during the square wave pulsed ohmic heating application is 35.46 V AC. The oscilloscope image of the PSU output is given in Figure 4.

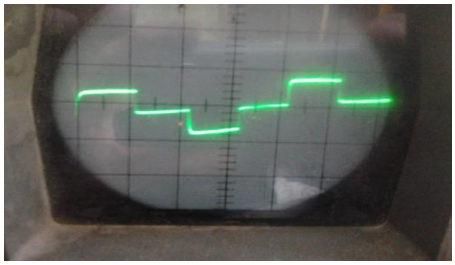

Figure 4. Oscilloscope image of the PSU output.

2.2.2

50 Hz alternating current has 20 milliseconds period. During 20 milliseconds period, there are two pulses one of which occurring at the positive direction of the axis and the other at the negative direction of the axis.

2.2.3 Electrical circuit and equipment of the ohmic heating system

Ohmic heating equipment set up is demonstrated in Figure 5. The system utilizes square wave form pulses.

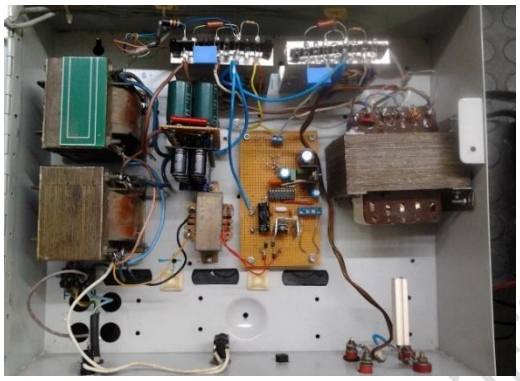

Figure 5. Ohmic heating equipment set up.

PSU is connected to the grid. There are three transformers in the circuit. The first transformer reduces 220 V AC whereas required level V DC is obtained via diode, capacitors and regulator and given to the microprocessor. The microprocessor runs the program consisting of the instructions set. In accordance with the program, square wave form pulses having 50 Hz and 20 milliseconds period is obtained from the microprocessor output. The second transformer reduces 220 V AC whereas required level V DC is obtained via diode and capacitors used in the circuit and it is used to supply the power transistors. The third transformer transfers the PEF signal obtained from the power transistors to the output. Diodes used in the circuit ensures AC/DC conversion. Power transistors enable to obtain square waveform pulses from the output of the microprocessor as well as ensuring transmission to the transformers. In the circuit, the 0.22 Ω resistance which is in series with the digestate is connected to the ends of the voltmeter and the current passes through it. The circuit also includes the oscilloscope as well as the frequency meter.

2.3 Samples

Samples used in this study are obtained from a biogas plant. The feedstock of the plant consists of chicken manure and chicken tissue waste. Samples are taken from the digester output before entering the decanter. pH (21°C) of the digestate samples generally varies between 7-7.5. The digestate approximately has 96% water content (w/w). The analysis results indicate that the digestate mainly has the following content such as; organic matter, organic carbon, humic acid, fulvic acid, organic and inorganic nitrogen, phosphorus pentoxide, potassium oxide, calcium oxide, magnesium oxide, iron, manganese and free amino acids with variable ratios depending on the process feedstock. To sustain the process stability as well as to enhance the efficiency, biogas plants can utilize co-fermentation and/or controlled additives application. Therefore, the content of the samples taken from a biogas plant can fluctuate in accordance with the tolerance ranges for fermentation. Consequently, the results obtained during this study is specific to the composition of the digestate samples originated from the related feedstock composition. Physical and chemical analysis of the digestate samples are given in Table 1.

Table 1. Physical and chemical analysis of digestate.

