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Chitosan-oligo-saccharide-ZnO-nanoparticle composite: A superior alternative to chitosan for biomedical applications

S. Visnuvinayagam[1]*, V. Murugadas[1], L.N. Murthy[2,3], A.A. Zynudheen[1], D. Karthikeyan[4] and R. Atchudan[5,6]

[1]*ICAR-Central Institute of Fisheries Technology, Cochin-682 029, India*
[2]*National Fisheries Development Board, Hyderabad, Budwel-500 052, India*
[3]*Mumbai Research Centre of ICAR-Central Institute of Fisheries Technology, Mumbai- 400 703, India*
[4]*Sapthagiri College of Engineering, Periyanahalli, Dharmapuri-635 205, India*
[5]*Department of Chemistry, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Chennai-602105, India*
[6]*School of Chemical Engineering, Yeungnam University, Gyeongsan, 38541, South Korea*

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***Corresponding Author Email :** visnuvinayagam@yahoo.co.in ***ORCiD:** https://orcid.org/0000-0001-9989-5374

Abstract

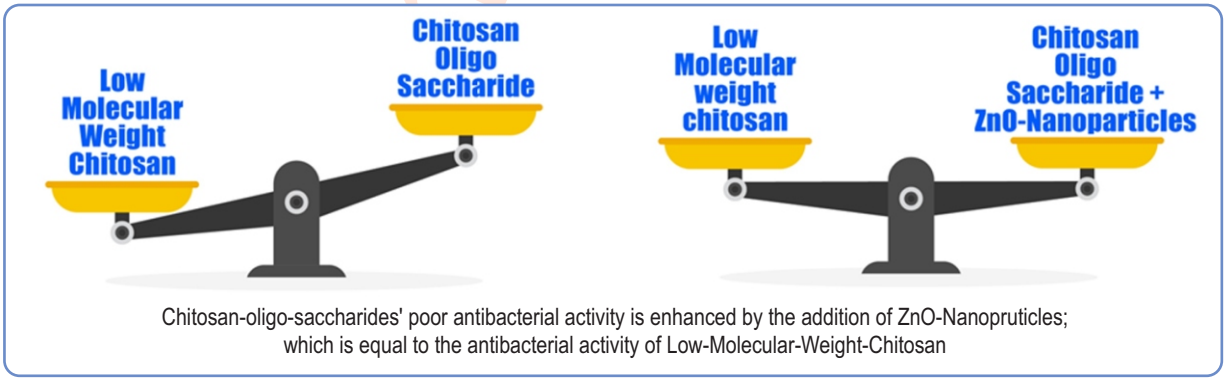
Aim: The present study was designed to improve the antibacterial activity of Chitosan Oligo-Saccharide (COS) by incorporating Zinc Oxide nanoparticles (ZnO-NPs). Additionally, the impact of various organic acids on chitosan (CH) was also examined.

Methodology: ZnO-NP was synthesised by sol-gel method and its nano-size was confirmed through various techniques such as UV-spectra, Scanning Electron Microscope and Fourier-transform infrared spectroscopy. Thereafter, the ZnO-NP-CH composites were prepared with a different molecular weight of chitosan, *i.e.,* COS, low, medium and high molecular weight of chitosan. The antibacterial activity of chitosan, including its ZnO-NP composite, was evaluated using different molecular weights with constant pH and deacetylation percentage. Additionally, chitosan was prepared in various organic acids to identify a suitable solvent.

Results: Most of the resistant bacteria became susceptible after the addition of ZnO-NP in all different molecular weight chitosan.

Interpretation: Chitosan Oligo-Saccharide antibacterial activity was improved at par with low molecular weight chitosan. Hence, the COS-ZnO-NP composite possessed a high antibacterial property of water-soluble nature, which will be useful for more food and biomedical applications.

