ISSN: 0973-3469, Vol.18, No.(1) 2021, Pg. 56-65

Material Science Research India

www.materialsciencejournal.org

Antibacterial Properties of Scallop Shell Derived Calcium Hydroxide Powders

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Abstract

Globally increased bivalve aquaculture production results in a vast amount of by-product discharges such as scallop shells. Utilization of these wastes to produce new products such as antibacterial agents can cooperate to reduce environmental problems and provide a high value-added product at a lower cost. In this study, scallop shells are heat-treated at 800°, 900°, 1000°, and 1100°C for 4 hours at atmospheric conditions. X-ray diffraction analysis revealed that calcium carbonate is the only inorganic phase in the powdered scallop shells. Ten weeks after the thermal treatment of the scallop shells, the calcium hydroxide phase was the only crystalline phase determined by X-ray diffraction analysis for the samples calcined at 1000° and 1100°C. At lower calcination temperatures, calcium carbonate and calcium hydroxide phases were co-existing in the samples. Scanning electron microscopy investigations depicted that using scallop shells as a starting material to synthesize nanometer-sized calcium hydroxide is achieved. It was determined that applied calcination temperature has a significant effect on the particle size of the obtained calcium hydroxide phase. Antimicrobial activity of calcined and uncalcined shell powders were tested against *Escherichia coli* and *Staphylococcus aureus*. No antibacterial activity was detected for the uncalcined scallop shell powders. However strong antibacterial activity was determined for the powders after subjection to calcination. Calcination of scallop shells is an environmentally friendly, readily applied, and low- cost approach to achieve nanometer-size calcium hydroxide that can be used as an inorganic antibacterial material in various composite systems.

Article History
Received: 27 November 2020
Accepted: 16 March 2021

Keywords
Antibacterial Activity;
Calcination;
Calcium Hydroxide;
Scallop Shell.

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Doi: http://dx.doi.org/10.13005/msri/180107

Introduction

Bivalve aquaculture production, notably clam, oyster, mussel, and scallop culture, has been continuously increasing globally over the past few decades.[1] Scallop shells are a by-product of the seafood industry that is cheap, abundant, and readily available. The annual discharge amount of the scallop shells is very high, and these wastes can cause environmental problems such as strong unpleasant smell and soil pollution due to the heavy metal content of these shells. Scallop shells can be used to produce various food additives, paving materials,and plastering.[2] Recently researches are focused on applications of scallop shells in various areas such as reinforcement for the polymer matrix composites,[3, 4] desulfurization agent,[5] filler in plywood adhesive,[6] skin protective material,[7, 8] anti-obesity agent,[9] formaldehyde adsorbent,[10] and adsorbent for radioactive substances such as Sr^{2+}.[11]

Seafood industry wastes are valuable materials that can be used as a starting material to synthesize engineering materials that can be used in a wide variety of industrial applications.[12-18] However, these materials are not utilized in most cases as a natural raw material source and are not recycled on a large scale to produce high-value-added industrial products. Recently intensive research is performed to utilize the scallop shells to achieve calcium-containing inorganic materials that can be used in various commercial applications.[2, 19-23] Scallop shells are used to synthesize various inorganic materials such as calcium oxide (CaO),[24] calcium hydroxide ($Ca(OH)_2$), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$),[25] calcium titanate ($CaTiO_3$),[26] and aragonite ($CaCO_3$).[27-29] Recently, investigated applications of scallop shell derived calcium-containing materials are as a catalyst,[19, 24, 30, 31] antibacterial agent,[2, 21-23] inactivating avian influenza virus,[32] limestone substitutes,[33] reinforcement for the composites,[22] and sources to improve soil.[20]