Analysis Paremeters	(Unit)	w/w (Analysis results)
pH (21 °C)	--	**7,3**
Density	g/cm³	**1,02**
Organic Matter (70 °C- 550 °C)	(%)	**2,6**
Humidity (70 °C)	(%)	**95,6**
Organic Carbon	(%)	**0,38**
Total Humic Acid+ Fulvic Acid	(%)	**0,49**
Organic Nitrogen	(%)	**0,41**
Total Nitrogen (N)	(%)	**0,48**
Nitrate Nitrogen (NO_3-N)	(%)	**<0,5^{RL}**
Ammonium Nitrogen (NH_4-N)	(%)	**<0,5^{RL}**
Total Phosphorus Pentoxide (P_2O_5)	(%)	**0,22**
Neutral Ammonium Citrate and Water Soluble Phosphorus Pentoxide (P_2O_5)	(%)	**0,22**
Phosphorus Pentoxide, only soluble in mineral acid (P_2O_5)	(%)	**0,0**
Total Potassium Oxide (K_2O)	(%)	**0,24**
Water Soluble Potassium Oxide (K_2O)	(%)	**0,23**
Total Calcium Oxide (CaO)	(%)	**0,53**
Water Soluble Calcium Oxide (CaO)	(%)	**0,08**
Total Magnesium Oxide (MgO)	(%)	**0,06**
Water Soluble Magnesium Oxide (MgO)	(%)	**0,04**
Total Iron (Fe)	(%)	**0,03**
Water Soluble Iron (Fe)	(%)	**0,002**
Total Manganase (Mn)	(%)	**0,003**
Water Soluble Manganese (Mn)	(%)	**<0,001^{RL}**

RL:Reporting Limit

Samples which are used during this study has water content of 95,6% (w/w). Due to high water content of the digestate,rapid moist heating concept which is considered to have significant effect on bacterial spore inactivation is obtained. Spores in high viscous matrixes are accepted to be protected from ohmic heating effects, thereby being highly resistant. Thus, high water content of the digestate is assumed to be advantageous in terms of providing low viscous environment.

pH is among the critical factors which effect success of microbial inactivation during moist heat applications [34]. Digestate utilized for this study, has relatively high pH. Due to the fact that, some species are adversely effected by low pH environment, inactivation results may change for digestate with lower pH values.

Free amino acids, organic nutrients and cations such as; ammonium, calcium, magnesium etc. which are present in the digestate content, may act as germination triggering agents for bacterial spores during ohmic heating, thereby effecting the inactivation success. Same components may also support outgrowth of vegetative cells, therefore effecting the obtained

results. Biogas digestate composition contains a mixture of unionized ammonia in equilibrium with ammonium ion. When the temperature and pH of the digestate are increased, this equilibrium is expected to shift to the advantage of free ammonia which causes not only the loss of the nutrient content of the digestate but also release of undesired ammonia emissions [34]. Therefore; due to relatively high pH and ammonia content of the utilised digestate samples, target temperature is determined not to exceed 70°C and holding times are minimized. Ionic content of the digestate has an impact on the electrical conductivity property. To perform ohmic heating applications, appropriate electrical conductivity ranges needs to be obtained. In this concept, though some moderate fluctuate may be encountered, digestate composition ensures required electrical conductivity ranges.

In literature, the presence of heavy metals have been reported to inhibite the activation of some spore species [34]. Thus, heavy metal concentration of the digestate to be processed via ohmic heating is among the important factors to sustain the proposed inactivation mechanisms of the spores.

According to some research, additional chemical effects that ocur during ohmic heating may contribute to microbial inactivation. It is anticipated that, due to formation of free oxygen and hydrogen, chloride, hydroxyl and hydroperoxide radicals along with metal ions, inactivation effect may be enhanced[34]. Therefore, digestate composition which is dependent on feedstock as well as digester operating conditions, may influence the achieved degree of microbial inactivation.

2.4 Microbial analysis method

Microbiological analysis are performed on the triplicate samples before and after the ohmic heating process. Initial and final states of vegetative cell and spore counts as well as reduction ratios of *Clostridium spp* and *Bacillus cereus* species in the untreated and ohmic heated samples are indicated.

Microbiological analysis procedure which is applied for *Clostridium spp* is provided below;

- 10 g of each digestate sample is weighed under aseptic conditions and then diluted in a ratio 1:10 with the addition of 90 mL Maximum Recovery Diluent. Obtained suspension is homogenized by Stomacher.
- Serial dilutions are prepared.
- Pour plate procedure is applied to inoculate each dilution into Iron Sulfite Agar medium. Pour plates are incubated anaerobically at 37°C for 24 hours.
- Following incubation, typical black colored *Clostridium* colonies are counted and expressed as colony forming units per gram, (cfu/g) based on the dilution factor.

To count the number of *Clostridium spp* spores;

- Homogenate is incubated in a water bath at 80°C for 15 minutes to inhibit Clostridium spp vegetative cells.
- Afterwards, by applying the same procedure steps as vegetative cells counting, the number of Clostridium spp spores are counted.