Key words: Antibiogram, Chitosan, Nanoparticles, Zinc oxide



Chitosan-oligo-saccharides' poor antibacterial activity is enhanced by the addition of ZnO-Nanopruticles; which is equal to the antibacterial activity of Low-Molecular-Weight-Chitosan

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Introduction

Chitosan (CH) is a glucosamine polymer known for its wide range of antibacterial and antioxidant properties (Abd El-Hack *et al.,* 2020). Due to its superior antimicrobial and biocompatible properties, researchers have reported encouraging findings regarding the extension of food product shelf life (Jeyakumari *et al.,* 2016) and its potential in biomedical applications (Wang *et al.,* 2020). Therefore, chitosan and its derivatives have achieved the self-affirmed generally Recognised as Safe (GRAS) status (GRN No. 443). The percent of degree of deacetylation and the molecular weight are important factors that contribute to enhanced antibacterial activity, as highlighted in a study by Kumirska *et al.* (2011). The majority of chitosan products found in the market have a deacetylation percentage of approximately 75% (Ospina *et al.,* 2015). A higher deacetylation percentage is associated with improved antibacterial activity, as demonstrated by Divya *et al.* (2017). Nevertheless, increasing the deacetylation percentage requires significant amount of chemicals, which can impose a substantial burden on the environment (Yadav *et al.,* 2019).

In this study, the antimicrobial activity of chitosan was enhanced by incorporating ZnO-NP. However, the antibacterial activity of chitosan can vary depending on its molecular weight (Meng *et al.,* 2012). According to a study by Naveed *et al.* (2019), LMW-CH showed higher antimicrobial activity than HMW-CH, MMV-CH and COS. Despite its limited antibacterial activity, COS is widely favoured in a range of biomedical applications over chitosan due to its water-soluble properties, which enhance its compatibility in the field of biomedicine (Lodhi *et al.,* 2014; Kaczmarek *et al.,* 2019). These applications include superior anticancer and anti-inflammatory properties (Azuma *et al.,* 2015). When it comes to food applications, it is crucial to prioritise the enhancement of antimicrobial activity. On the other hand, in biomedical applications, antimicrobial properties are an additional criterion for consideration. In view of this, in the present investigation, the antimicrobial activity of COS was assessed after the incorporation of zinc oxide nanoparticles (ZnO-NP). According to previous findings, the incorporation of ZnO-NP into chitosan significantly boosts its antibacterial activity (Visnuvinayagam *et al.,* 2019). However, data on the ZnO-NP integrated COS (ZnO-NP-COS) is limited, therefore, the present investigation was hypothesized to enhance COS composite antibacterial activity by incorporating ZnO-NP and making them equal to LMW-CH antibacterial activity.

Hitherto, in most researches acetic acid has been used to dissolve chitosan for antibacterial activity and limited literature is available on chitosan dissolved in organic acids (Chen *et al.,* 2009). To the best of the author's knowledge to date, reports on chitosan with ZnO-NP combination with different organic acids are meagre, hence, this study would also explore a better understanding of chitosan antimicrobial activity in different acids in the presence of ZnO-NP-Chitosan. Here, the GRAS listed food-grade organic acids viz. Propionic acid (582.3081), Lactic acid (582.1061), Citric acid (582.1033), Malic acid (582.1069), Acetic acid (582. 1005), Tartaric acid (582, 1099) and Benzoic acid (582.3021) (Davidson, 2005) and one non-food-grade organic acid, Oxalic acid were tried with chitosan for suitable antimicrobial activity.

Materials and Methods

Test material: Chitosan (Hi-Media, Mumbai) obtained from shrimp shells with ≥ 75-degree deacetylation was used for the study. Chitosan (1%) was prepared in 1% propionic, lactic, citric, malic, oxalic, acetic, tartaricand benzoic acid (HiMedia, Mumbai) to assess the antimicrobial properties. Different molecular-weight chitosan HMW-CH (311-375kDa), MMV-CH(190-310kDa), (50-189kDa) and COS (>3.9kDa) were obtained from a commercial chemical dealer (SRL, Kolkata).

Preparation of zinc oxide nanoparticles (ZnO-NP): ZnO-NPs were prepared as per the method of Visnuvinayagam *et al.* (2019) with minor modifications. A 500 ml of 1 M zinc nitrate was added dropwise into 500 ml of 3M NaOH with constant stirring at 70°C and kept for 16-24 hrs undisturbed for settlement. Further, these settled nanoparticles were centrifuged at 8000 rpm for 5 min and the ZnO-NP was washed repeatedly and kept in a vacuum oven overnight at 70°C. This dried ZnO-NP was used for further characterization studies.

Preparation of zinc oxide nanoparticles (ZnO-NP-CH) composite: The ZnO-NP-CH was prepared according to the method outlined by Visnuvinayagam *et al.* (2019; 2021). Chitosan (1 g) was accurately weighed in a beaker and combined with 1% acetic acid solution, which was then subjected to constant stirring using a magnetic stirrer. In parallel, ZnO-NP 1 g was introduced into 1% acetic acid in a separate beaker and thoroughly mixed to achieve a uniform dispersion. ZnO-NP was added dropwise to chitosan to achieve a uniform composite, and the pH was adjusted with glacial acetic acid.

