Antibacterial Activity against *Streptococcus mutans* of Brass-Ash-Derived Zinc Oxide Nanoparticles

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Received 22 November 2020 • Revised 20 March 2021 • Accepted 20 April 2021 • Published 1 May 2021

Abstract:

Background: In recent years, popularity has been grown into management of industrial wastes or chemical by-products, and brass ash is the one to be in consideration. With its high amount of zinc oxide (ZnO), this fly ash is able to be processed for ZnO which provides a lot of useful medical applications, especially antimicrobial activity against certain kinds of bacteria.

Objective: This study aims to examine whether antibacterial activity against *Streptococcus mutans* (S. mutans) of waste-derived ZnO is obtained by which size of the milled particles.

Method: Once the ash was obtained, it was then purified for ZnO using chemical precipitation method. Subsequently, purified ZnO (S_0) was milled in a high energy ball miller under a variety of milling durations: untreated, 0.5-hour milled ($S_{0.5}$), and 1.0-hour milled (S_1). Characterization of ZnO was done through x-ray fluorescence (XRF) analyzer for element composition, x-ray diffraction (XRD) machine for determination of crystallographic parameters, as well as scanning electron microscope (SEM) for particle size distribution. Purified and milled ZnO was, at last, tested for its antibacterial activity against *S. mutans* using microbroth dilution method compared to commercial ZnO (C), and the result was interpreted using a microplate reader.

Results: ZnO at 99% purification was synthesized in the process. Its size means were 123.99 nm for untreated ZnO, and 104.41 nm and 76.22 nm respectively after being milled. Of all the sizes compared, optimal antibacterial activity, eventually, was acquired from ZnO in size of 76.22 nm (milled for an hour), which its minimal inhibitory concentration (MIC) was 28.125 μg/ml.

Conclusion: The smaller the size of ZnO, the greater the antibacterial activity is. Aside from the main objective, chemical precipitation process could purify ZnO and size distribution was affected by milling time. For further study, the author suggests that synthesized ZnO

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GMSMJ 2021; 1 (2): 99-108
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should be incorporated into ZnO-containing dental products and be tested for its significant change in properties. Also, testing of brass-ash-derived ZnO on other bacteria is essential for further research.

Keywords: Zinc oxide, *Streptococcus mutans*, Antibacterial activity

Introduction

Brass, nowadays, is a widely-used alloy owing to its advantages, for example, excellent corrosive resistance, polishing and finishing characteristic, and easy manufacturing process[1]. Nevertheless, during smelting, a large portion of brass slags are formed, causing a leak into environment if not properly managed. When milled and pulverized, these slags are typically called "brass ash" or "brass fume", containing a large amount of ZnO, a smaller amount of CuO, and some other impurities—e.g., SiO_2 or PbO[2]. Despite their harmfulness, recovery of valuable compounds present is feasible through several methods[3-6].

As brass ash has a great deal of ZnO contents, when purified, applications of ZnO come into varieties. To illustrate, it is used in ceramic products, electronic compartments, additive in fertilizers or animal feed, vitamin supplements, and sunscreen[7]. In biomedical aspects, it is also applied to aid bio-imaging, bio-sensors, and delivery of drugs or genes[8]. Similarly, with or without the presence of light activation, ZnO, specifically in nanoscale, is synthesized to utilize its antimicrobial effect and it is proved to be effective by lots of researches[9-11]. Many of dental products are a good example of an application of this property: for instance, intermediate restorative material (IRM) applies the property in combination with the strength of ZnO as a temporary tooth restoration with bactericidal activity[12]. Endodontists are also another example to apply the powder as well as eugenol as a sealer during tooth obturation with the effect[13].

Major antibacterial mechanisms of ZnO are reported in the following 3 methods, including release of reactive oxygen species (ROS), Zn^{2+} release, and direct contact to bacterial cell. These mechanisms can cause damage to cell structures critically, leading to leakage of cellular contents and eventually cell death[9].

Even if ZnO is claimed to provide antibacterial effect against various species of bacteria, it is not yet elucidated that the one synthesized from brass ash and milled for different sizes are effective against *S. mutans*, the strain which is significantly attributed to dental caries among young population[14].

Caries mechanisms of *S. mutans* are initiated when acquired pellicle starts coating the tooth surface. This, then, allows some kind of bacteria, including *S. mutans*, to colonize and grow. The strain, in the presence of fermentable carbohydrates, is capable of producing acid, thereby creating acidic environment—which cariogenic bacteria favor, and demineralizing tooth structure—which, in the end, leads to dental caries[15].