Microbiological analysis procedure which is applied for Bacillus cereus is provided below;

- 10 g of each digestate sample is weighed under aseptic conditions and then homogenized with Maximum Recovery Diluent.
- Homogenate is incubated in a water bath at 80°C for 15 minutes to inhibit vegetative cells.
- Serial dilutions are prepared.
- Using standard plating method, 0.1 mL of the prepared dilutions is dispensed into two parallel petri dishes and transferred to the previously poured Mannitol Egg Yolk Polymyxin Agar plates and spread homogenously with a sterile L stick.
- Following 15 minutes period to allow the medium to absorb the inoculum, the petri dishes are turned upside down and incubated at 30°C for 18-24 hours.
- At the end of the incubation period, petri dishes containing 15-150 colonies are counted. Due to lecithinase activity, *B.cereus* colonies are identified as pink, large colonies surrounded by a precipitation zone.

3 Research findings and discussion

3.1 Ohmic heating process parameters

Prior to the outdoor experiments, initial temperature of the samples are recorded as 21°C. The power unit is started and allowed to run until the samples reached the target temperature 70°C. To retain the sample temperature at 70°C, power unit can be re-energized and run according to signals received from the thermometer. One trial includes 5 minutes holding time at 70°C whereas during the other trial, the samples are hold at 70°C for 15 minutes. $V_{effective}$ which is applied between the electrodes is 35.46 V. Frequency is 50 Hz. Voltage gradient between the electrodes is 2.72 V/cm. Sample height dependent electrode surface area is 0.00425 m^2. Data regarding sample temperature and average voltage on 0.2 ohm resistance V(0.2) versus time is provided on Table 2. Current and electrical conductivity of the sample versus time are provided on Table 3.

Table 2. Ohmic heating process parameters.

t (min)	$V_{0.2}$ (Volt)	T(°C)
0	0.45	21
2	0.49	25
4	0.53	29
6	0.55	32
8	0.58	36
10	0.60	40
12	0.62	45
14	0.65	50
16	0.68	55
18	0.70	59
20	0.72	62
22	0.73	65
24	0.74	69

26	0.74	71
28	0.75	75

Table 3. Ohmic heating process parameters.

t (min)	I (A)	σ (S/m)
0	2.25	1.941
2	2.45	2.113
4	2.65	2.286
6	2.75	2.372
8	2.90	2.502
10	3.00	2.588
12	3.10	2.674
14	3.25	2.803
16	3.40	2.933
18	3.50	3.019
20	3.60	3.105
22	3.65	3.149
24	3.70	3.192
26	3.70	3.192
28	3.75	3.235

3.2 Microbiological analysis

The results of the microbiological analysis on the untreated and ohmic heating treatment applied samples are given on Table 4, Table 5, Table 6 and Table 7. Log reductions achieved for average CFU/mL counts of *Clostridium spp* vegetative cells are given on Table 4 and *Clostridium spp* spores are given on Table 5. Results obtained on *B.cereus* vegetative cells and spores are given on Table 6 and Table 7 respectively. A1, A2, A3, C1, C2, C3 represents the identical treated sample groups. Ohmic heating applications utilizes square waveform pulses accompanied by 5 minutes and 15 minutes holding times at 70°C target temperature.

Table 4. Results for *Clostridium spp* vegetative cells.

Samples	*Clostridium spp.* Vegetative (CFU/mL)	Mean	Log Reduction
Untreated	$8.5x10^5$	$8.5x10^5$	1.650
A1- 5 min	$1.9x10^4$	$1.9x10^4$	
A2- 5 min	$1.7x10^4$		
A3- 5 min	$2.2x10^4$		
C1-15 min	$1.2x10^4$	$1.6x10^4$	1.725
C2-15 min	$1.1x10^4$		
C3-15 min	$2.7x10^4$		

Table 5. Results for *Clostridium spp* spores.

Samples	*Clostridium spp*. Spores (CFU/mL)	Mean	Log Reduction
Untreated	$3.3x10^4$	$3.3x10^4$	0.987
A1- 5 min	$1.2x10^3$	$3.4x10^3$	
A2- 5 min	$5.2x10^3$		
A3- 5 min	$3.9x10^3$		
C1-15 min	$2.3x10^3$	$5.0x10^3$	0.819
C2-15 min	$6.3x10^3$		
C3-15 min	$6.5x10^3$		

Table 6. Results for *B.Cereus* vegetative cells.

