

## ANTIBACTERIAL EFFECT OF ZnO CRYSTALS ON FOODBORNE PATHOGENS: AN OPTIMIZATION STUDY

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### ABSTRACT

In this study, antibacterial effect of ZnO particles was investigated using the Taguchi method. ZnO crystals with different size, and BET specific surface areas were produced in the laboratory. Bacteria of *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 15313) and *S. Enteritidis* (ATCC 13076) were developed and disk diffusion method has been used to determine antibacterial activity of ZnO crystals on these bacteria. ZnO crystals prepared were found to be effective on these bacteria, and comparative results were obtained. It has been determined that the crystal particles of different properties of the same material have different effects on the bacteria. The results show that the Sample N2 on *S. aureus* at 0.1 g.mL<sup>-1</sup> concentration was optimized.

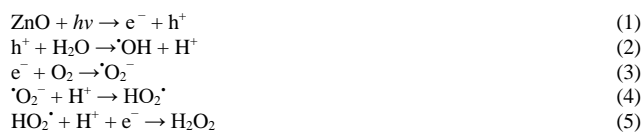
**Keywords:** Antibacterial effect, Zinc oxide, Taguchi, Design of Experiment

### INTRODUCTION

A large proportion of microorganisms including bacteria, viruses, molds, parasites, etc. lead to contagious epidemics. Bacteria such as Enterococcus, Staphylococcus, and Streptococcus are thought to cause very serious outbreaks worldwide (Hirota *et al.*, 2010). Microorganisms have undergone genetic changes under the influence of technological, economic and cultural developments. It is also known that microorganisms increase resistance to antibiotics being used. For this reason, it is necessary to develop methods to combat microorganisms. The use of metal oxides on microorganisms is also one of these methods. It is also an advantage to have different applications of metal oxides at the same time (Frederickson *et al.*, 2005). The antibacterial effect of a substance is expressed as inhibitory effect of its on bacteria's growth. To talk about the antibacterial effect of a substance, the substance must either kill the bacteria or stop its development. These substances can be organic or inorganic compounds. Organic and inorganic antibacterial agents are compared, the most important advantages of inorganic compounds over organic compounds are that they are more resistant to high temperature and pressure during processing, are effective even at low concentrations, and have a long shelf life. There are many inorganic materials showing antibacterial properties: Silver (Ag), Titanium (Ti), Zinc (Zn), Magnesium (Mg), and Calcium (Ca) etc. Besides, they must never show the toxic properties. Therefore metal oxide materials, are showing antibacterial properties, are used in a wide range of areas, which have positive effects on human health (Polat & Fenercioğlu, 2014; Raghupathi *et al.*, 2011). Among them, zinc oxide (ZnO) is an antibacterial material frequently used in (Rosi & Mirkin, 2005). ZnO is one of the five zinc compounds listed on the list of safe additives by the US Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) (Premanathan *et al.*, 2011). In result of studies on the mechanism of antibacterial effect of ZnO, three basic theories have been stated:

The first of these theories is the production of reactive oxygen species (ROS). It was known that the aqueous suspensions of ZnO, increase the amount of ROS, mostly hydroxyl radicals, hydrogen peroxide, and free oxygen. In this situation, it is thought that the antibacterial effect of ZnO is caused by the damage generated by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on microorganisms (Ohira *et al.*, 2008; Sirelkhatim *et al.*, 2015). The process has defined in the literature as follows: when exposed the ZnO to the ultraviolet and visible light, electrons and electron holes (e<sup>-</sup>, h<sup>+</sup>) are formed (Eq. 1) and the formed hole breaks down the bond of the H<sub>2</sub>O molecule then cause OH<sup>-</sup> and H<sup>+</sup> radicals have formed in the ZnO suspension (Eq. 2). Dissolved oxygen molecules in suspension are converted to superoxide radical anions (•O<sub>2</sub><sup>-</sup>) (Eq. 3). The anion reacts with H<sup>+</sup> to form the HO<sub>2</sub>• radical (Eq. 4). Subsequently, it reacts with hydrogen ions to form the H<sub>2</sub>O<sub>2</sub>

molecule (Eq. 5). The formation of ROS from ZnO particles is given by the equations as below.



Sawai and colleagues investigated the antibacterial effect of ZnO on *E. coli* and the role of H<sub>2</sub>O<sub>2</sub> in the mechanism of the antibacterial effect. They have found that with the increasing concentration of H<sub>2</sub>O<sub>2</sub>, the killing effect on bacteria increases. As a result of the experiments, it was observed that ZnO and H<sub>2</sub>O<sub>2</sub> showed parallel results, thus it was thought that the antibacterial effect of ZnO is caused by the mechanism of H<sub>2</sub>O<sub>2</sub> production (Sawai *et al.*, 1998).

In fact, in 1996, Sawai and colleagues conducted a study to prove the presence of active oxygen species produced by metal oxides. In their study, magnesium oxide, calcium oxide and zinc oxide were used as metal oxides. It was determined by oxygen electrode analysis that H<sub>2</sub>O<sub>2</sub> was produced from ZnO powders, and also the chemical luminescence method has been shown to produce active oxygen from all three metal oxide powders. In the chemical luminescence analysis, the resistance effect ordering was determined as CaO, MgO and ZnO, and this order is consistent with antibacterial effect sequencing. In this case it is understood that one of the causes of the antibacterial effect is also the produced active oxygen (Sawai *et al.*, 1996).