The scallop shells' phase content is reported as 98–99 % calcium carbonate, trace inorganic materials, and 1–2 % organics.[9] It is well known that at elevated temperatures, organics are removed from the structure and calcium carbonate ($CaCO_3$) inorganic phase converted to another phase, CaO. Achieved CaO has a strong antimicrobial activity, and it can also be used to produce various inorganic phases. Calcium hydroxide ($Ca(OH)_2$) ceramic material is achieved via hydration of CaO, and it is known that $Ca(OH)_2$ is a bactericidal material and is an effective candidate to be applied as a filler for the root canal in endodontic treatment applications.[34] Both CaO and $Ca(OH)_2$ and their composites with various materials can also be used as a catalyst for various reaction systems.[35-40]

In the current study, the aim was to synthesize an antibacterial inorganic material via heat treatment of waste scallop shells at four different temperatures (800°, 900°, 1000°, and 1100°C) at a constant dwell time, 4 hours. Characterization of the heat-treated scallop shells were carried out via X-ray diffraction and scanning electron microscopy investigations. The antimicrobial effects of the powdered as-received scallop shells and heat-treated scallop shells on gram-positive, and gram-negative bacteria were assessed through viable cell counts.

Experimental Studies

Reagents and Chemicals

Nutrient Broth (Merck) and agar powder (Himedia) were used for the microbiological tests. The bacterial isolates *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were a kind gift from Dr. Aslihan Kurt Kizildogan, 19 Mayis University, Samsun, Turkey. Distilled water was used throughout experiments.

Scallop shells were collected from marine region of Mugla/Turkey. Collected shells were physically cleaned and washed with water to remove the impurities. Cleaned shells were rinsed with distilled water and dried in an oven overnight before the calcination process. Calcination studies were performed in air atmosphere at 4 different temperatures (800°, 900°, 1000° and 1100°C) for 4 hours. Applied heating and cooling rate was 10°C/min. Ten weeks after calcination process samples were ground to the powder form in an agate mortar for the XRD analysis.Phase content of the samples were performed by X-ray diffraction (XRD, Bruker AXS/Discovery D8), using monochromatic Cu-K_α radiation (λ=1.5406Å). Microstructure of the scallop shells and calcined scallop shell samples were investigated with a scanning electron microscope (SEM, Carl Zeiss/Gemini 300) in a secondary electron (SE) image mode.

Antimicrobial Activity Assay

The grounded as-received scallop shells and calcined scallop shells were sterilized at 121°C for 20 min prior to being used for antibacterial experiments. The bacterial strains, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were grown in a nutrient broth for 18 h at 37°C. The cells were diluted to 10^7 CFU/ml (colony forming units) using nutrient broth and added into test tubes containing samples (0.2 %). The samples were incubated for 24 h at 37°C with agitation (200 rpm). At the end of the incubation period, samples were taken and diluted in saline and then grown on nutrient agar plates. Bacterial colonies were counted after 24 h of incubation at 37°C, and enumeration was performed in CFU/ml.

