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Antibacterial activity of zinc oxide nanoparticles against *Vibrio harveyi*

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ABSTRACT

Antibacterial activity of zinc oxide (ZnO) nanoparticles against *Vibrio harveyi* was studied with different sizes of synthesised (S_70 and S_83) and commercially available (C_50 and C_100) ZnO particles. The synthesised nanoparticles were prepared without (S_70) and with poly vinyl alcohol (PVA) (S_83). The synthesised nanoparticles were characterised by X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy dispersive X-ray spectra (EDX). In XRD, the sharp intense peaks at 2θ confirm the crystalline structure of ZnO. Both synthesised and commercially available ZnO nanoparticles were compared with bulk ZnO particles at various concentrations for their antibacterial activity. ZnO nanoparticles showed enhanced antibacterial activity as compared to bulk ZnO and the antibacterial efficiency was indirectly proportional to size and directly proportional to concentration up to 100 ppm. Capping of ZnO nanoparticles with PVA did not show any significant improvement in the antibacterial property.

Keywords: Antibacterial activity, Aquaculture, *Vibrio harveyi*, Zinc oxide nanoparticles

Introduction

The increase in global demand for shrimps has led farmers to adopt intensive culture practices that are detrimental to pond water quality. Stress resulting from crowding and poor water quality leads to enhanced susceptibility of cultured shrimps to several bacterial and viral diseases (Colorni *et al.*, 1981; Ruangpan and Kitao, 1991; Venkateswaran *et al.*, 1991). One of the major bacterial diseases in shrimp aquaculture is luminous vibriosis, caused by Gram-negative luminous bacteria, most commonly *Vibrio harveyi* (Kraxberger *et al.*, 1990; Lightner *et al.*, 1992; Boonyaratpalin *et al.*, 1993; Owens, 1993; Liu *et al.*, 1996; Alvarez *et al.*, 1998). It is recognised as an opportunistic pathogen, which can become virulent under stress conditions resulting in large-scale mortality in hatcheries and in all stages of cultured penaed shrimps (Lavilla-Pitogo *et al.*, 1990; Jiravanichpaisal *et al.*, 1994; Manefield *et al.*, 2000; Vaseeharan and Ramasamy, 2003; Austin and Austin, 2007; Haldar *et al.*, 2010). The symptoms of vibriosis include loss of appetite, slow growth, high mortality and luminescence of the bodies of infected shrimps (Lavilla-Pitogo *et al.*, 1990; Jiravanichpaisal *et al.*, 1994; Karunasagar *et al.*, 1994).

Antibiotics have been widely used in shrimp aquaculture to combat vibriosis (Baticados and Paclibare, 1992). However, there has been public concern over the use of antibiotics as they can lead to the development of drug resistant bacteria, thereby reducing drug efficacy. Moreover, the accumulation of antibiotics both in the environment and in shrimp tissue is potentially risky to consumers and the environment (Alderman and Hastings, 1998). Such adverse effects have prompted scientists to explore alternatives to replace antibiotics in controlling diseases in shrimp.

Plant based products, extracts from tropical plants (Wei *et al.*, 2008) and plant oils have been traditionally used to control bacterial infections as alternatives to antibiotics. Organic antibacterial material is often less stable particularly at high temperatures and pressures compared to inorganic material (Sawai, 2003). As a consequence, inorganic materials such as metal and metal oxides have attracted much attention over the past decade due to their ability to withstand harsh process conditions (Wang *et al.*, 1998; Hewitt *et al.*, 2001; Fu *et al.*, 2005; Makhluf *et al.*, 2005; Zhang *et al.*, 2007).

Recently, the use of nanoparticles has caught the attention of researchers with a wide variety of successful applications in various sectors. They have been successfully used in removal of metabolites and contaminants from polluted water (Joo and Cheng, 2006;

Kim *et al.*, 2006; Celebi *et al.*, 2007). There are studies on silver nanoparticles against shrimp pathogenic bacteria *viz.*, *Vibrio harveyi* (Vaseeharan *et al.*, 2010) as well as on oxides of aluminium, iron, magnesium, cerium and zirconium nanoparticles (Ravikumar *et al.*, 2011).

