AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

In vitro antagonistic properties of copper nanoparticles and probiotic *Bacillus subtilis* against pathogenic luminescent *Vibrio harveyi*

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Abstract. Aquaculture industry especially shrimp farming in Iran and other parts of the world has been facing various disease that among various agents, pathogenic bacteria has emerged as important because it causes large economic losses to the shrimp farming industry. This study investigated the antagonistic properties of copper nanoparticles (34 mg/L), probiotic *Bacillus subtilis* (10^8 CFU/mL), and combination of these two against with pathogenic bacteria, luminescent *Vibrio harveyi* in vitro condition in order to investigate possible use of probiotic *B. subtilis* and copper nanoparticles in the diet for growth and immunity enhancement in *Litopenaeus vannamei*. This study reports that in Well diffusion test and Disc diffusion test against pathogenic bacteria luminescent *V. harveyi* maximum inhibition showed by combination of suspension of probiotic *B. subtilis* and copper nanoparticles the amount of (W = 22.54±1.72 and D = 16.37±2.9) whereas minimum inhibition was copper nanoparticles the amount of (W & D = 0). Results indicated the possible synchronic use of probiotic *B. subtilis* and copper nanoparticles in shrimp diet.
Key Words: Well diffusion test, Disc diffusion test, immunity, shrimp diet, *L. vannamei*.

Introduction. Shrimp aquaculture is a major industry throughout many countries in the Asia-Pacific region and Latin America. FAO has reported disease outbreaks as a major inhibitor factor for development of aquaculture industry worldwide (Alexandra 1991; Lavilla-Pitogo et al 1998; Martin et al 2004; Subasinghe et al 2001).

White shrimp *Litopenaeus vannamei* (Boone 1931) was considered an important farmed species. Since 2003, it ranks first in the production of farmed species. Accordingly, due to the low costs of food and high compatibility to be gradually replaced by other species were widely farmed in the world (FAO 2005).

In the recent two decades, aquaculture and specifically shrimp culture rapidly developed. Currently, the aquaculture industry in Iran and other parts of the world has been facing serious problems due to microbial and viral diseases. Vibriosis, especially luminous disease, has caused serious loss in shrimp hatcheries (Mirbakhsh et al 2013) that leaded to the use of commercial antibiotics for the treatment and prevention of shrimp diseases resulting in undesirable effects and there were followed hidden negative results. Among the main problem arises for example the issue of antibiotic resistance. Thus, the demand for environment-friendly sustainable aquaculture is increasing (Gatesoupe 1999) and following the use of materials to enhance the growth and immune response of aquatic organisms. In the recent two decades, for preventing aquaculture problems, probiotics coming as a solution in this industry. Probiotics are non-pathogenic microorganisms that can be used instead of antibiotics as a bio-control agent (Fuller 1978; Gatesoupe 1999; Mishra et al 2001). The mechanism of action of probiotics has been investigated, which improving the of host life quality and prevent from infectious diseases, but the mechanism is not precisely specified. Probiotics in aquaculture have

been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species; and enhancement of nutrition of host species through the production of supplemental digestive enzymes (Thompson et al 1999; Verschuere et al 2000). One of the most recommended bacterial probiotics in shrimp culture belonged to *Bacillaceae* family (Ziaei-Nejad et al 2006; Liu et al 2010; Jiqiu et al 2009). *Bacillus* spores is used as a bio-control agent to cut down vibriosis in shrimp culture industry (Rengipipat et al 2000; Skjermo & Vadstein 1999) and its use is recommended in the diet of shrimps.

Antagonistic activity is a common phenomenon in the bacterial world. Bacterial species bacterial prevent of growth and spread other micro-organisms. Probiotics such as *Bacillus subtilis* produced Tyrothricin, Bacitracin, Gramicidin S, and Polymyxin polypeptides against to a wide range of positive and negative gram bacteria also that by showing the inhibition effect on pathogenic vibrio. Of course main reason for antagonistic activity of *Bacillus* is producing antimicrobial proteins and antibiotics (Perez et al 1993).

The history of the probiotics use as a food supplement for the cultural animals refers to decade of 1970, and in fact the use of probiotics or beneficial bacteria to replacing with existing pathogens in culture by antagonistic processes. Antagonism of *Bacillus* bacteria and luminescent vibriosis pathogenic agent in *in vitro* conditions have been reported by many researchers (Gullian et al 2004; Pai et al 2010).