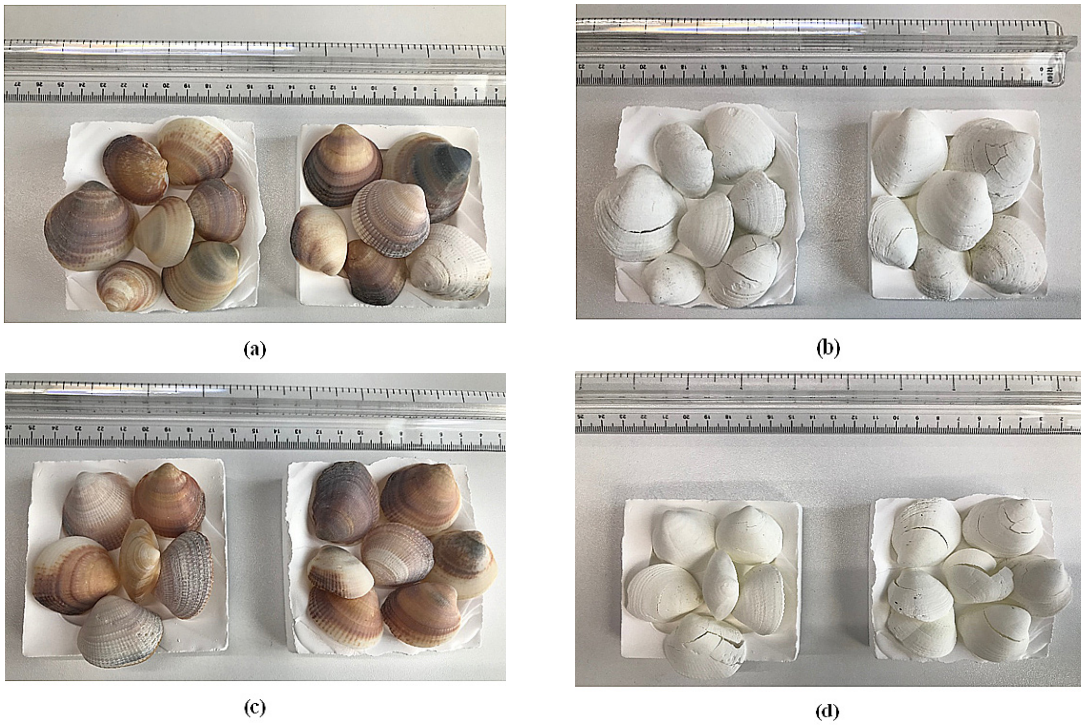


Fig. 1: Macroscopic images of the scallop shells. (a) Before calcination and (b) after calcination at 800°C for 4 hours. (c) Before calcination and (d) after calcination at 1100°C for 4 hours

Results and Discussion

Macroscopic images of the as-received scallop shells before and after calcination at two different temperatures, 800° and 1100°C, are given in Fig. 1. Durig the calcination process organic content of the scallop shells are removed from the structure and a crystalline material is achieved as confirmed by the XRD analysis.

Phase analysis revealed that powdered as-received scallop shells contain only calcium carbonate phase (Fig. 2). This result is consistent with the literature reporting that scallop shells consist of calcium carbonate as an inorganic phase.[41] Sawai *et al*.[41] applied heat treatment to the scallop shells at 600°, 700°, 800°, 900°, and 1000°C for 1 hour. They reported XRD analysis results of the as-received and heat-treated scallop shells. Calcium carbonate was determined as an inorganic phase for the as-received and heat-treated scallop shells up to 700°C.[41] Sawai *et al*.[41] reported that CaO was formed firstly at 800°C and at higher temperatures stronger CaO peaks were detected.

SEM investigations of the powdered as-received scallop shells are given in Fig. 3. Powdered scallop shells have a dense structure, and due to the applied milling procedure, fine particle size and relatively wide particle size distribution was achieved (Fig. 3). The powdered as-received scallop shells' particle size is changing from a few micrometers size down to nanometer size.