Among the inorganic metal oxides used as nanoparticles, ZnO nanoparticles exhibit strong antibacterial activities against a broad spectrum of bacteria (Sawai, 2003; Adams *et al.*, 2006; Huang *et al.*, 2008; Jones *et al.*, 2008). It has been found to have bactericidal property against *Salmonella* (Jin *et al.*, 2009), *Streptococcus, Staphylococcus* (Hunang *et al.*, 2008) and *E. coli* (Zhang *et al.*, 2007; Padmavathy and Vijayaraghaven, 2008). ZnO nanoparticles and nanowires are nontoxic, biosafe and biocompatible as it degrades into mineral ions and absorbed by the body with in few hours (Zhou *et al.*, 2006). There is limited research on the antibacterial activity of metal oxide nanoparticles on shrimp pathogenic bacteria. Hence, this study aims at evaluating ZnO nanoparticles for their antibacterial property against *Vibrio harveyi.*

Materials and methods

Preparation of nanoparticles

In this study, antibacterial activity of two commercially available zinc oxide nanoparticles (C_50 and C_100) and two synthesised ZnO nanoparticles, without capping (S_70) and with PVA capping (S_83) were evaluated against *Vibrio harveyi*. ZnO nanoparticles of C_50 and C_100 were purchased from SIGMA and the primary size of the nanoparticles given by manufacturer were <50 nm and <100 nm.

ZnO nanoparticles of S_70 and S_83 were prepared by hydrolysis method (Huang *et al.*, 2008). Ten grams of zinc acetate (99%) was dissolved in ethylene glycol medium by heating to 80°C. Under vigorous stirring, 50 ml water was added and the solution was heated to 95°C. The temperature was maintained for 4 h to allow ZnO nanoparticles to precipitate (S_70). In order to increase the stability of synthesised nanoparticles, 0.05M PVA was added as capping material to zinc acetate in ethylene glycol medium as described above. The mixture was then heated under stirring to a temperature of 90°C to get capped ZnO nanoparticles (S_83).

Bacterial cultures

For antibacterial experiments, *V. harveyi,* was selected as the target organism. All materials and petri-plates were autoclaved before the experiment. The pathogenic bacteria *V. harveyi* was isolated from aquaculture sediment and cultured using the selective medium Thiosulfate-citrate-bile salts-sucrose agar (TCBS). The bacterial strain was identified using standard biochemical methods (Holt *et al.*, 1994) and confirmed by molecular methods (Oakey *et al.*, 2003). The density of bacterial cells in the liquid culture was estimated by optical density (OD) measurements at 600 nm and was maintained at 0.75 ± 0.05 - 0.8 ± 0.05, which is the ideal optical density of the cells (Padmavathy and Vijayaraghaven, 2008).

Antibacterial test

The relative antibacterial activity of ZnO suspensions of commercially available (C_50 and C_100) and synthesised nanoparticles (S_70 and S_83) were studied under different concentrations. Preliminary trials were conducted with different concentrations between 10 and 1000 ppm with 100 ppm intervals to decide Minimum Inhibitory Concentration (MIC) level. The efficiency of selected concentrations was compared by zone of inhibition test by well diffusion method. For this, 20 ml of Tryptone Soya Agar (TSA) was poured in well-rinsed autoclaved petriplates and 1.0 ml of active bacterial culture (0.78 OD) was homogeneously spread in the agar plates. Selected concentrations of ZnO nanoparticles solution (40µl) were filled in the wells, prepared by cutting the agar by gel puncture. The plates were incubated at 37°C for 18-22 h. The zone size was determined by measuring the radius of the zone.

Statistical analysis

One way analysis of variance was used to determine the significance ($p<0.05$) on bactericidal efficiency between different concentrations and particle sizes. Analysis was done using SPSS (V) 16 (Statistical Package for Social Sciences, Chicago, IL, USA) software package.

Results and discussion

Confirmation of ZnO nanoparticles

Structural characterisation of synthesised nanoparticles was studied by X-ray diffraction (XRD) at Anna University, Chennai. The sharp intense peaks of ZnO (S_70) at 2θ values of 31.64, 34.33, 36.08, 47.21, 56.16 62.49 and 67.49 deg corresponding to (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3) and (1 1 2) planes of ZnO (Fig. 1a) confirmed the crystalline nature of ZnO. Similarly the sharp intense peaks of ZnO with capping material (S_83) at 2θ values of 31.74, 34.36, 36.17, 47.32, 56.30, 62.48, 65.97, 67.52 and 68.65 deg corresponds to (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0), (1 1 2) and (2 0 1) planes of ZnO (Fig. 1b) confirmed the crystalline structure of ZnO.

More information on the shape, size distribution and morphology of the synthesised nanoparticles were analysed using scanning electron microscopy (SEM)