Zhang *et al.* have examined the antibacterial effect of ZnO nanoparticles on the presence of ZnCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. ZnO suspension of 1.25·10<sup>-3</sup> M resulted in a considerable decrease in bacterial colony count, whereas ZnCl<sub>2</sub> did not show antibacterial effect. H<sub>2</sub>O<sub>2</sub> has provided 100% reduction in bacterial count (Zhang *et al.*, 2010).

Yamamoto investigated the effect of different sizes of ZnO particles on *E. coli* and *S. aureus*. ZnO powder ranging in particle size 0.1-0.8 μm was prepared by heating ZnO to 1400 °C and crushing. Antibacterial activity has been determined by measuring the electrical conductivity. As a result of the study, it was determined that the antibacterial activity increased as the particle size decreased. This situation is explained by the increase in ZnO surface area in contact with microorganisms as the particle size decreases. It can also be explained as increase in the amount of H<sub>2</sub>O<sub>2</sub> produced from the ZnO surface. It has been observed that the effect of ZnO particle size is less on *S. aureus*. The reason is that bacterial cell surfaces are based on both different chemical composition and structure. On the cell surface of *E. coli*, there are three layers including lipid A, lipopolysaccharide and peptidoglycan, while only the peptidoglycan layer is present on the surface of *S. aureus* cell (Yamamoto, 2001).

Electrostatic interactions appear as the second theory in the literature. ZnO particles are bound to the bacterial cell membrane under the influence of electrostatic forces. In the meantime, ROS increase oxidative stress, causing great damage to the cellular structure, leading to cell death (Saliani *et al.*, 2016). The last theory, called release of Zn<sup>+2</sup> ions, is free Zn<sup>+2</sup> ions damage the cell structure. Zn<sup>+2</sup> ions have a significant effect on destroying bacterial cells by causing mechanical damage to the cell wall, leading to serious damage to the amino acid and enzyme structure (Sirelkhatim *et al.*, 2015; Song *et al.*, 2010).

The sensitivity of gram-negative bacteria to ROS is less than that of gram-positive bacteria. The fact that both cell walls have different polarities is one of the reasons for this situation; gram-positive bacteria have lower negative charge on the cell walls. Thus, free radicals, superoxide, and peroxide ions penetrate the cell better, easily damage the cell and cause cell death (Espitia *et al.*, 2012). Another reason for this is the structural differences in the structure of both cells. Gram-negative bacteria contain a three layer cell wall structure. In the top layer, there are membrane proteins, pores, phospholipids and lipopolysaccharides. Peptidoglycan is in the middle layer. In the bottom layer is a cytoplasmic membrane (phospholipid 40%, and protein 60%). Cause of electrostatically interaction between gram-negative bacteria and the ZnO particles is the lipopolysaccharides in the top layer of the cell wall. As a result of the electrostatic interaction, the cell wall begins to degrade; ion diffusion takes place and cell depletion occurs (Dutta *et al.*, 2012).

ZnO is an inorganic compound which is soluble in acid and alkaline but insoluble in water and alcohol, it looks like white powder. Produced mostly by combustion of zinc vapor, ZnO is used in many areas of the industry. Cosmetics such as moisturizers, makeup materials, creams, lotions, powders are produced using ZnO. The main reason for the use of ZnO is that it is especially effective in keeping contact with the skin of the cosmetic products. Its ability to effectively reduce UV radiation for UVA and UVB is another reason for its use (Moezzi *et al.*, 2012). It is known that in the food sector for protective purpose against foodborne pathogens and for preventing microbial contamination in the food packaging industry, in dental treatment materials, and in medical creams, it is used because of its antibacterial property (Galstyan *et al.*, 2018).

There are many methods applied in production of ZnO: hydrothermal synthesis (Yu & Yu, 2008), chemical vapours deposition (De Filipo *et al.*, 2018), sol-gel (Ivanova *et al.*, 2010), thermal decomposition (Sheikhshoae *et al.*, 2018), electrochemical (Jose *et al.*, 2018), chemical precipitation (Akin & Oner, 2012), etc. Among these methods, chemical precipitation method is preferred because it is a low cost and easily applicable method.

Antibacterial activity of a substance is expressed as the inhibitory effect on the bacterial growth or the killing effect on bacteria. For the measure of antibacterial activity, antibacterial susceptibility tests are used. Disc diffusion test is suitable for testing many pathogenic bacteria. It is the one of the oldest and most commonly used antibacterial susceptibility test methods. In addition, many antibacterial agents is suitable for the test and no special equipment is required. Paper discs impregnated with a certain amount of antibacterial agent are placed on the surface of agar plates spreading standard suspension prepared from the test microorganism. As the discs dissolve after a while and become diffused toward the agar, the inoculated microorganism also starts to multiply. At the end of a given incubation period, a circular inhibition zone occurs around which bacteria cannot reproduce. The more susceptible the microorganism is to the antibacterial substance, the greater the inhibition zone formed around the disc. The diameter of the inhibition zone is measured in mm and evaluated according to standard zone tables and the sensitivity of the microorganism to the antibacterial substances used is determined (Gülay, 2002).