But another method to deal with pathogenic agents from far time, is the use of heavy metals as a disinfection method to prevent growth of microorganisms (Kim 1998). With the expansion of human knowledge in various fields and the emergence of nanotechnology, significant progress in this case has been made and it is because they are made of nanoparticles with diameters less than 100 nm, the surface area is increased and it also has anti-microbial properties which has increased more than 99%. Due to the high antimicrobial effects of this substance, have been recently used in many commercial-medical products, and of course this new method is also considered in aquaculture. Nanoparticles with regard to its unique physical and chemical properties, is used in the many fields as biological, environmental and aquaculture. Therefore attracted much attention from scientists and researchers but to choose of functional nanoparticles is important that nanoparticles used to have any adverse effect on the aquatic medium.
Scientist from the Russian Academy of Sciences have reported that young carp and sturgeon exhibited a faster growth rate (30% and 24% respectively) when they were fed iron nanoparticles (Prochorov et al 2002).

Crustaceans have haemocyanin containing copper as oxygen-carrying pigment in haemolymph. Copper has functions in hematopoiesis and in numerous copper-dependent enzymes, including lysyl oxidase, cytochrome c oxidase (CCO), ferroxidase, tyrosinase, and superoxide dismutase (SOD). In this research the copper nanoparticles have been used, because they have important activities in crustaceans such as respiratory and antioxidant activity (Dallinger 1977). Davis et al (1993) reported that requirement of *L. vannamei* in the basal diet containing 2 mg copper kg^{-1} of diet, copper is not enough to meet the physiological needs of shrimp. Their results showed that because the copper from seawater can't provide physiological needs for western white shrimp, a source of food needs for maximum growth and adequate mineral structure, which with a diet of 34 mg copper kg^{-1} supply lead to weight gain and increase copper content in hepatopancrase of shrimp. So copper nanoparticles in addition to the lack of a negative effect in shrimps can have a positive effect on growth.

The suggested use of copper nanoparticles in the diet of shrimp to improve growth and survival of juvenile shrimp is an important issue that needs to be reviewed and laboratory studies are needed. Copper is the main element which oxygenating haemolymph in shrimp, belonging to the group of micro-nutrients and heavy metals. Until now non research has been reported on the use of copper nanoparticles in shrimp diets. Given to the emerging nature of nanotechnology yet has not been done accurate assessment of possible dangers on other biological systems, so therefore before performing this propose *in vivo* conditions is needed to study and investigate *in vitro* conditions.

About bactericidal effect of nanoparticles, Rupareli et al (2008) reported the antimicrobial properties of silver nanoparticles with size of 3.32 nm, copper nanoparticles with a size of 9.25 nm with various concentrations against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. Was determined that antimicrobial activity of silver nanoparticles in comparison with copper nanoparticles were 40-50 % higher and also that bactericidal efficiency of nanoparticles depends on many factors and is not only dependent on the structure of the bacterial cell membrane, which leads to the conclusion of more needs for research.

The aim of this study was to investigate the effect of copper nanoparticles with the proposed concentration of the probiotic *B. subtilis* spores and viable cells of *B. subtilis* in the shrimp diets, and to determine that use of combination of these two components, and to see if despite to the bactericidal properties of nanoparticles it will lead lower the effect of probiotic *B. subtilis* or not? The aims also include comparison and evaluation of the antagonistic effects of probiotic *B. subtilis* and copper nanoparticles, and the combination of these two against pathogenic bacteria luminescent *Vibrio harveyi* isolated in shrimp farm in Bushehr in *in vitro* conditions.

Material and Method. The strains used in this work were a virulent strain of luminescent *V. harveyi* isolated from infested shrimps from the Iran Shrimp Research Center. Biochemical tests and partial 16S rDNA gene sequence analyses as a pathogenic strain of *V. harveyi* was approved for studies by the gene bank of Iranian Research Organization for Science and Technology (IROST), and an identification code was prepared (PTCC 1755).

Strain of *B. subtilis* as a probiotic bacteria used in this study was purchased from Biochem Germany Company.