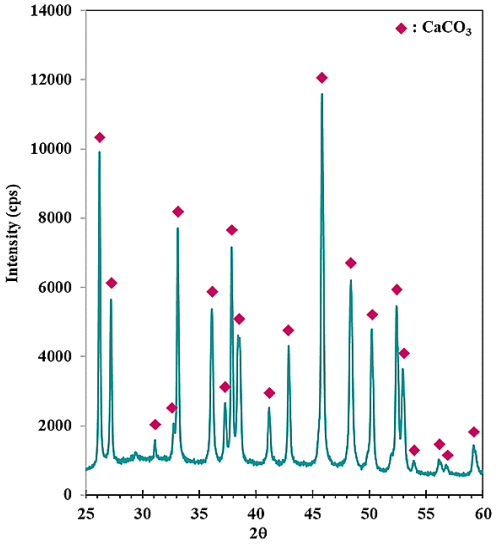


Fig. 2: XRD analysis of the powdered scallop shells

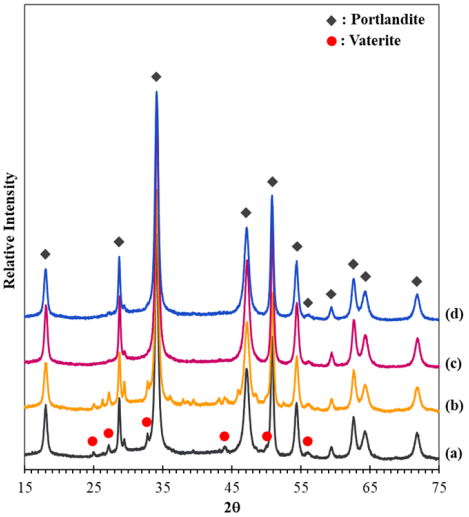
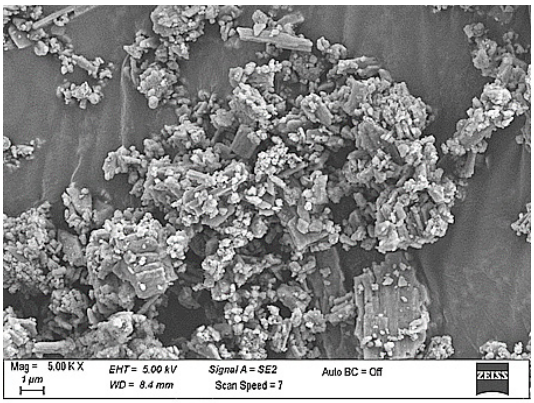
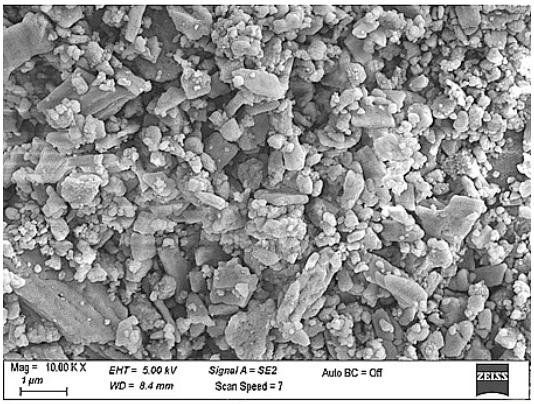


Fig. 4: XRD analysis of the scallop shells calcined at (a) 800°C, (b) 900°C, (c) 1000°C, and (d) 1100°C for 4 hours



(a)



(b)

Fig. 3: SEM images of the powdered scallop shells at (a) 5000X and (b) 10000X

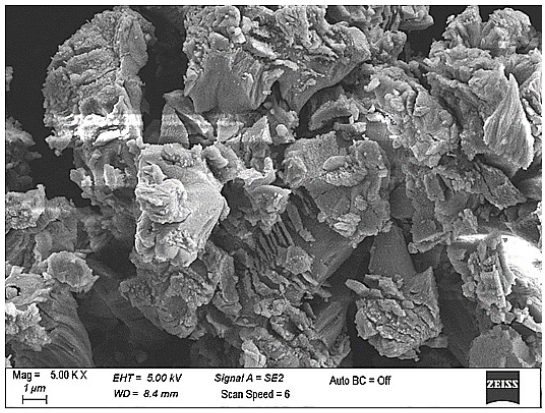
Heat-treated scallop shells XRD analysis was performed approximately ten weeks after the heat treatment process. The heat-treated samples were stored at atmospheric conditions in a closed box. XRD analysis results revealed that the calcium hydroxide ($Ca(OH)_2$) phase (portlandite, PDF 00-044-1481) was formed in all four calcined scallop shell samples. During the scallop shells' heat treatment at >800°C, calcium carbonate ($CaCO_3$) phase converted to CaO via an endothermic reaction[42] given below.

$$CaCO_{3(s)} \rightarrow CaO_{(s)} + CO_{2(g)} \Delta H_{298K} = 178 \text{ kJ/mol} \quad ...(1)$$