Characterization of ZnO-NP by UV-Visible spectroscopy: ZnO-NP and ZnO bulk particles were analyzed using ultraviolet-visible (UV-Vis) spectroscopy (25 lambda, Perkin Elmer).

Characterization of ZnO-NP by Scanning Electron Microscopy: ZnO nanoparticles were mixed with ethanol and directly placed over the stab. Thereafter,ethanol was evaporated by the Infra-Red lamp. After the completion of drying process, the stab was subjected to ultra-thin coating of gold using a sputter coating machine and directly examined under a Scanning Electron Microscope (Philips XLD 3D model) at 2000X to 10000X for determining the size and shape.

Characterization of ZnO-NP by FTIR: ZnO nanoparticle was tested in the Nicolet™ iS™ 10 FTIR Spectrometer and the characteristic absorption peak was noted.

Biological materials: The following bacterial cultures *P. aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC-10536), *V. cholerae* (ATCC 14033), *Salmonella* Typhimurium (ATCC 23564), Methicillin- Resistant *Staphylococcus aureus* (ATCC 43300), *L. monocytogenes* (ATCC 13932), *B. subtilis* (ATCC 6633), *B. cereus* (ATCC 11778), *S. aureus* (ATCC 25923) were procured from the American Type Culture Collection (ATCC) located in Manassas, Virginia, USA, for the purpose of conducting a well diffusion-based antimicrobial assay.

Antibiogram: Antibiogram was carried as per Asbel *et al.* (2024) standard well diffusion technique using Muller-Hinton Agar. Test bacterial culture viz., *Escherichia coli, Salmonella* sp., *Vibrio cholera, Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Bacillus subtilis,* Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* were inoculated into Nutrient Broth (BHI) and incubated at 37°C for 2–4 hrs; further,the cultured organisms were introduced into a test tube containing sterile normal saline solution until a turbidity of 0.5 McFarland standard was observed. After submerging a sterile cotton swab into the test culture, it was transferred to pre-prepared Muller-Hinton Agar plates. Diameter well measuring 5-6 mm was formed using a cork borer (HiMedia, Mumbai) and the bottom of the agar was sealed a with sterile molten agar to avoid leakages. The prepared chitosan/HMW-CH/MMW-CH/LMW-CH/COS and its ZnO-NP combination were added into the wells, and thereafter the plates were incubated at 37°C. After 24hr, the halo zone was measured using a standard antibiotic zone scale (HiMedia, Mumbai).

Results and Discussion

Chitosan with various molecular weights were produced by enzymatic or chemical degradation, including HMW-CH, MMV-CH, LMW-CH and COS. Despite the potent antibacterial activity exhibited by the first three products, their low solubility in water rendered them unsuitable for use in food and diverse biomedical applications. Conversely, despite high solubility in water, COS exhibited limited antibacterial efficacy as a result of its substantial hydrolysis. Therefore, the objective of the current investigation was to enhance the antibacterial efficacy of chitosan with varying molecular weights through the integration of ZnO-NP.

In nanotechnological delve, the confirmation of nano-size is considered most important to get suitable results. In this regard, in the present exploration, ZnO-NP was synthesized by the sol-gel method and subjected to UV-spectra, Scanning electron microscope (SEM), and Fourier-transform infrared spectroscopy (FTIR) for the nano-size authentication. During the initial screening process, the synthesized ZnO-NP was checked for maximum wavelength (λ_{max}) in the range of 200–700 nm in a UV-visible spectrophotometer and observed for blue shift. The reduction in λ_{max} value of prepared ZnO-NP was compared with normal bulk ZnO particle (commercial Analytical grade ZnO) (Visnuvinayagam *et al.,* 2019). In the present investigation, the λ_{max} value of ZnO-NP was around 354 nm (Fig. 1) whereas the λ_{max} value of ZnO- bulk particles was around 380 nm, which indicates blue shift due to difference in size (Fig. 1). As a result of small sized nanoparticles, *i.e.,* due to the reduced size of nanoparticles,