Samples	*B.Cereus* Vegetative (CFU/mL)	Mean	% Increase
Untreated	$3.7x10^2$	$3.7x10^2$	55%
A1- 5 min	$4.2x10^2$	$5.7x10^2$	
A2- 5 min	$8.0x10^2$		
A3- 5 min	$5.1x10^2$		
C1-15 min	$3.9x10^2$	$4.5x10^2$	23%
C2-15 min	$4.2x10^2$		
C3-15 min	$5.6x10^2$		

Table 7. Results for *B.cereus* spores.

Samples	*B.Cereus* Spores (CFU/mL)	Mean	%Reduction
Untreated	$9.0x10^1$	$9.0x10^1$	18%
A1- 5 min	$5.0x10^1$	$7.3x10^1$	
A2- 5 min	$7.0x10^1$		
A3- 5 min	$1.0x10^2$		
Untreated	$9.0x10^1$	$9.0x10^1$	%Increase
C1-15 min	$2.0x10^2$	$1.2x10^2$	37%
C2-15 min	$7.0x10^1$		
C3-15 min	$1.0x10^2$		

3.3 Statistical analysis

All of the experiments are carried out independently in triplicate and three analyses per replication at least were done. The significant differences in the experimental data among the 5 minutes and 15 minutes holding times inactivation treatments on *Clostridium spp* vegetative cells and spores to be evaluated using paired samples t -test. Two tailed t-test is performed to identify the p value between the two groups. Regarding tests, two-sided p value which is less than 0.05($p<0.05$) is accepted to be statistically significant whereas p value which is higher than 0.05 is evaluated as the difference between the two groups is statistically insignificant. The tests are performed to compare the log reductions achieved for *Clostridium spp* vegetative cells and spores during 5 minutes and 15 minutes holding times which are presented on the Table 8 and Table 9 respectively with standard deviation and variance values as well. A1, A2, A3 represents the identical sample groups.

Table 8. T-test results for *Clostridium spp* vegetative cells.

Log reduction ±SD	*Clostridium spp* vegetative 5 minutes	*Clostridium spp* vegetative 15 minutes
A1	1.650	1.850
A2	1.699	1.888
A3	1.587	1.498
SD	0.056	0.215
Variance	0.003	0.046
p two tailed	0.401	

The two tailed p value is calculated as 0.401. According to the conventional criteria, the difference is statistically not significant. Thereby, it can be concluded that application of 15 minutes duration instead of 5 minutes period of ohmic heating doesn't have a significant impact on the inactivation of *Clostridium spp* vegetative cells suspended in the sample biogas digestate.

Table 9. T-test results for *Clostridium spp* spores.

Log reduction ±SD	*Clostridium spp* spores 5 minutes	*Clostridium spp* spores 15 minutes
A1	1.439	1.156
A2	0.802	0.719
A3	0.927	0.705
SD	0.337	0.256
Variance	0.113	0.065
p two tailed	0.068	

The two tailed p value is calculated as 0.068. According to the conventional criteria, the difference is statistically not significant. Thereby, it can be concluded that application of 15 minutes duration instead of 5 minutes period of ohmic heating doesn't have a significant impact on the inactivation of *Clostridium spp* spores suspended in the sample biogas digestate.

3.4 Energy consumption

According to the system design, power unit is allowed to run until the samples reach the target temperature. As soon as the target temperature is obtained, power unit is stopped. The heating chamber is insulated to minimize the heat losses. As a result, during the 5 minutes holding time at the target temperature, no significant temperature drop has been observed. Therefore, it is not required to re-energize the power unit so there has been no additional energy consumption of the system during 5 minutes holding time.

Electrical energy consumed during the microbial inactivation experiments via ohmic heating utilizing square wave form pulses with 5 minutes holding time is calculated. Each batch processed 500 g digestate sample. Calculation is based on Equation (3).

$$P = I_{average} V \quad (3)$$

P= 3.13*35.46 =111.192 Watt

P : Power (Watt)

$I_{average}$: Average current (A)

V : Voltage (V)

Alternating current has 20 milliseconds period with two pulses one of which is at the positive direction and the other one occurring at the negative direction of the axis. During 26 min period for the sample to reach approximately 70°C, pulsed voltage is applied for 13 min, remaining 13 min to be the gap period. Accordingly, energy input in kWh is calculated as 0.0240 kWh/ 500 g.