Sirelkhatim *et al.* have examined the antibacterial effect of ZnO nanoparticles produced by the French process on *E. coli*. Particles with a diameter of 800 nm were produced by application at 700 °C for 1 hour. The main purpose of this study is to investigate the mechanism of antibacterial action and to evaluate the usability of ZnO nanoparticles for food-borne diseases and food packaging. The "tube dilution method" was used to determine the antibacterial activity. As the ZnO concentration increased, bacterial inhibition was increased. Field Emission Scanning Electron Microscopy (FESEM) images have been shown that ZnO nanoparticles did not penetrate into the cell membrane, but bacterial growth was inhibited. Thus, it has been understood that the antibacterial effect of ZnO is directly related to the production of ROS. This result shows that the toxic effect of ZnO nanoparticles is applicable only to microorganisms and that it can be used in terms of food safety (Sirelkhatim *et al.*, 2015).

Dutta *et al.* have investigated the antibacterial effect of thioglycerol-modified ZnO produced by wet chemical method against *E. coli*, a gram-negative bacterium. Studies to confirm that the antibacterial effect of ZnO nanoparticles originate from the produced ROS was made in the presence of histidine antioxidants. Histidine is known to be a cleansing agent for hydroxyl radicals and free oxygen and has no antibacterial activity. As a result of the studies carried out, it was observed that the antibacterial effect increased as histidine amount decreased (Dutta *et al.*, 2012).

In the study worked by Ohira *et al.*, the specific crystal structure of ZnO was obtained from an aqueous solution formed by mixing zinc chloride, tri-ethanol amine and thio-urea, and it has been compared with commercial ZnO with regard

to the antibacterial effect on *E. coli* and *S. aureus*. As the ZnO concentration increased, antibacterial activity has been increased for both ZnO. It has been determined that the antibacterial effect of commercial ZnO is much stronger. It has been thought that the cause of this difference is related to the surface area (Ohira *et al.*, 2008).

Jalal *et al.* examined the antibacterial effect of ZnO dispersed in glycerol on *E. coli*. Zinc acetate and sodium hydroxide have been mixed at room temperature and heated by microwave (2.45GHz, 850W) to synthesize ZnO. The antimicrobial effect of prepared ZnO was determined by disk diffusion method. As concentration and application time increased, it has been observed that ZnO was a very good antibacterial agent (Jalal *et al.*, 2010).

Zhang *et al.* have investigated the effect of zinc oxide particle size and concentration on the antibacterial activity. *E. coli* was selected as the target microorganism. Experiments have shown that the antibacterial activity increases with increasing the ZnO particle concentration and with decreasing the particle size. When ZnO (2417 nm) with the largest particle size was used, antibacterial activity was observed to be negligible (Zhang *et al.*, 2007).

Selvam and Sundrarajan have investigated the antibacterial effect of cotton fabrics materials that coated by Poly-N-vinyl-2-pyrrolidone (PVP) and ZnO. 100% bacterial reduction was observed when ZnO was used at 20 mg.L<sup>-1</sup>. ZnO used in this study was produced by chemical precipitation method. The obtained ZnO was coated with cotton-dry-cure method on cotton fabrics (Selvam & Sundrarajan, 2012).

Selvam *et al.* have investigated the antibacterial effects of zinc oxide (ZnO), titanium dioxide (TiO<sub>2</sub>) and silver oxide (AgO) nanoparticles on cotton fabrics. Experiments have been performed on *S. aureus* and *E. coli*. Studies indicate that ZnO has the strongest antibacterial effect with a 100% decrease. Reduction in the number of bacteria of TiO<sub>2</sub> and AgO has 90% and 85%, respectively (Selvam & Sundrarajan, 2012).

Azam *et al.* reported the antibacterial effect of zinc oxide (ZnO), copper oxide (CuO), and iron oxide (Fe<sub>2</sub>O<sub>3</sub>) on gram-negative *E. coli* and *P. aeruginosa* and gram-positive *S. aureus*, and *B. subtilis* bacteria. Antibacterial activity was detected by disk diffusion method. The results of X-ray diffraction (XRD) and transmission electron microscopy (TEM) revealed that ZnO nanoparticles had a smaller particle size than CuO and Fe<sub>2</sub>O<sub>3</sub>. The strongest antibacterial effect was seen by ZnO on *B. subtilis* with formation of 25 mm inhibition zone. Under the same conditions, CuO causes 21 mm zone formation, while Fe<sub>2</sub>O<sub>3</sub> causes 15 mm zone formation. The diameters of inhibition zone around the *E. coli* bacteria were 19, 15, and 3 mm for ZnO, CuO, and Fe<sub>2</sub>O<sub>3</sub>, respectively. Similar results were obtained for *P. aeruginosa* and *S. aureus*. According to these results, ZnO shows the strongest antibacterial effect (Azam *et al.*, 2012).

In a study by Adams *et al.*, the antibacterial effect of TiO<sub>2</sub>, SiO<sub>2</sub> and ZnO on *E. coli* and *B. subtilis* was investigated. The measurement of the antibacterial activity was carried out for a concentration range between 10 and 5000 ppm of each metal oxide. As a result of the experiments, it has determined that ZnO has the highest antibacterial effect, while SiO<sub>2</sub> has the lowest effect (Adams *et al.*, 2006).

Yamamoto *et al.* have prepared MgO-ZnO solid material in air for 3 h at 1400 °C and used the material to measure antibacterial activity for *E. coli* and *S. aureus* by electrical conductivity. At the end of the studies, it was observed that the antibacterial effect decreased as the ratio of ZnO in material increased (Yamamoto *et al.*, 2000).