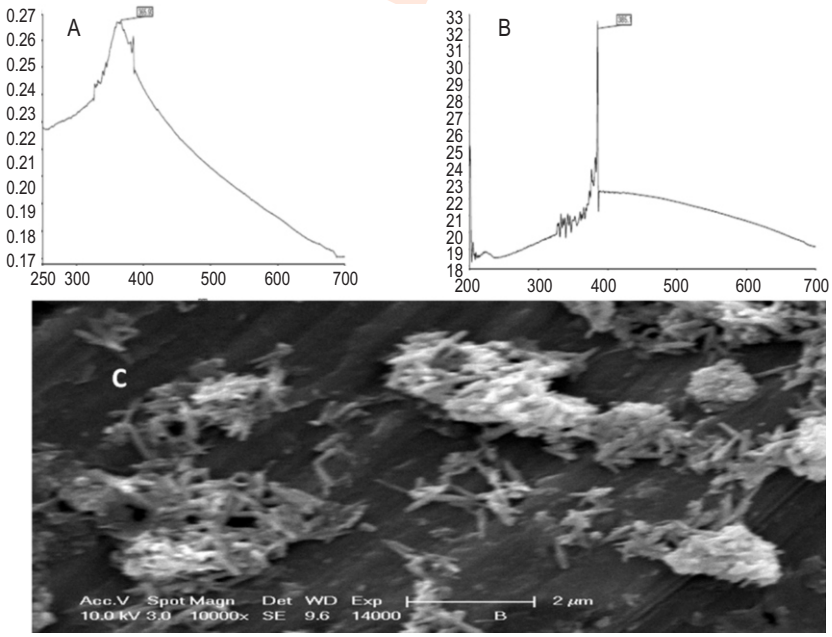

Fig. 1: Characterization of ZnO-NP. (A) Zinc oxide nanoparticle λ_{max} is 354 nm. (B) Zinc oxide bulk particle λ_{max} is 380 nm and (C) Zinc oxide Nanoparticles images in Scanning electron microscope.

Table 1: Enhancement of zone of inhibition in agar well diffusion assay (in mm) for different molecular weights of chitosan after addition of ZnO-nanoparticles

Pathogenic bacteria	Chitosan High Mol. Wt		Chitosan Medium Mol. Wt		Chitosan Low Mol. Wt		Chitosan oligosaccharides	
	CH	ZnO-NP-CH	CH	ZnO-NP-CH	CH	ZnO-NP-CH	CH-OS	ZnO-NP-COS
P. aeruginosa	32.5±0.5^{A}	38.5±0.5^{B}	31.0±1.0^{A}	38.5±0.5^{B}	38.0±1.0^{B}	35.5±0.5^{B}	20.5±0.5^{C}	38.5 ±0.5^{B}
E. coli	20.0±1.0^{A}	31.5±0.5^{B}	21.0±1.0^{A}	28.0±1.0^{B}	24.0±1.0^{AB}	29.5±0.5^{B}	13.5±0.5^{C}	29.5±0.5^{B}
V. cholerae	34.0±1.0^{A}	31.5±0.5^{AD}	22±1.0^{B}	34.5±0.5^{AD}	28.5±0.5^{C}	36.0±1.0^{D}	19.5±0.5^{E}	27.0±1.0^{C}
Salmonella	21.5±0.5^{A}	24±1.0 A^{BC}	23.1±1.0^{ABC}	31.0±1.0^{CD}	25.5±0.5^{B}	30.5±0.5^{CD}	14.5±0.5^{E}	27.5±0.5^{A}
MRSA	33.5±1.5^{A}	36.5±0.5^{A}	35.0±1.0^{A}	42.5±0.5^{B}	36.5±1.5^{A}	45.0±1.0^{B}	18.0±1.0^{C}	41.5±1.5^{B}
L. monocytogenes	33.5±1.5^{A}	40.5±0.5^{B}	33.0±1.0^{A}	40.5±0.5^{B}	42.0±1.0^{B}	43.5±1.5^{B}	31.5±0.5^{A}	42.5±1.5^{B}
B. subtilis	41.5±0.5^{A}	45.0±2.0^{A}	42.0±1.0^{A}	45.5±1.5^{A}	43.0±1.0^{A}	45.0±2.0^{A}	26.5±1.5^{B}	42.5±1.5^{A}
B. cereus	36.0±1.0^{A}	40.5±0.5^{B}	36.5±0.5^{A}	38.5±0.5^{AB}	40.5±0.5^{B}	39.5±0.5^{B}	18.5±0.5^{C}	38.0±1.0^{AB}
S. aureus	35.0±0.5^{A}	41.0±2.0^{B}	36.5±1.5^{A}	40.0±1.0^{B}	36.0±1.0^{A}	40.0±1.0^{B}	22.5±0.5^{C}	28.0±1.0^{D}