3.5 Interpretation of microbial analysis results

Mechanisms which take part in the microbial inactivation via ohmic heating have still not been fully explored yet. Due to their complex and protective structure, determination of the inactivation mechanisms of bacterial spores should become a future research subject. Though, this paper aims to present possible inactivation mechanisms as a review of our literature research. Analysis results regarding to 5 minutes holding time at 70°C indicate that 97.72% and 89.5% decrease in the number of *Clostridium spp* vegetative cells and endospores is achieved respectively. Due to the impact of square wave form pulsed ohmic heating, it is hypothesized that the proteins which are located on the spore coat and responsible for retaining the dormant state undergo reversible denaturation along with structural changes in the macromolecules. As a result, permeability of the spore to be triggered to some extent. Therefore, it is estimated that the synergistic effect of unlethal temperature and low voltage electric field with square wave form pulses promote the penetration of heat and electric field effects into the inner parts of the spore structure.

3.5.1 General acknowledgment suggests that similar to the vegetative cell membrane, both inner and cytoplasmic membrane of the spore damages due to heat. Therefore, average heat treatment provided during OH to be sufficient to obtain permeability of the outer membrane of the spore.

As mentioned above, permeability of the coat can also be triggered due to synergistic effect of unlethal temperature and low voltage electric field. Accordingly, heat and square wave form pulses which reach cortex, also estimated to trigger the permeability of this section to some extent. Consequently; the spore core is rehydrated via intake water from the digestate. Rehydration degree will be in accordance with the water activity difference of the spore core and the digestate. It is concluded that 89% of the *Clostridium spp* spores to be subject to degradation of their structural integrity as a result of OH treatment with 5 minutes holding time at 70°C, accompanied by other specified process parameters.

Analysis results regarding to 15 minutes holding time at 70°C indicate that 98% and 84% decrease in the number of *Clostridium spp* vegetative cells and endospores is achieved respectively. It is observed that increasing holding time to 15

minutes doesn't have a significant effect on the reduction of vegetative cells of *Clostridium spp*. 15 minutes holding time instead of 5 minutes has seen to decrease the reduction ratio of the *Clostridium spp* spores. However, if two results to be evaluated in terms of log reduction, there is no significant difference. 5 minutes holding time results in 0.98 log reduction for *Clostridium spp* spores whereas 15 minutes holding time results in 0.81 log reduction. Even if the temperature of the digestate doesn't reach the boiling point, increased temperature leads the bond structure of water content in the product tend to open and some fractions begin to demonstrate vapor characteristics. Such fractions will be able to penetrate through the inner parts of the spore structure. Thereby, spore core is hypothesized to be rehydrated to some extent which decreases heat resistance of the spores. According to the analysis results regarding to 5 minutes holding time at 70°C; 55% increase in the count of *B.cereus* vegetative cells and 18% decrease in the number of *B.cereus* spores is recorded. A slight decrease in the number of spores accompanied by a significant increase in the number of vegetative cells suggests that this ohmic heating application might have triggered germination and following outgrowth of *B.cereus* spores in the digestate. The result to be interpreted as some of the *B.cereus* spores in the digestate have completed germination process which is followed by outgrowth. Based on literature research, it is hypothesized that germination may have occurred due to the following conditions;

- Square wave form pulsed ohmic heating to induce activation of *B.cereus* spores and enhance germination rate of the vegetative cells.
- Germination specific lytic enzymes to be activated as a consequence of the collapse of the spore cortex. According to some studies, heat activation and electrical pulses application can result in Ca-DPA leakage from the spore core. Calcium which is originated from the Ca-DPA leakage is responsible for the cortex collapse.
- During the application of square wave form pulsed ohmic heating, cortex lytic enzymes belonging to *B.cereus* spores to be activated. Cortex lytic enzymes which are located on the spore coat, are assumed to have direct role on the spore germination process.
- Activated cortex lytic enzymes; CwlJ and SleB are supposed to trigger the degradation of spore's cortex peptidoglycan. At the same time, activated lytic enzymes enhance germination to be completed as well as supporting transition to the outgrowth phase [35].
- During the application of square wave form pulsed ohmic heating, spore enzymes to be activated.
- Free amino acids, organic nutrients and cations such as; ammonium, calcium, magnesium etc. are present in the digestate content. When the spores which are suspended in the digestate are activated, related digestate content may act as germination triggering agents with respect to their binding affinities with the spore's germination receptors.
- There is a weak electrostatic interaction between the germination enzymes and the spore core. During the spore activation, germination enzymes are released from the breaking bonds.
- During heat activation of the spores, due to the change in the permeability of the spore coat and breaking of the bonds between DPA and enzymes, DPA release occurs. It is hypothesized that DPA content of the core is reduced to the critical threshold level at which germination can begin [36].
- During heat activation of the spores, protease enzyme is activated and amino acids such as l-alanine which is known to be a germination triggering agent for *B.cereus* spores, is released [37].