The medium required in this work were TSA (Trypticas Soy Agar), TSB (Trypticase Soy Broth), NA (Nutrient Agar), and MHA (Mueller Hinton Agar). The bactericidal effect of *Bacillus* and nanoparticles against *Vibrio*, have been performed in Muller Hinton Agar medium, because the amount of inhibitor factors such as sulfonamides, trimethoprim and tetracycline is very low and also the amount of cations Ca^{2+} and Mg^{2+} is regulated in this medium.

Copper nanoparticles were purchased from the Iranian Nano-materials Company, with an average size of 18.36 nm and generally spherical shape, a product of American US Nano.

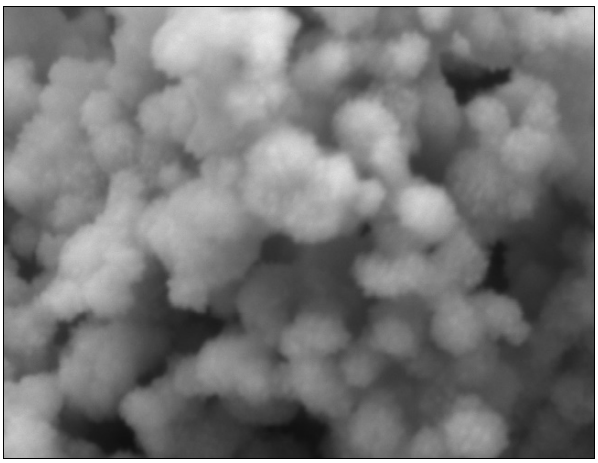

Figure 1. SEM's Copper Nanoparticles.

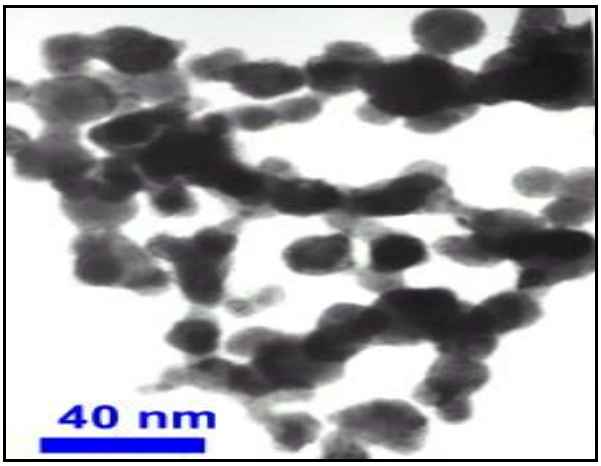

Figure 2. TEM's Copper Nanoparticles.

Copper nanoparticles suspension with density of 34 mg L^{-1} was utilized, prepared with normal saline solution for 1 hour in a shaker to obtain a homogeneous suspension.

For production of active culture from pathogenic bacteria of luminescent *V. harveyi*, culture was initiated on the TSA medium with 2.5% NaCl, with incubation for 24 hours at 30°C, then colonies was obtained by salt water (2.5% NaCl) dissolved to a

concentration reached half McFarland, and to product suspension of probiotic spores of *B. subtilis* with concentration of 10^8 CFU mL^{-1} which was dissolved in the NSS.

The combination of copper nanoparticles with probiotic *B. subtilis* (as spores or bacterial extract), were prepared with concentration of 34 mg copper nanoparticles L^{-1} and *B. subtilis* 10^8 CFU mL^{-1}.

To obtain cell-free extracts (culture supernatant fluid), the spore suspension of *B. subtilis* on NA medium was incubated for 24 hours, bacterial colonies were transferred to trypticas soy broth and then incubated for 72 hours in a shaking incubator at 30°C. *B. subtilis* culture (3 days old) was centrifuged at 9600 rev min^{-1} for 15 min (Vaseeharan & Ramasamy 2003) and the supernatant fluid was filtered through a 0.22 µm membrane filter (Sartorius, Bedford, MA, USA).

Challenge test: challenged of *B. subtilis*, copper nanoparticles and combination of these two with luminscent *V. harveyi* by Well diffusion test and Disc diffusion test.

1. Challenged spores of *B. subtilis*, copper nanoparticles and combination of these two with luminescent *V. harveyi:* After preparing the suspension of luminescent *V. harveyi* with concentration of 0.5 Mc Farland, a culture on MHA was performed. In Well diffusion test, the wells created then 50 µl spore suspension of probiotic bacteria *B. subtilis*, and copper nanoparticles and combination of these two was added in to wells. In disc diffusion test, discs with approximately 6 mm diameter impregnated with the suspensions were placed on MHA medium.