Values are given as Mean±S.E. Means with different superscripts indicate significant differences at a 5%. Comparison between different molecular weights of chitosan with each bacteria

the absorption edge was systematically pushed to a lower wavelength (Gupta *et al.,* 2015; Dobrucka and Dugaszewska, 2016) and also due to the size quantization effect (Koch *et al.,* 1985). A previous study also reported that the bulk and nanoparticles exhibited λ_{max} values around 385 and 365 nm, respectively (Visnuvinayagam *et al.*, 2019).

Eventhough UV spectra is a trouble-free and cost-effective technique, it is only a preliminary step to scrutinize the nano size. But, SEM analysis not only verify the nano-size, but also the shape of the NP can be evaluated. In the present study, the SEM images showed that the ZnO-NP was nano-flake (NF) shaped with an average width of 50 -70 nm and length 70 to 90 nm (Fig. 1). Besides, in the present investigation, the size of the ZnO-NP was also confirmed by Fourier-transform infrared spectroscopy (FTIR) for better precision. FTIR images showed characteristic absorption for ZnO-NP at 417 cm^{-1} (Fig. 2). Similarly, Alwan *et al.* (2015) also reported that absorption at 417 cm^{-1} is a characteristic of ZnO-NP. In the same way, in the present study characteristic absorption was observed at 452 cm^{-1} similar to Patil *et al.* (2016) (Fig. 2). Therefore, based on the above size authentication, the synthesized ZnO was nano sized, which could be used to find the effect on different molecular weights as well as different organic acids.

In the present study, the antimicrobial activity was observed based on the well diffusion assay, and the perusal of results showed that the addition of ZnO-NP resulted in augmentation in antimicrobial activity observed in all the MW chitosan, however, but it varied in each bacteria. In *Bacillus subtilis*, all chitosan and its composite showed equal inhibitory activity, except for COS (Fig. 3; Table 1). The probable reason could be that *B. subtilis* is highly susceptible to most of the antimicrobial agents (Kong *et al.,* 2018). While comparing the zone of inhibition, it has been observed that the COS activity was significantly lower in all bacterial species (Fig. 3 and Table 1), especially while comparing with LMW, the zone of inhibition was significantly lower (P<0.05). Interestingly, after adding ZnO-NP to COS, *i.e.,* (ZnO-NP-COS), the antibacterial activity of COS drastically increased, *i.e.,* up to 16 mm higher zone of the zone of inhibition compared to COS. Statistically, while comparing with LMW-CH, it was equal to LMW-CH; even it was on par with ZnO-NP-LMW-CH composite activity (no significant difference at 5% level). Correspondingly, for *L. monocytogenes,* around 10 mm increase in the zone size was observed for COS, which was equal to the LMW-CH and its composite. A similar result was observed for MRSA, *P. aeruginosa, V. cholerae, Salmonella* and *E. coli*. Based on the statistical analysis, for each bacteria, there was a significant (P<0.05) increase in the antibacterial activity of COS after the addition of ZnO-NP, which was equal to LMW chitosan, *i.e.,* no significant difference (P>0.05) was observed in terms of antimicrobial activity between LMW and ZnO-NP-COS. Even though variation in the antibacterial activity was observed between each bacterial species, in general around 6-16 mm higher inhibition zone was observed after the addition of ZnO-NP. So, the enhanced antibacterial activity of ZnO-NP-COS can be exploited in food and biomedical applications. Also, it was observed that the addition of ZnO-NP leads to equal antibacterial activity of HMW-CH, MMW-CH and LMW-CH.