Vasanthi and colleagues have examined the antibacterial activity of tin (Sn) doped ZnO produced by spray pyrolysis on *E. coli*. Experiments were carried out by measuring the optical density at 600 nm for each sample containing Sn at different ratios (0-10%). As a result, it was observed that the antibacterial effect increased as Sn ratio increased (Vasanthi *et al.*, 2013).

Li *et al.* have prepared films from a mixture of chitosan, Ag<sup>+</sup> and ZnO, and investigated the antibacterial effects of their on *B. subtilis*, *E. coli*, *S. aureus*, *Penicillium*, *Aspergillus*, and *Rhizopus*. Studies have shown that the chitosan/Ag<sup>+</sup>/ZnO blend has a much stronger antibacterial effect than the chitosan/Ag<sup>+</sup> and chitosan/ZnO blends (Li *et al.*, 2010).

ZnO in three different forms were prepared by Jin *et al.*: ZnO quantum dots (QD) powder, Polystyrene (PS) ZnO QD nanocomposite film and Polyvinylpyrrolidone (PVP) coated ZnO QD. They have studied the antibacterial effects of these materials using the agar diffusion method and *L. monocytogenes*, *S. Enteritidis*, and *E. coli* O157: H7 were targeted microorganisms. Experiments were performed in culture medium and liquid egg white. It was observed that for all three microorganisms ZnO-PVP application was more effective, as the ZnO concentration increased, antibacterial activity increased. One of the results obtained that ZnO-PS film is not effective on bacterial growth inhibition. This situation is explained as: because of ZnO was not released through the nanocomposite film, it was not diffused in the agar, and therefore no inhibition effect has been obtained (Jin *et al.*, 2009).

Liu and Kim examined the antibacterial effects of nanocomposites consisting of chitosan, polyethyleneglycol (PEG), ZnO, and Ag<sup>+</sup> materials on *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* by disk diffusion method. In their work, the strongest antibacterial effect were shown by the nanocomposite consisting of chitosan/PEG/ZnO/Ag<sup>+</sup>. It has been observed that as the ratio of Ag<sup>+</sup> ion and ZnO

in the mixture increases, the antibacterial effect also increases (Liu & Kim, 2012).

Rekha et al. have investigated the antibacterial effect of pure ZnO and Mn doped ZnO in terms of microorganisms of *E. coli*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *B. subtilis* and *S. aureus*. It was observed that the antibacterial activity increases with the ratio of the Mn used as the additive increases (Rekha et al., 2010).

Li et al. have investigated the antibacterial effect of ZnO coated polyvinylchloride (PVC) on foodborne pathogens by the agar diffusion method. As a result of the study, it was determined that as the amount of ZnO nanoparticles used increases, the inhibition zone diameter around the bacteria increases and the activity on *S. aureus* is higher than *E. coli*. It has been determined that the empty PVC films used for control do not show any antimicrobial effect (Li et al., 2009).

Tayel et al. have investigated the antibacterial effect of zinc oxide by disk diffusion method for foodborne pathogens *Salmonella typhimurium* and *S. aureus* bacteria. Experiments have shown that as the ZnO particle size decreases and the ZnO concentration increases, the antibacterial activity increases and thus the inhibition zone diameter around the bacteria increases (Tayel et al., 2011).

Fang and colleagues have studied antibacterial agents that act on six pathogenic bacteria that cause oral infections: *Streptococcus mutans*, *S. mutans*, *Actinomyces viscosus*, *Lactobacillus casei*, *Staphylococcus aureus* and *Candida albicans*. They used two different ZnO complexes, magnesium hypochlorite and three different Ag<sup>+</sup> compounds as antibacterial agents. A dilution method was used to determine the antibacterial activity. Experiments have shown that all inorganic materials used have antibacterial activity, but complexes containing silver and zinc have a much stronger effect (Fang et al., 2006).

In practice, the use of ZnO, which is used for many purposes and for multiple purposes, has been a matter of curiosity on the cancer. Wahab et al. have investigated the effect of ZnO on HepG2 and MCF-7 cancer cells. HepG2 leads to liver cancer development, while MCF-7 causes breast cancer development. When the concentration of ZnO used was 2.5-5 µg.mL<sup>-1</sup> and there is no effect was observed on the cells. Nevertheless, when the concentration reached 10-25 µg.mL<sup>-1</sup>, it has been observed that the cancer cells were damaged and their growth stopped. This study has proven that ZnO is a cheaper inorganic material that can be used in the treatment of cancer (Wahab et al., 2014).

Queiroz and colleagues examined the antibacterial effect of zinc oxide and the other three substances. These four items were chosen so that they could be used in dental canal treatment. *Kocuria rhizophila*, *Enterococcus faecalis*, *Streptococcus mutans*, *E. coli* and *S. aureus* bacteria were selected as target microorganisms and disc diffusion method was used. Studies have shown that ZnO and eugenol filler exhibit the highest antibacterial effect against *K. rhizophila* (32.67 mm) (de Queiroz et al., 2009).

As you seen, the previous studies had proved the antibacterial effect of ZnO. Again, with the studies done by scientists, it is a known fact that the size of various crystals, including ZnO, is reduced to the nanoscale thus the antibacterial effect is increased. How the antibacterial effect changes when the size of ZnO, which is the most encountered hexagonal rod morphologic structure have sizes more than nano-scale, is aimed to be clarified with this study.