As a result, this research is aimed initially to derive ZnO nanoparticles (NP) from industrial brass ash using chemical precipitation technique, subsequently to mill the powder under varied conditions so that the particles are at different sizes, and to test antibacterial activity of ZnO against *S. mutans*. using microdilution in the end[16,17].

Method and Experimental Procedure

1) Purification and Synthesis of ZnO

1.1) Analyze components contained

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in a sample of the obtained brass ash with XRF spectrophotometer (HORIBA, MESA 500-W) and its corresponding software (MESA).

1.2) 40 g of brass ash powder is then leached with 250 ml of 23% w/w NH_4Cl solution, producing the solution with undissolved precipitate. This step is done under constant stirring for 1 hour and a temperature of approximate 90ºC using a magnetic hot plate stirrer (Wisdom LABORATORY INSTRUMENTS, WiseStir).

1.3) A vacuum filter (IM-TECH, 2 Stages Vacuum Pump) is applied with suction flask and Buchner funnel to filter out the precipitate from step 1.2). Note that the filter papers (Whatman, FILTER PAPER 4) should be moistened with a few drops of water to ensure vacuum filtration.

1.4) Add 3 g of finely powered Zn metal to the remaining liquid part.

1.5) Add 5-10 drops of 1000 ppm polymethyl methacrylate (PMMA) as an anti-flocculating agent.

1.6) Again, a vacuum filter is applied to filter out the precipitate (occurred in 1.4).

1.7) The Zn-containing solution is left to cool at room temperature (25ºC to 35ºC) for another 2 hours.

1.8) Filter the suspension to collect the white precipitate formed.

1.9) Re-suspend the solid in water at around 90ºC with continuous stirring for a period of 1 hour.

1.10) After alcohol wash and repeated filtration, the solid is then dried at 70ºC in a hot air oven (memmert, UF 110) for 1 hour. The white precipitate in this step is expected to be ZnO.

1.11) Re-analyze the precipitate using x-ray fluorescence spectrophotometer for its elemental composition and x-ray diffractometer (PANalytical, X' Pert-PRO MPD) in combination with X'Pert High-Score Plus programme for its crystallographic parameters. Be reminded that commercial ZnO (M Dent, Zinc Oxide) is also included for examination as a reference.

2) Size Particulation for ZnO NP[18-20]

2.1) Measure initial particle size of purified ZnO powder using SEM (LEO, LEO 1450VP) with an aid of ImageJ programme.

2.2) Equally divide the mass of ZnO into 3 groups: one for no treatment (S_0) with commercial ZnO (C) as a reference, and the other two for 0.5-hour ($S_{0.5}$) and 1.0-hour milling time (S_1) orderly. Of the 2 groups, each will be milled using a high-energy ball mill under the following specifications: 125-ml ZrO_2 container, 0.3-mm-diameter ZrO_2 milling ball (275 g in total), spin at 1200 rpm, and 95% v/v Ethanol (C_2H_5OH) as medium.

2.3) To isolate milled particles from milling balls, a sieve of a size smaller than the milling balls is used. Here, a 250-micron mesh aperture is applied to complete this step.

2.4) It is suggested that the milled particles be kept in a hot air oven until they are dry or free of the milling media.

2.5) Similarly to 2.1), re-analyze the size distribution of milled ZnO particles from SEM images using ImageJ program.

3) Testing of ZnO NP for Antibacterial Activity against *S. mutans* Using Microbroth Dilution Method[16,17]

Antibacterial activity testing in this research is modified from the suggested guidelines and protocols from Clinical and Laboratory Standards Institute (CLSI) and uses MIC as a representation of antibacterial activity using microdilution[16,17]. For preparation of antibacterial agents in detail see Table 1

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Table 1 Selected scheme of preparation of antibacterial agents

Groups/ Conditions	Agents/Code	Solvents	Diluents	Concentration (µg/ml)
Group A: Primary Test and Report	**Ampicillin** T.P.DRUG LABORATORIES (1969), STERILE AMPICILLIN SODIUM, 100% equivalent to ampicillin	0.9% NaCl	0.9% NaCl	8
Group B: Primary Test and Report Selectively	**Vancomycin** SIGMA Life Science, Vancomycin hydrochloride from *Streptomyces orientalis*, 90% equivalent to vancomycin	Water	Water	16
Commercially Made	C	DMSO	DMSO	7200
Brass-Ash-Derived	S_0	DMSO	DMSO	7200
	$S_{0.5}$	DMSO	DMSO	7200
	S_1	DMSO	DMSO	7200