Analysis results regarding to 15 minutes holding time at 70°C indicate that 23% and 37% increase in the count of *B.cereus* vegetative cells and spores are recorded respectively. It is observed that the increase ratio of vegetative cells during 15 min holding time to be less than the ratio obtained via 5 minutes holding time. During the trials, power unit is allowed to run till the samples reach the target temperature and if required, it is re energized to retain the sample temperature at 70°C. The power unit runs according to the signals received from thermometer.

3.5.2 Due to turn on/off mode, during the holding time period of 5 minutes or 15 minutes, the effect of temperature will be much more significant than electrical pulses. Therefore, 10 minutes additional holding time will cause *B.cereus* vegetative cells to be exposed to additional heat effect accompanied by increased reduction ratio with respect to 5 minutes holding time. According to the obtained data, the effect of additional holding time with turn on/off mode brings about additional heat effect primarily which in turn increases the reduction ratio of the vegetative cells of *B.cereus*. Due to the fact that the rate of germination which is then followed by outgrowth is higher than the inactivation rate of ohmic heating on the vegetative cells, the number of *B.cereus* living cells is increased as a result of ohmic heating which is applied on the digestate with related operating parameters. Besides, 70°C - 15 min application resulted in higher inactivation on *B.cereus* vegetative cells with respect to 70°C- 5 min which is attributed to the extended heat exposure.

On the other side, 70°C - 15 min application resulted in 37% increase in the number of *B.cereus* spores in the digestate. This result might indicate that extension of the heat application period to cause stress on the vegetative cells of the *B.cereus*, thereby triggering sporulation. At this point, it can be concluded that there might be a threshold period during which the transition to sporulation occurs at 70°C application.

During this study, low voltage is applied between the electrodes. Voltage level selection is dependent on the equipment design and the ammonium-ammonia equilibrium in the digestate. Due to the fact that, the equipment is a small laboratory scale design, it is preferred to work under low voltage gradients. Higher voltage gradients could enhance the microbial inactivation degrees, nevertheless such system will require an electrical circuit with much bigger size transformers which will not be applicable for a laboratory scale work. Increase in the final product temperature could also improve the microbial inactivation degree. However, final product temperature as 70°C is determined in accordance with the pH value to obtain increased nutrient retention with minimum ammonia emissions during the heating period.

4 Results

As a result of this study, a laboratory scale ohmic heating set up operating with low voltage and low frequency as well as utilizing square waveform pulses is designed. The obtained ohmic heating system design is applied to the biogas digestate with operating parameters 70°C target temperature, 2.72 V/cm voltage gradient, 50 Hz frequency, $V_{effective}$: :35,46 V AC, square waveform AC with 20 msn period cycle. Applications with 5 minutes and 15 minutes holding times at the target temperature resulted in different responses of each reference microbial species. Obtained microbiological analysis suggests that, ohmic heating with the provided operating parameters can effectively inactivate both vegetative cells and spores of *Clostridium spp* which are suspended in the related biogas digestate composition, thereby can be utilized for hygienization of the digestate in terms of *Clostridium spp*. In contrary, identical ohmic heating parameters have an opposite effect on the vegetative cells and spores of *B.cereus* suspended in the same environment.

4.1.1 Each research on the microbial inactivation of ohmic heating should be evaluated as a specific case. Assessment needs to be done by considering both internal and external factors as well as their interactions with each other. Therefore, the results obtained in this study are specific to digestate based internal factors as well as applied process parameters.

In literature, mechanisms which take part in the microbial inactivation via ohmic heating have still not been fully explored yet. Due to their complex and protective structure, determination of the inactivation mechanisms of bacterial spores should become a future research subject.

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6 Author contribution statements

In the scope of this study, 1 st author contributed to the preparation of this paper in terms of formation of the idea, performance of the literature review and the experiments, the assessment of the obtained results. 2nd author contributed to the critical evaluation of the scientific work and the paper.

7 Ethics committee approval and conflict of interest statement

There is no need to get ethics committee permission for the prepared article. There is no interest conflict with any person or institution in the prepared publication.

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