## MATERIAL AND METHODS

### Materials

Zinc nitrate hexahydrate, Zn(NO<sub>3</sub>)<sub>2</sub>.6H<sub>2</sub>O and hexamethylene tetramine (HMT-C<sub>6</sub>H<sub>12</sub>N<sub>4</sub>) used in ZnO production were purchased from Sigma-Aldrich. Different polymers have been used as additives during ZnO production. The type II water used for doing experiments and cleaning experimental was produced with the Merck Millipore Elix Essential.

When the antibacterial activity was determined, the disc diffusion method was performed using Mueller-Hilton Agar (Biolife), Nutrient Broth (Merck), Mc Farland No 0.5. The microorganisms and the nutrient media for their development are summarized in Table 1: Baird Parker (Salubris), Fraser Broth (Difco™) + PALCAM Agar (Difco™), Buffered Peptone Water (Merck) + Selenite Cystin Broth (Salubris) + Brilliant Green Agar (Salubris).

**Table 1** Microorganisms and used mediums

Bacteria	Used medium
<i>S. aureus</i>	Baird-Parker Agar
<i>L. monocytogenes</i>	Fraser Broth + PALCAM Agar
<i>S. Enteritidis</i>	Buffered Peptone Water + Selenite Cystine Broth + Brilliant Green Agar

### Methods

In this study, ZnO particles with different properties were produced by using chemical precipitation method and the antimicrobial effect of the produced ZnO powder material was investigated on the bacteria of *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 15313), and *S. Enteritidis* (ATCC 13076). The reason why the chemical precipitation method is preferred is that the ZnO crystal

structure is easy to control in the presence of additives. Also, this method is preferred for synthesizing ZnO because it is cheap and requires less equipment than the other methods.

### Experimental Design

Experiment planning is done for optimization using Taguchi experimental design method. The L<sub>9</sub> (3<sup>3</sup>) orthogonal array was used as the experimental plan. In this way, the number of experiments is reduced and the ZnO concentration, ZnO sample, and the optimization of the targeted bacterial parameters will be possible. In addition, the main effect graphs and statistical methods were used, which makes it easier to evaluate the data obtained after the experiment. The Minitab program was used for all calculations and graphical drawings.

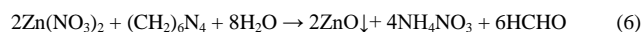
Factors used in Taguchi experimental design are: type of ZnO sample, concentration, and bacteria. Three levels were studied for each factor (Table 2). Table 2 shows the experimental design made according to the Taguchi L<sub>9</sub> (3<sup>3</sup>) orthogonal array. Totally, nine experiments were performed with three replications to improve reliability.

**Table 2** Taguchi design for experiments

Experiment	ZnO (A)	[ZnO] (B) g.mL <sup>-1</sup>	Bacteria (C)
1	N1	0.1	<i>L. monocytogenes</i>
2	N1	0.075	<i>S. aureus</i>
3	N1	0.05	<i>S. Enteritidis</i>
4	N2	0.1	<i>S. aureus</i>
5	N2	0.075	<i>S. Enteritidis</i>
6	N2	0.05	<i>L. monocytogenes</i>
7	N3	0.1	<i>S. Enteritidis</i>
8	N3	0.075	<i>L. monocytogenes</i>
9	N3	0.05	<i>S. aureus</i>

### Synthesis and characterization of ZnO samples

All of the ZnO producing procedure was performed with a 1000 mL double-jacket glass reactor. Reactor was kept at 95 °C for 1.5 h. The temperature control of the reactor was carried out with a circulating water bath (Polyscience - AP07R-20-A12E). The cryostat has been controlled by a probe that measures the temperature inside the reactor used. Reactor has been mixed using a magnetic stirrer. Both solutions of HMT and Zn(NO<sub>3</sub>)<sub>2</sub>.6H<sub>2</sub>O were prepared to have molarities of 0.03 M in the reactor after mixing. Different polymers were used as additives in synthesis (Eq. 6). The crystals obtained as a result of the experiments were filtered through a 0.22 µm porous membrane and washed with pure water. The resulting precipitate was dried in a vacuum oven at 60 °C for 48 hours.



This method of ZnO production can be found on many scientific sources and is a production method used quite often (Akin & Oner, 2012; Atayev et al., 2015; Bekat & Öner, 2016).

SEM, XRD and BET analyzes were used in characterization studies. XRD analysis is important to understand whether the material is ZnO. SEM analysis is used to determine the morphology of the material. Furthermore, the average length and width values can be found with the measurements made from these SEM images. During this size analysis, two hundred crystals were measured for each value determined. Multi-point BET analysis has been used to measure the BET specific surface areas of the materials produced. Analyzes were performed with Quantachrome NovaTouch LA.

### Determination of Antibacterial Activity

*S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 15313), and *S. Enteritidis* (ATCC 13076) strains to be used in the determination of antibacterial activity were provided as pure culture (Microbiologist). Each microorganism has been developed and activated in its unique medium.

*S. aureus* occurs naturally in the mucous tissue covering the nose and throat cavity. It is found in skin, abscesses, pimples and boils. For this reason, the presence of these bacteria in food is an indicator of the lack of hygiene in food enterprises. It is a gram-positive and facultative anaerobic bacteria. Optimum growth temperatures are 30-37 °C. During the study, the bacteria in question (studied) were incubated in Baird Parker Agar medium at 35 °C for 48 h. While doing colony selection, black-gray, bright, smooth colonies on Agar are selected (Zangerl & Asperger, 2003).