Abbreviations: NSS, normal saline solution; DMSO, dimethyl sulfoxide

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Results

1) XRF and XRD results

Table 2 XRF results

Type of ZnO	Result			
	Element	**Mass (%)**	**Oxide Form**	**Mass (%)**
Brass Ash	Zn	73.024	ZnO	90.897
	O	18.910	-	-
	Cu	3.335	CuO	4.175
	Cl	3.151	-	3.151
	Pb	1.191	PbO	1.283
	Ca	0.148	CaO	0.207
	Br	0.133	-	0.133
	Fe	0.107	Fe2O3	0.153
C	Zn	79.993	ZnO	99.573
	O	19.690	-	-
	Na	0.276	Na2O	0.373
	Fe	0.026	Fe2O3	0.037
	Ni	0.014	NiO	0.018
S_0	Zn	79.722	ZnO	99.235
	O	19.513	-	-
	Cl	0.765	-	0.765

From Table 2, XRF analysis of the ash manifests lesser percentage of ZnO (90.897%) when compared with that of C and S_0. XRF result also indicates that the reference powder contains 99.573% of ZnO by mass with some other compounds accounting for 0.427%. Likewise, that of S0 shows 99.235% purity with only 0.765% chlorine contamination.

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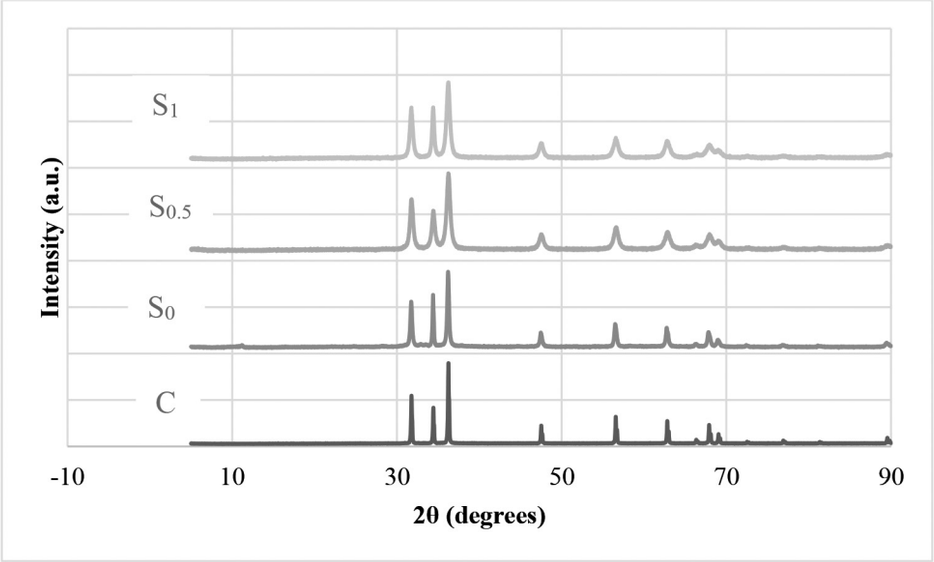

Figure 1 Stacked XRD results

The investigation is carried out with usage of Cu Kα radiation (1.5418740 Å) using continuous Gonio scanning mode at 2θ ranging from 4.9980 to 89.9999. After examination of 2 kinds of ZnO—one for reference (shown as blue, C) and the other 3 for synthesized ZnO (S_0, $S_{0.5}$, S_1), their XRD patterns match well with the reference peaks. These sorted diffractograms reveal that both types of ZnO are indexed greatly to hexagonal wurtzite structure with the same crystallographic parameters.