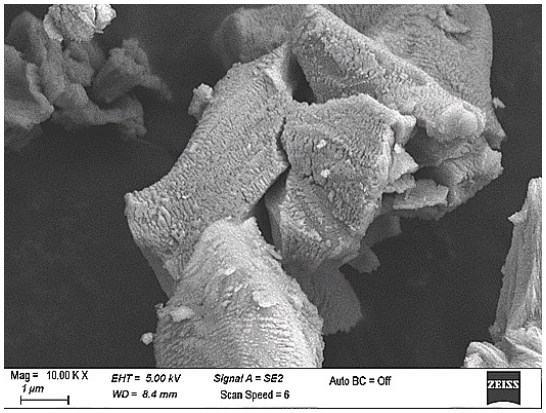
Calcium carbonate has a very low hygroscopicity[43] however, calcium oxide has a hygroscopic nature. It is known that when scallop shell-derived CaO is exposed to atmospheric conditions, $Ca(OH)_2$ phase is formed.[44] The hydration/dehydration reaction of CaO, also suitable for the thermochemical energy storage systems,[45] is given in Eq. 2. Mihara *et al.*[44] reported that three months after the heat treatment of the scallop shells, XRD analysis revealed that CaO reacted with the atmospheric moisture and $Ca(OH)_2$ phase was formed at the 900°C heat-treated scallop shell samples.

$$CaO_{(s)} + H_2O_{(g)} \leftrightarrow Ca(OH)_{2(s)} \Delta H_{298K} = -104 \text{ kJ/mol} \quad ...(2)$$

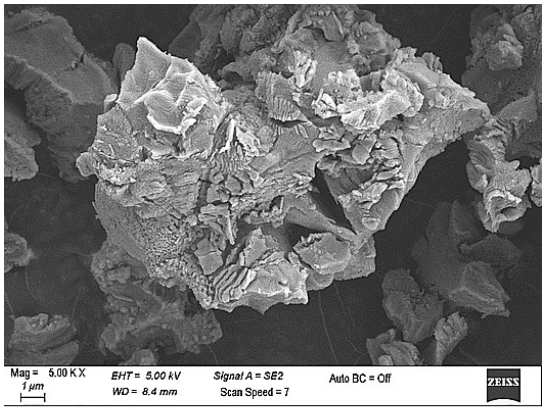
XRD analysis showed that the calcination temperature is a significant parameter on the phase content. When samples were calcined at 800° and 900°C calcium hydroxide ($Ca(OH)_2$) and $CaCO_3$ (vaterite, PDF 00-069-0001) phases were co-existing. At higher calcination temperatures vaterite phase was disappeared.



(a)



(b)



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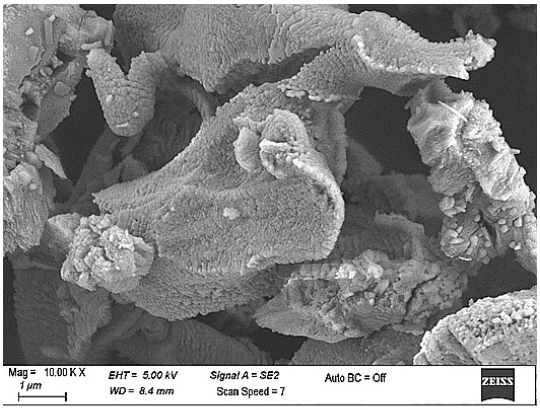
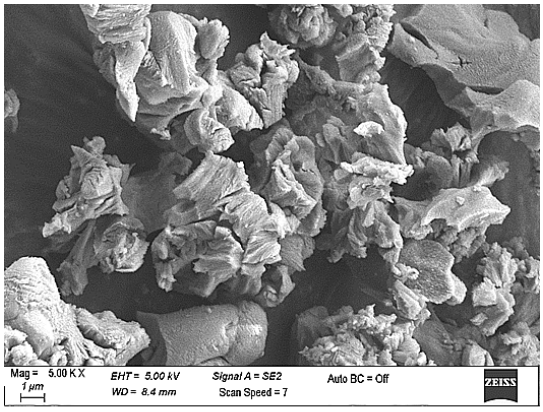