*Listeria monocytogenes* is an important pathogen in terms of public health, which can survive even under unfavorable conditions such as cooling, freezing, heating and drying processes, which can develop at the refrigerator temperature that can be widely distributed to the environment. They cause a disease called Listeriosis. *L. monocytogenes* is a gram positive, facultative anaerobic, encapsulated and non-spore bacteria. The optimum growth temperature is generally 35-37 °C, and the strains can develop in a wide temperature range of 1-45 °C. For *Listeria*



analysis, biochemical tests are carried out for pre-enrichment, enrichment, selective solids cultivation, investigation of colonies and identification. In Fraser Broth medium, it is incubated at 30 °C for 24 h and thus pre-enriched. Then, on the prepared Palcam Agar medium, it is spread and incubated at 30 °C for 48 h hours (Forsythe & Hayes, 2000).

*Salmonella*, is a rod-like gram-negative, facultative anaerobic bacteria, and can lead to typhoid, paratyphoid and food poisoning. The optimum growth temperature is 37 °C. In the classical method, *Salmonella* has pre-enrichment, selective enrichment, selective solidification, biochemical tests and serological confirmation, which takes 7 days. In this process, it is incubated for 24 h at 35 °C in buffered Peptone Water. Then, it is inoculated with 1 ml to Selenit Broth's medium, and incubates 24 h at 35 °C. It is spread in Brilliant Green agar and incubated at 37 °C for 48 h. Transparent and smooth colonies are selected.

Microorganisms developed in their own environments are inoculated into Nutrient Broth medium for the determination of antibacterial activity. Smooth colonies selected with the help of a loop are transferred to the broth medium, and incubate at 37 °C (Adams & Moss, 2000).

In the antibacterial activity tests, a standard 0.5 McFarland solution (108 microorganisms.mL<sup>-1</sup>) is used. McFarland standards have been developed to determine the number of bacteria in a liquid medium. The standard turbidity tubes developed by McFarland using barium chloride and sulfuric acid contain a degree of turbidity equivalent to the amount of bacteria that is sown on a liquid medium. The bacterial suspension to be compared with the McFarland standard is compared on black bands on a white surface under good light. If the tube containing the bacterial suspension and the blur of the McFarland tube are equal, it is assumed that the number of targeted bacteria in the suspension is obtained. If the turbidity is not equal, then the suspension of the turbidity is achieved by adding bacteria to the bacterial suspension or by diluting the suspension.

The sample taken from this suspension was inoculated to the Mueller-Hinton Agar surface. For this purpose, a sterile swab was immersed in the adjusted inoculum and turned several times by pressing the inner upper wall of the tube to drain excess fluid. The surface of Mueller-Hinton Agar plate was sown by drawing three times. After each drawing, the plate has been rotated by 60 ° to obtain an equal inoculation. The 6 mm diameter sterile discs were placed on the agar by providing aseptic conditions through the forceps. In this process, there was 22 mm distance between the discs and 14 mm from the edge of the petri dish to ensure that the zones will not overlap. The ZnO suspension was adsorbed onto the disc with a micropipette (100 µL). The one empty disk impregnated pure water for control purposes, and it is placed on the agar. The media were then incubated at 35 °C for 24 hours. The inhibition zones formed as a result of the incubation were measured with a ruler and antibacterial activity was determined.

**RESULTS AND DISCUSSION**

In the Figure 1, by the comparison of the XRD analyses with the ZnO pattern, it has observed that the materials are pure ZnO. It is understood from the SEM images that the morphology of the material is in hexagonal rod structure (Figure 2, 3, and 4). Average sizes for samples N1, N2, and N3 have measured and calculated from the SEM images. The average size of the sample N1 has found as 1174×1689 nm (Figure 2). In Figure 3, it was calculated that sample N2 has 200×1822 nm. Finally, in the Figure 4, average size of sample N3 was obtained as 280×1350 nm. BET specific surface areas of the sample N1, N2, and N3 were measured 4.17, 3.10, and 1.53, respectively. Characterization information of samples is summarized in Table 3.

**Table 3** Properties of used samples as antibacterial agent

Sample	Average	Width/Length	BET Specific Surface
	Width × Length (nm)	Ratio	Area (m <sup>2</sup> /g)
N1	1174×1689	0.6951	4.17
N2	200×1822	0.1098	3.10
N3	280×1350	0.2074	1.53

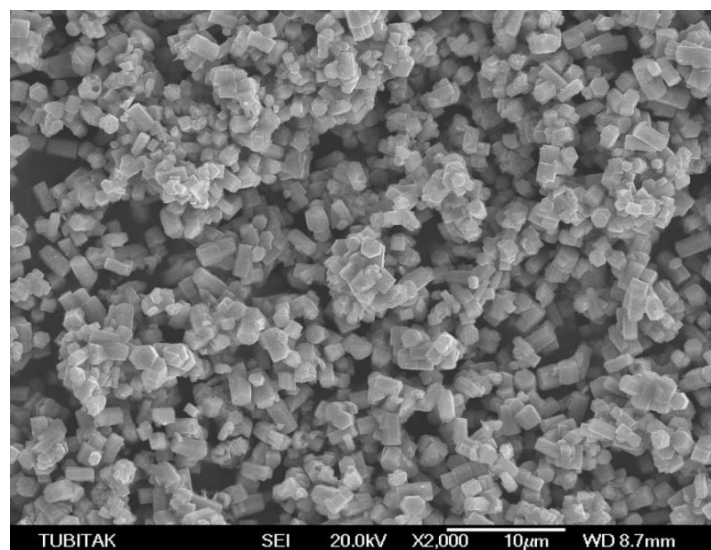


Figure 2 SEM image of sample N1.