2) ZnO Size Distribution and Related MIC Interpretations

Table 3 ZnO size distribution with corresponding MIC

ZnO Category	Mean Size (nm)	Interpreted MIC (μg/ml)
C	194.450	112.5
S_0	123.991	112.5
$S_{0.5}$	104.410	56.25
S_1	76.221	28.125

Based on the information provided by Table 3, trend of MIC can be interpreted that when particle size of ZnO is reduced, its MIC, which represents the antibacterial activity, tends to increase. As the size is reduced to 76.221 nm, the MIC is optimal (28.125 μg/ml, n = 3)

Discussion and Conclusion

Chemical precipitation technique used to purify ZnO yields high content of ZnO precipitate up to 99% purification with its dimensions nearly reaching nanoscale[21]. To optimize the purification procedure, other remaining precipitates, acquired through vacuum filtration, should be purified

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additionally so that it makes purification by-products of use and applicable[3,4]. For analytical process, it is suggested that XRF and XRD examinations of these precipitates as well as the latter investigation for brass ash should be incorporated for detailed verifying methods. Note that every device and instrument used should be calibrated with its own reliable appliances before used, and the notion is also included for other steps performed in the experiment. For large scale production of ZnO from brass ash, even if this experiment exhibits cost-effectiveness in the process, productivity and time taken for synthesis, still, are supposed to be improved.

Mean size of milled ZnO is affected by total milling time the powder is milled in the machine[20]. Nonetheless, it cannot be concluded that the size is, as well, influenced by milling ball size, ball material, etc. Thus, the author recommends studying other effects on size of the compound for the purpose of generating guided milling conditions to produce desired particle size. As there are many shapes of obtained ZnO, when the size, especially in rod shape, is reported, the author is suggested that length of the particle should be measured and added to the result in addition to its width. For more convenient and acceptable way of size interpretation, the author advises that a particle size analyzer be applied[22]. This will improve the accuracy level of size distribution as crystallite size, not agglomerated particle size, is appraised. Finally, a change in color of ZnO from white to soft yellow is noticed together with an increase in surrounding temperature after milled. It is discussed that whether hydrogen incorporation, oxygen deprivation, or a combination of multiple factors is the cause of this thermo-chromic phenomenon of ZnO[23].

According to the evaluation of antibacterial activity against *S. mutans*, the compound at all sizes exhibits the activity. This is especially the greatest for ZnO powder with the smallest size compared (S_1), revealing that its optimal MIC is equal to 28.125 μg/ml. The MIC results of these powders correlate with those present in other literatures, which the value ranges from 0.156 to 806.18 μg/m[9,10]. However, the process of antibacterial testing used in those studies is completely not the same as the one used in this study, and minimal bactericidal concentration (MBC) is suggested to be incorporated. More replications as well as controls should be performed to acquire a more dependable result. Additionally, it is still dubious about how MIC is interpreted if there is no clear inoculated well present, as ZnO and the inoculum both can make the medium turbid. Another recommendation for this part of experiment is to apply CAMHB with supplemental 2 – 5% v/v LHB following the suggested protocol instead of MHB used here; yet, there some reasons why MHB is used as medium of choice for susceptibility testing. To begin with, it shows acceptable batch-to-batch reproducibility for susceptibility testing. In addition, not only does it support satisfactory growth of most pathogens, it is also low in inhibitors that affect the results of some drugs. In the last place, a large body of data and experience has been gathered about tests performed with this medium[16].

Further study in this field of research is suggested on in-vivo antibacterial testing or testing of a change in properties after ZnO NP synthesized from brass ash is integrated into dental products or some other medical applications as additional features of nanoparticles have been widely reported due to their larger surface area to volume ratio[5,8]. Besides, even if antibacterial activity of ZnO against various strains of bacteria is reported, the test of these brass-ash derivatives against other bacteria, except *S. mutans*, is somehow recommended[9-11].

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Acknowledgement

I would like to thank Dr. Arnon Jumlongkul and Associate Professor Durunee Wattanasiriwech, my research advisors and also Dr. Kamonnaree Chotinantakul, Dr. Pathamet Khositharattanakool, Dr. Sivaporn Sivasinprasasn, Dr. Siwaporn Praman, Dr. Surachet Woottisin and Dr. Thaneeya Hawiset, as Research Committee, for giving me plenty of instructions for this research.

My special thanks were extended to Mrs. Sirikarnnapa Krajangcharoensakul and Ms. Paweena Saleechanta of Scientific and Technological Instruments Center (STIC) for laboratory training in antimicrobial testing and bacterial preparation—Moreover, they included Ideal Chemicals (Thailand) Pvt. Ltd. for providing a satisfactory amount of brass ash used in the experiment at free of charge.

Eventually, I would like to thank my parents and my sister for their encouragement, care, willingness to help, as well as their financial support as usual throughout the study.

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