(d)



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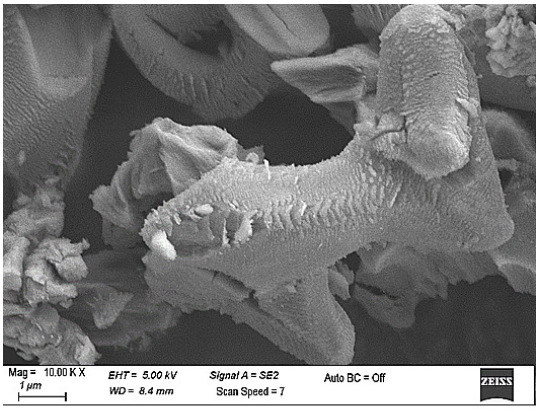

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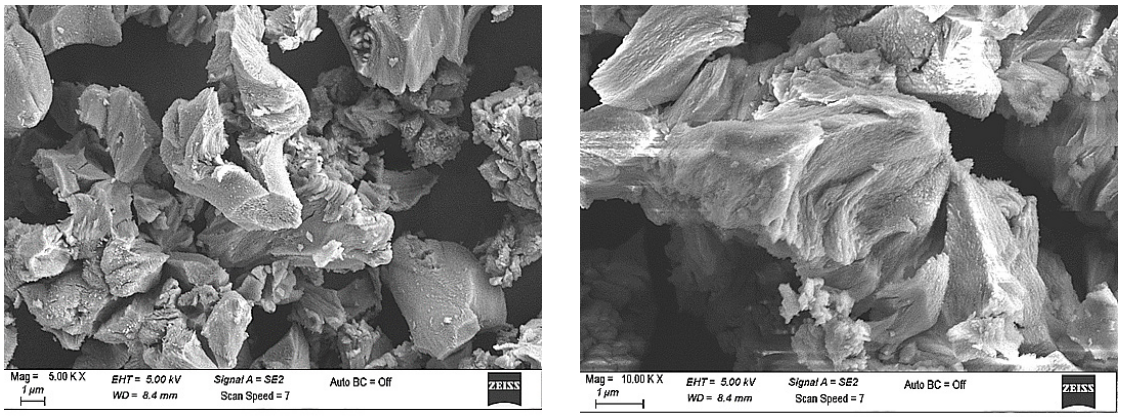

(g) (h)

Fig.5: SEM analysis of the scallop shells calcined at (a-b) 800°C, (c-d) 900°C, (e-f) 1000°C, and (g-h) 1100°C for 4 hours

Scanning electron microscopy investigations of the heat-treated scallop shells (Fig. 5) revealed that the particle size and distribution of the obtained calcium hydroxide phase slightly depends on the applied heat treatment temperature. Microstructural investigations revealed that the particle size and distribution of the heat-treated samples are very fine and homogenous for all samples. These nano meter sized calcium hydroxide powders can be used as a starting material to synthesize various ceramic materials such as calcium silicates and calcium phosphates.

Table 1: Effect of grounded scallop shell and heat-treated scallop shell powders on *E. coli* and *S. aureus* viability

Number of viable cells (CFU/ml)		
Samples	***S. aureus***	***E. coli***
SS	$1.7x10^{10}$	$3.1x10^{9}$
SS-800	ND	ND
SS-900	ND	ND
SS-1000	ND	ND
SS-1100	ND	ND

SS: powdered scallop shell, SS-800: powdered scallop shell heat -treated at 800°C, SS-900: powdered scallop shell heat-treated at 900°C, SS-1000: powdered scallop shell heat-treated at 1000°C, SS-1100: powdered scallop shell heat-treated at 1100°C, ND: <5.