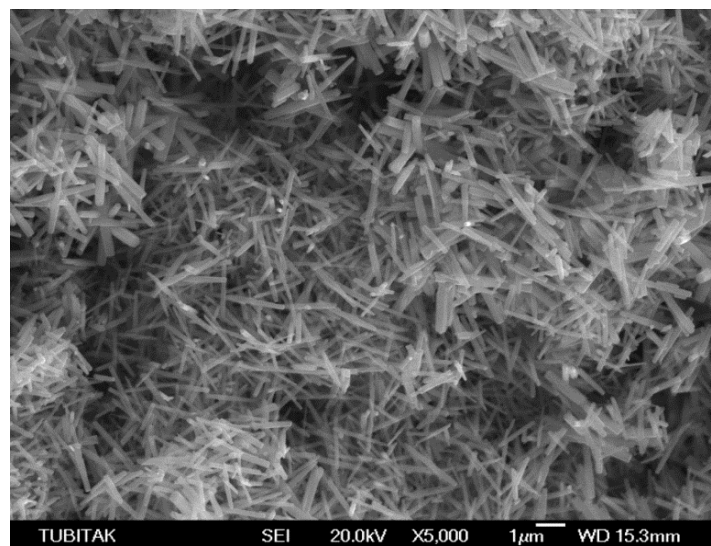


Figure 3 SEM image of sample N2

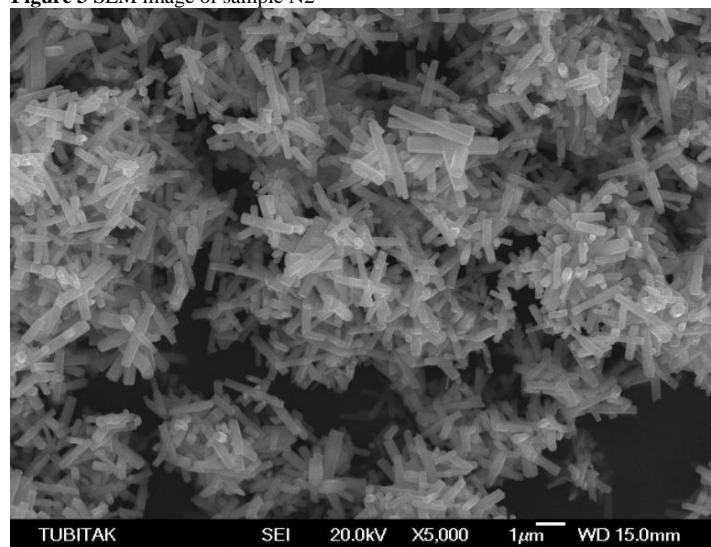


Figure 4 SEM image of sample N3

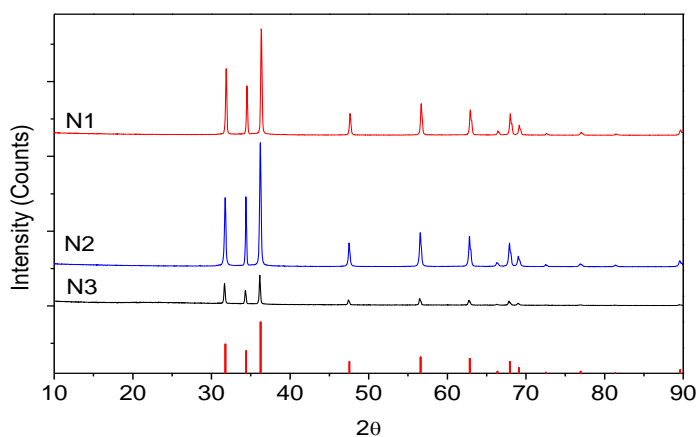


Figure 1 XRD analysis of samples

As a summary of the study, all test results are given in Table 4. Because of Taguchi L<sub>9</sub> (3<sup>3</sup>) experimental design used, the table is not enough to make comments, but also statistical calculations are needed for interpretation. Table 3 shows the properties of three different types of ZnO samples produced for use in antibacterial activity assays. Morphologically, the three samples of ZnO particles are hexagonal form, but vary in size and surface area.