2. Challenged extract of live *B. subtilis*, copper nanoparticles and combination of these two with luminescent *V. harveyi:* Almost alike the previous challenge with the exception that in this case we used the cell-free extract instead of spore suspension and a volume of 50 µl of *Bacillus* cell-free extracts was introduced into the wells of the agar medium or on paper disc and incubated for a period of 24–48 h at 30°C (Mishra et al 2001).

At the first all cultured mediums were incubated for 2 hours at 15°C and then for 24-72 hours at 30°C. Antibacterial activity was defined as the diameter (mm) of the clear inhibitory zone formed around the well and disc.

The results were analyzed by the One-Way ANOVA test and Duncan Post-Hoc test to determine differences ($P<0.05$) between testing groups. All statistics were performed with IBM SPSS for Windows, version 19 (SPSS Inc, Chicago, IL, USA).

Results and Discussion. The results of well diffusion test showed that probiotic spores of *B. subtilis* (22.3±2.6 mm), and the combination of *B. subtilis* and copper nanoparticles (22.9±1.7 mm) have a significant inhibition activity against luminescent *V. harveyi* in comparison with copper nanoparticles (Figure 3 & Table 1), however, these results were confirmed in disc diffusion test.

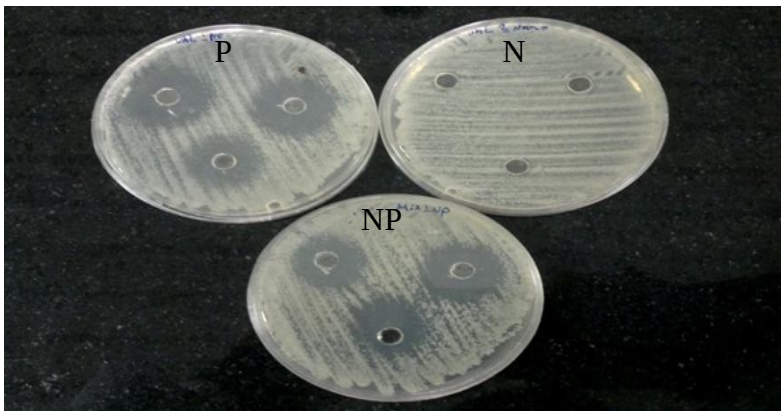

Figure 3. Inhibition zones around the wells after 24 hours.

Investigating the inhibitory diameter zone around wells after 72 hours, were indicated reduction in antibacterial activity against *Vibrio* bacteria (Figure 4).

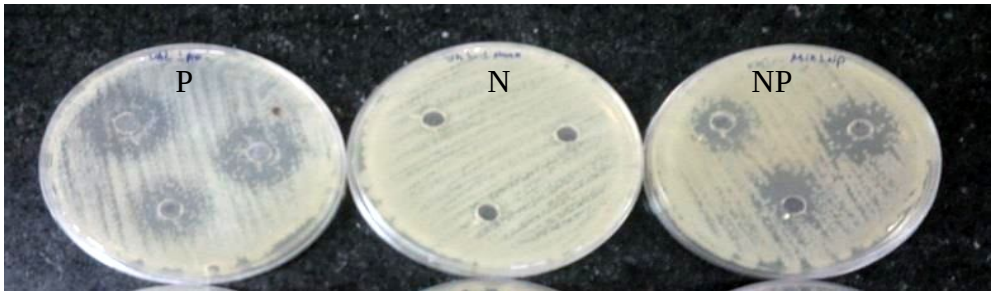

Figure 4. Inhibition zones around the wells after 72 hours.

In challenge extract, probiotic bacteria *B. subtilis*, copper nanoparticles and copper nanoparticles combined with probiotic bacteria against luminescent *V. harveyi,* specific inhibition zones were not formed around the wells of containing copper nanoparticles but the combination of probiotic and copper nanoparticles against luminescent *V. harveyi* showed significant antagonistic effect.