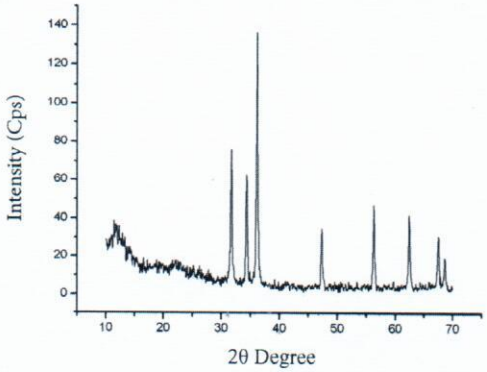


Fig. 1a. XRD pattern of S_70 ZnO NPs

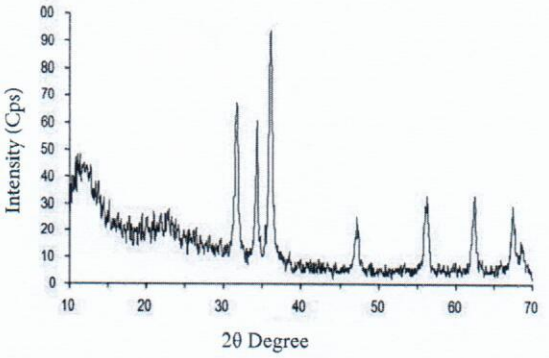


Fig. 1b. XRD pattern of S_83 ZnO NPs

at Anna University, Chennai Fig. 2 (a) and Fig. 2 (b) shows the SEM images of the synthesised particles without capping (S_70) and with capping material (S_83) respectively.Size of nanoparticle ranged from 50-85 nm and 70-90 nm with mean size of 70 nm and 83 nm for nano ZnO and nano ZnO with capping material (PVA) respectively.

The elemental composition of the synthesised sample was confirmed by energy dispersive x-ray spectra (EDX) (Fig 3a and 3b). The spectra showed that zinc content was more in S_70 (61.48%) as compared to S_83 (46.89%) and this difference significantly contributed in its antibacterial activity.

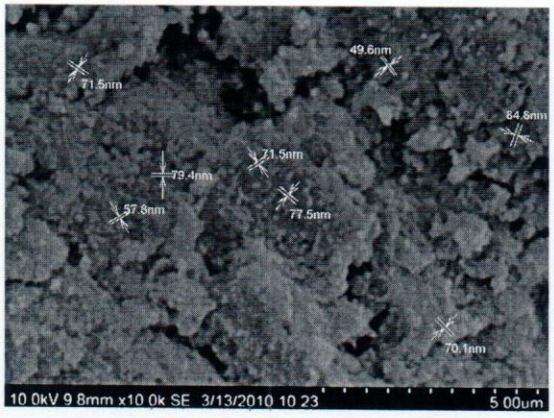


Fig. 2a. SEM image of S_70 ZnO NPs

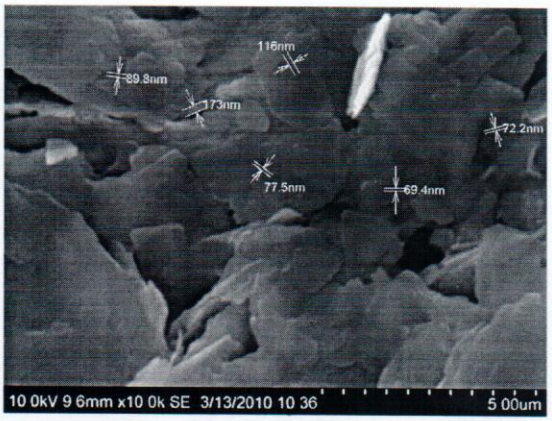
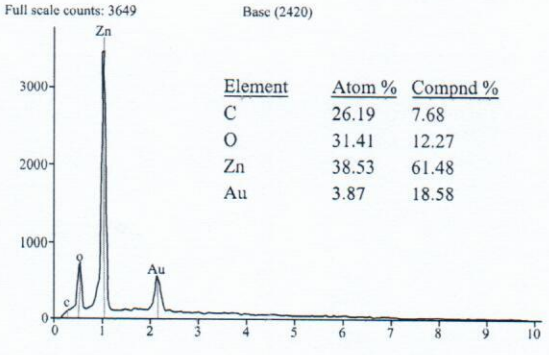
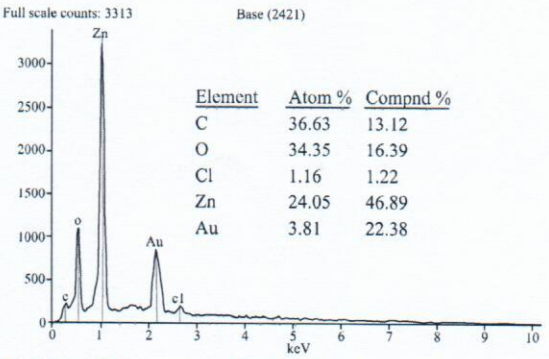


Fig. 2b. SEM image of S_83 ZnO NPs



Element	Atom %	Compnd %
C	26.19	7.68
O	31.41	12.27
Zn	38.53	61.48
Au	3.87	18.58

Fig. 3a. EDAX Spectrum of S_70 ZnO NPs



Element	Atom %	Compnd %
C	36.63	13.12
O	34.35	16.39
Cl	1.16	1.22
Zn	24.05	46.89
Au	3.81	22.38