**Table 4** Responses according to Taguchi L<sub>9</sub> (3<sup>3</sup>) Experimental Design

Experiment	Zone Diameter (mm)			Average diameter (mm)
	Repetition			
	1	2	3	
1	11	13	9	11
2	9	10	6	8.3
3	3	1	2	2
4	14	11	11	12
5	4	2	5	3.7
6	5	6	7	6
7	4	5	7	5.3
8	6	3	8	5.7
9	9	7	8	8

The experimental responses obtained in the Taguchi Experimental design are converted the signal/noise (S/N) ratio for interpretation. Three different situations are considered to calculate the Signal to Noise ratio. These states and the formulas used are given below:

The formula used when the result we want to achieve is the minimum:

$$\frac{Signal}{Noise} = -10 \cdot \log \left[ \frac{1}{n} \sum_{i=1}^n (y_i^2) \right] \quad (7)$$

The formula used when the result we want to achieve is the maximum:

$$\frac{Signal}{Noise} = -10 \cdot \log \left[ \frac{1}{n} \sum_{i=1}^n \left( \frac{1}{y_i} \right) \right] \quad (8)$$

When the result we want to reach is the best when it is nominal, the formula used is:

$$\frac{Signal}{Noise} = -10 \cdot \log \left[ \frac{y_{ort}^2}{s^2} \right] \quad (9)$$

In these equations, y is the performance characteristic, s is the variance, i is the experiment sequence, n is the total number of experiments. In this study, the Eq. 8, which is used in the case of "the greatest value is the best", is preferred. In Table 5, "delta" value is the difference between the biggest and the smallest values between the levels; the "rank" value refers to the order of these differences. According to this ranking, it can be determined which factor is the most important factor. As a result of the studies, it is seen that all three factors are effective on the result.

**Table 5** Average values response table calculated according to Taguchi L<sub>9</sub> (3<sup>3</sup>) Experiment Design; A: Type of ZnO sample, B: ZnO concentration, C: Targetted microorganism

Level	A	B	C
1	7.100	<b>9.433</b>	7.567
2	<b>7.233</b>	5.900	<b>9.433</b>
3	6.333	5.333	3.667
Delta	0.900	4.100	5.767
Rank	3	2	1

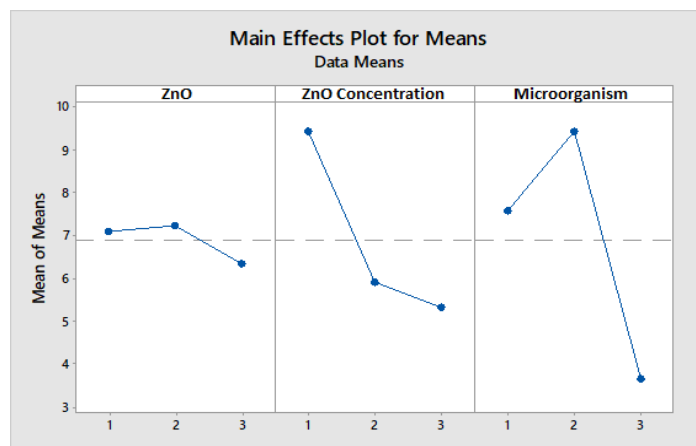
It was seen that the investigation of the responses obtained in both tables, the Level 2 (Sample N2) for the factor A; Level 1 (0.1 g.mL<sup>-1</sup> concentration) for the factor B; Level 2 (*S. aureus*) for the factor C are the most effective levels.

Hypothesis testing has been performed to investigate the effect of factors on response. According to the hypothesis test, the zero hypothesis is that H0 factors are ineffective on the response, otherwise H1 hypothesis is effective on the response of the factors. P values were calculated by using Minitab program for hypothesis testing. The H0 hypothesis was considered valid if the P value was greater than 0.05 for the 95% confidence level; otherwise the H1 hypothesis was valid (Table 6).

**Table 6** Variance Analysis

	Sum of Squares	Average of The Squares	F Value	P Value	Degree of Freedom
ZnO Sample	1.42	0.71	0.68	0.59	2
Concentration	29.62	14.81	14.31	0.06	2
Microorganism	51.95	25.11	25.11	0.04	2

Calculated P values; 0.59 for the ZnO sample; 0.06 for the concentration; for the targeted microorganism, it is 0.04 (Table 8). In this case, the most important factor affecting the result is the target microorganism. *S. aureus* is the most sensitive bacteria to ZnO particles. The most resistant bacteria are *S. Enteritidis*. Although the P value for the effect of ZnO concentration is close to the limit, it is understood from the graphs that it is effective on the result (Figure 5). As expected, of the ZnO concentration increase around the bacterial colony leaves an increase in the inhibition zone diameter.



**Figure 5** Main effect graphs based on Taguchi experimental design; values of 1, 2 and 3 in x axis shows the levels of factors

## CONCLUSIONS

The results obtained as a result of the study are listed as follows: The Taguchi experimental design approach was successfully used to investigate which properties were effective on antibacterial mechanism of ZnO.

All synthesized ZnO samples were effective on bacterial colonies. It was seen that the targeted microorganisms, *S. aureus* rather than the others is most affected by ZnO particles. Another result is *S. Enteritidis* was affected minimum.

As we can expect it was seen that antibacterial effect was increased by increasing concentration of ZnO. It was found that 0.1 g.mL<sup>-1</sup>, maximum concentration was most effective.

N3 sample with the lowest BET value of 1.53 m<sup>2</sup>/g shows the lowest antibacterial effect. This sample size has measured 280 nm×1350 nm. This situation proves in the same way with the literature that antibacterial effect decreases with decreasing BET surface area.

Despite N2 sample has BET value of 3.1 m<sup>2</sup>/g, second high BET surface area, it has the lowest size with 200 nm. It was obtain the highest antibacterial effect. This means, when the two samples have been synthesizes for the antibacterial usage, if the BET surface areas were close to each other, the antibacterial effect has depend on the aspect ratio.

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