Effect of scallop shell powder and heat-treated scallop shell powder on viability of *E. coli* and *S. aureus* is given in Table 1. The number of *S. aureus* and *E. coli* cells incubated with scallop shell powder for 24 h were $1.7x10^{10}$ CFU/ml and $3.1x10^{9}$ CFU/ml respectively. On the other hand, number of *S. aureus* cells co-cultivated with shells calcined at 800°, 900°, 1000° and 1100°C were 1-5 CFU/ml. Growth of *E. coli* cells were totally inhibited that were co-cultivated with the shells calcined at 800-1100°C. Scallop shells calcined at all temperatures significantly inhibited growth of both the Gram negative *E. coli* and the Gram positive *S. aureus* compared to the number of viable cells incubated with the powdered as-received scallop shells. Oikava *et al*.[46] calcined shells of oyster, scallop, and

clam and determined their antibacterial activities via total aerobic counts and *E. coli*. According to aerobic counts 4100 cells were determined in one millilitre of culture containing uncalcined scallop powder and 720 cells per milliliter were detected in the culture containing calcined scallop powder. The number of *E. coli* cells co-cultured with uncalcined scallop was 62 whereas no cells were determined in the culture containing calcined scallop. The authors have verified antibacterial activity of the calcined scallop shells by viable count which is in accordance with the results obtained in this study. Watanabe *et al*.[47] heated scallop shells and obtained nanoparticles,the main component of which was calcium hydroxide. Particles of different sizes were incubated with *E. coli* and the number of viable cells was counted to determine their antibacterial activity. The nanoparticles inhibited growth of *E. coli* in a dose dependent manner. Both Oikava *et al*.[46] and Watanabe *et al*.[47] reported the strong alkalinity of the aqueous solutions of the calcined powders to be responsible for the antibacterial activity. Calcium hydroxide dissociates into calcium and hydroxyl ions in an aqueous environment. The rapid release of hydroxyl ions resulting in a highly alkaline environment inhibits the growth of most microorganisms. Antimicrobial activity of calcium hydroxide is reported to be dependent on several mechanisms such as damage to the cytoplasmic membrane, inhibition of DNA replication and denaturation of proteins.[48-50]

Conclusions

- Using scallop shells as a starting material to synthesize nanometer-sized ceramic materials via calcination is an environmentally friendly, readily applied, and low-cost approach.
- XRD analysis showed that the calcination temperature is a significant parameter on the phase content. When samples were calcined at 800° and 900°C calcium hydroxide and vaterite phases were co-existing. At higher calcination temperatures vaterite phase was disappeared.
- Microstructural investigations revealed that the particle size and distribution of the heat-treated samples are very fine (nanometer size) and homogenous for all samples. SEM investigations revealed that the achieved calcium hydroxide particle size slightly depends on the applied calcination temperature.
- Calcined scallop shells strongly inhibited the growth of *E. coli* and *S. aureus*. Calcium hydroxide dissociates into calcium and hydroxyl ions in an aqueous environment. The rapid release of hydroxyl ions resulting in a highly alkaline environment inhibits the growth of most microorganisms. Antimicrobial activity of calcium hydroxide is reported to be dependent on several mechanisms such as damage to the cytoplasmic membrane, inhibition of DNA replication and denaturation of proteins.[48-50]
- The results verified the potential of calcined shells as potential antimicrobial agents that can be used in various composite systems.

Acknowledgment

This project has been supported by the Foundation for Scientific Research Projects of Bursa Technical University (Project Number: 200COVID03). The authors wish to thank Seyda TAVSANOGLU (Mugla, Turkey) for supplying the scallop shells and Porland Porcelain (Bilecik, Turkey) for supplying the ceramic substrates. The authors gratefully acknowledge Bursa Technical University Central Research Laboratory (Bursa, Turkey) for the XRD and SEM analysis.

Funding

This project has been supported by the Foundation for Scientific Research Projects of Bursa Technical University (Project Number: 200COVID03).

Conflict of interest

The authors declare that she/he has no conflict of interest regarding the publication of this article.

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