Fig. 3b. EDAX Spectrum of S_83 ZnO NPs

Antibacterial activity of ZnO nanoparticles

The presence of an inhibition zone in well diffusion assay clearly indicates the biocidal action of ZnO by disrupting the cell membrane. The rupture of bacterial cell wall could be due to the surface activity of ZnO in contact with the bacterial membrane surface (Zhang *et al.*, 2007; Jiang *et al.*, 2009). The contact between nanoparticles and bacterial cell is initiated by surface charges on the particle (Neal, 2008) and the electrostatic interaction between the bacterial surface and nanoparticle (Stoimenov *et al.*, 2002). This interaction was confirmed by Zhang *et al.*, (2010) through electrochemical measurements.

After the contact with bacterial membrane, high rate of surface oxygen species generated from ZnO nanoparticles (Yamamoto *et al.*, 2000) leads to the death of bacteria by chemical interactions between hydrogen peroxide and membrane proteins (Zhang *et al.*, 2010). The damage to the cell membrane directly leads to the leakage of cytoplasmic contents (Sharma *et al.*, 2010), minerals, proteins and genetic materials causing cell death (Liu *et al.*, 2009).

Among the concentrations studied in the preliminary trials, there was no consistent increase in antibacterial activity of ZnO nanoparticles beyond 100 ppm. Hence, further trial was restricted to concentrations 10 and 100 ppm. The zone of inhibition test by well diffusion method indicated that both commercially available (C_50 and C_100) and synthesised nanoparticles (S_70 and S_83) exhibited antibacterial property at 100 ppm whereas at 10 ppm, only commercially available ZnO of 50 nm (C_50) had significant antibacterial property (Fig. 4). The bactericidal difference in the concentration might be due to the difference in generation of H_2O_2 in ZnO and this generation was linearly proportional to their ZnO particle concentration (Sawai *et al.*, 1996). The variation in the concentration of H_2O_2 under different concentration of ZnO nanoparticles were observed by Yamamoto *et al.* (2000).

Size of nanoparticles is found to play a vital role in determining the antibacterial activity. In this study, smaller size of nanoparticles showed higher bactericidal activity. This is supported by the finding of Padmavathy and Vijayaraghavan (2008). Among the nanoparticles, the efficiency of antibacterial property at 100 ppm concentration varied with respect to size of the particles. The maximum efficiency was observed for C_50 followed by S_70, S_83 and C_100. The efficiency of C_50 is significantly ($p<0.05$) higher when compared to other nanoparticles (Fig. 4).

The higher bactericidal activity of smaller sized nanoparticles could be due to the large surface area to volume ratio and the surface activity of ZnO. The

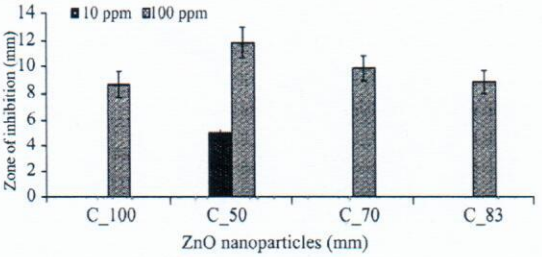


Fig. 4. Mean of inhibition zone diameters (mm) with SD in well diffusion assays of ZnO nanoparticles

generation of hydrogen peroxide (H_2O_2) depends strongly on the surface area of ZnO which results in more oxygen species on the surface and the higher antibacterial activity of the smaller nanoparticles (Yamamoto *et al.*, 2008). The use of poly vinyl alcohol (PVA) as a capping agent to increase the stability of nanoparticles and control the particles sizes did not show any significant improvement on antibacterial activity. Similar result was observed by Zhang *et al.* (2010).

To study the enhanced antibacterial property of ZnO nanoparticles, it was compared with bulk ZnO and the results showed that C_50 has 51.7% more efficiency over bulk, followed by S_70, S_83 and C_100 (Table 1). This could be due to the fact that nanoparticles are more abrasive in nature than bulk ZnO, thus contributing to the greater mechanical damage to the cell membrane resulting in enhanced bactericidal effect (Padmavathy and Vijayaraghavan, 2008).

Table 1. Enhanced antibacterial activity of nanoparticles at 100 ppm (% efficiency over bulk)

Commercially available ZnO NPs		Synthesised ZnO NPs	
C_100	C_50	S_70	S_83
34.1	51.7	42.3	35.6

Synthesised nanoparticles were confirmed by their characteristics. Both commercially available and synthesised zinc oxide nanoparticles have enhanced antibacterial property over bulk and their efficiency is indirectly proportional to size and directly proportional to concentration upto 100 ppm. The results of the present study clearly indicate that antibacterial property of ZnO nanoparticle has the potential to control *Vibrio harveyi* in aquaculture systems.

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