Table 1

Antagonistic properties of probiotic *B. subtilis*, copper nanoparticles, and the combination of these two against *V. harveyi luminescent*

Suspensions	*Diameter of inhibition zone (Mean±SD)*
Spores of *B. subtilis*	W= 22.3±2.6^{a}; D= 12.89±3.28^{A}
Extract of *B. subtilis*	W= 20.29±0.74^{a}; D= 12.22±1.27^{A}
Spores of *B. subtilis &* copper nanoparticles	W= 22.9±1.7^{a}; D= 13.1±0.4^{A}
Extract of *B. subtilis &* copper nanoparticles	W= 22.54±1.72^{a}; D= 16.27±2.9^{A}
Copper nanoparticles	W= 8.73±0.47^{b}; D= 0^{B}

W= Well diffusion test, D= Disc diffusion test.

Results of the investigation indicated that probiotic suspension and combination of probiotic and copper nanoparticles showed significant difference toward copper nanoparticle suspension. This indicated that these two had more antagonism activity against luminescent *V. harveyi*, but comparison of the probiotic suspension and copper nanoparticles suspension showed no significant differences (Table 1).

In this research, as well as studies of other researchers (Jiqiu et al 2009; Mirbakhsh et al 2013; Ziaei-Nejad et al 2006) on the antagonistic activity of probiotic bacteria *B. subtilis* on exposure to pathogenic *Vibrio* bacteria luminescent *V. harveyi* emphasize a significant antagonistic effect. As well as Yilmaz et al (2006) reported that Bacillus species have a wide range of antimicrobial activities against gram-positive and gram-negative bacteria, and eventually can inhibiting pathogenic *Vibrio* bacteria such as luminesent *V. harveyi*.

In the exposure of copper nanoparticles suspension against luminescent *V. harveyi* was determined that copper nanoparticles have a slight antagonistic activity against pathogenic bacteria. This is possible, because of the low concentration of copper nanoparticles in suspension. But the highest antagonistic effect against luminescent *V. harveyi* bacteria was obtained by suspension combination of probiotics *B. subtilis* and copper nanoparticles. In this study we used the copper nanoparticles (with proposed concentration in the diet of shrimp) in combination with probiotic bacteria *B. subtilis* against pathogenic bacteria luminescent *V. harveyi* which is among the first studies and previous research has not been performed in this regard.

A better efficacy of probiotic bacterium *B. subtilis* against pathogenic bacteria *Vibrio* is possible because copper nanoparticles have increased the antagonistic effect of probiotic bacteria against pathogenic bacteria. So that the copper nanoparticles showed slight antagonistic effect against pathogenic bacteria luminescent *V. harveyi*. The same slight effect is possible thereby increasing inhibition of luminescent *V. harveyi* bacteria by the combination of copper nanoparticles and probiotic *B. subtilis* suspension.

According to Diaz-Visurraa et al (2011), the mechanism of metal nanoparticles when they are attached on the bacterial cell surface could be described as a two phase process:
1) Physical phase: binding the nanoparticles to cell wall, the disaggregation of the exopolysaccharide matrix, cell separation, elongation and their rearrangement into small clusters;
2) Time-dependent molecular and cellular phase: the disruption of cell membrane is become predominant and increase roughness of the predominant cell membranes, amorphous mass with the perforation of the cell wall ultimately causes the release of intracellular material.

However, the mechanisms of metal nanostructure in bacteria cell which causing structural changes still not clearly elucidated. Therefore it could be possible that the effect of copper nanoparticles on bacteria *B. subtilis* have contributed to the release of intracellular material and leaded to increased antagonistic effect of probiotic *B. subtilis* against luminescent *V. harveyi*.

Conclusions. According to the results of this study, the antimicrobial properties of copper nanoparticles in combination with bacteria *B. subtilis* is used as probiotic in shrimp diet not only exhibited antagonistic effect of probiotics *B. subtilis* on exposure to luminescent *V. harveyi* bacteria but also increased the antagonistic effect of probiotic bacteria, although the differences according to Duncan's multiple mean test was not significant but gives the possibility of a concurrent use of the probiotic *B. subtilis* and copper nanoparticles in shrimp diet for research *in vivo* conditions.

Acknowledgements. This work was supported by Behbahan Khatam alanbia University of Technology, Iran.

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Received: 03 May 2015. Accepted: 12 June 2015. Published online: 22 June 2015.
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How to cite this article:
Ziaei-Nejad S., Salehi L. M., Ghaednia B., Johari S. A., Aberomand A., 2015 *In vitro* antagonistic properties of copper nanoparticles and probiotic *Bacillus subtilis* against pathogenic luminescent *Vibrio harveyi*. AACL Bioflux 8(3):445-452.