Among the combinations, ZnO-NP-LMW-CH possessed superior antimicrobial activity. The hierarchy of the antibacterial activity of varying-sized chitosan and its nano-composite was in the order: ZnO-LMW-CH< ZnO-MMW-CH<ZnO-HMW-CH< LMW-CH< ZnO-COS< HMW-CH<MMW-CH<COS. The activity of LMW-CH also enhanced, which was superior to other CH and its composites. Previous studies have reported that the inclusion of ZnO enhances positive charge on the amino group of chitosan, thereby facilitating the attachment of chitosan to interact with the negatively charged cell wall. Upon attachment, ZnO-NP nanoparticles release reactive oxygen species that are conjugated with Zn ions, leading to disruption of bacterial cell wall, and ultimately resulting in bacterial death (Summer *et al.*, 2024). In the present study, the chitosan was prepared with different food-grade organic acids for better understanding. Based on the

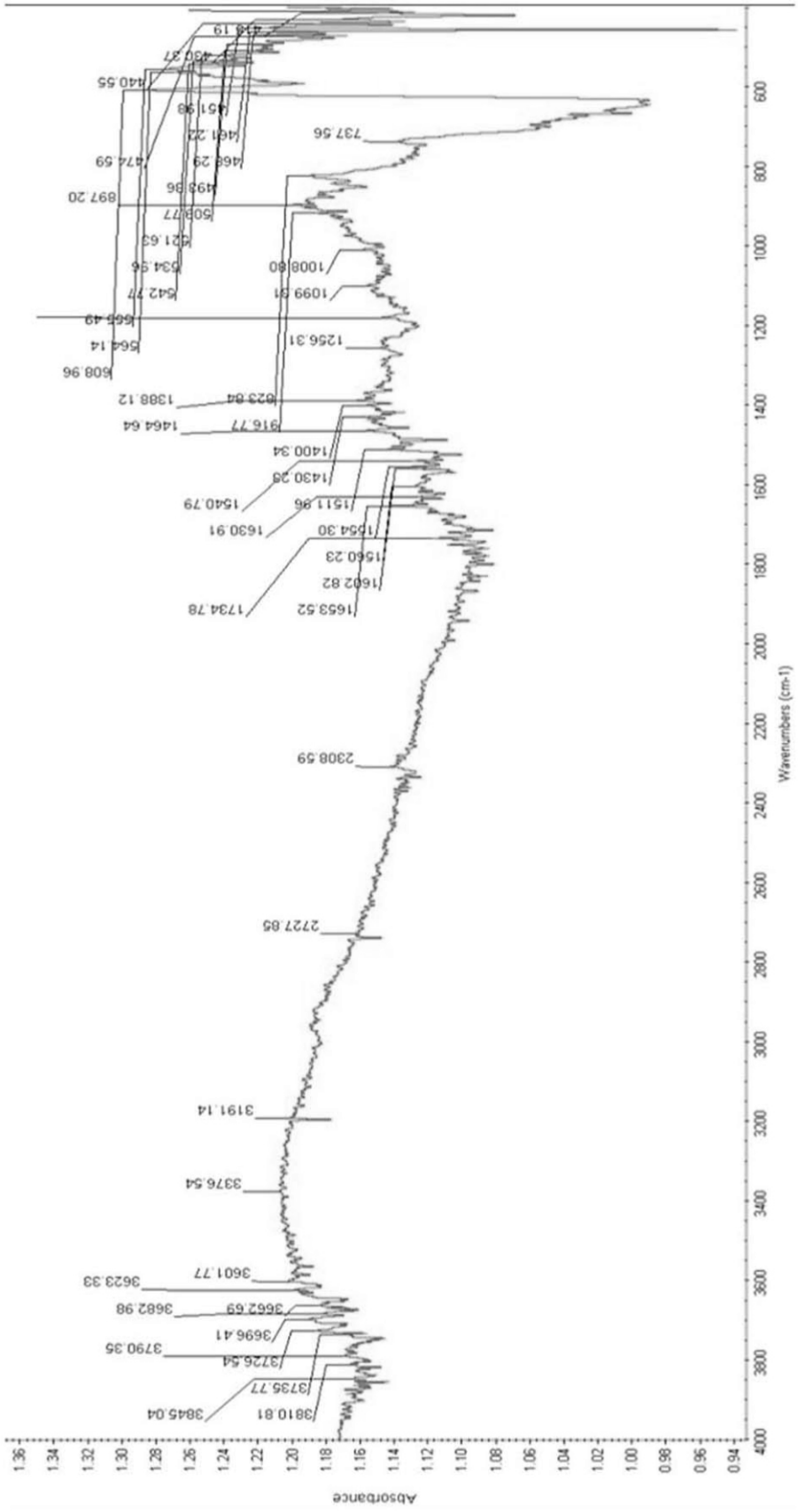

Fig. 2: FTIR image of zinc oxide nanoparticles.

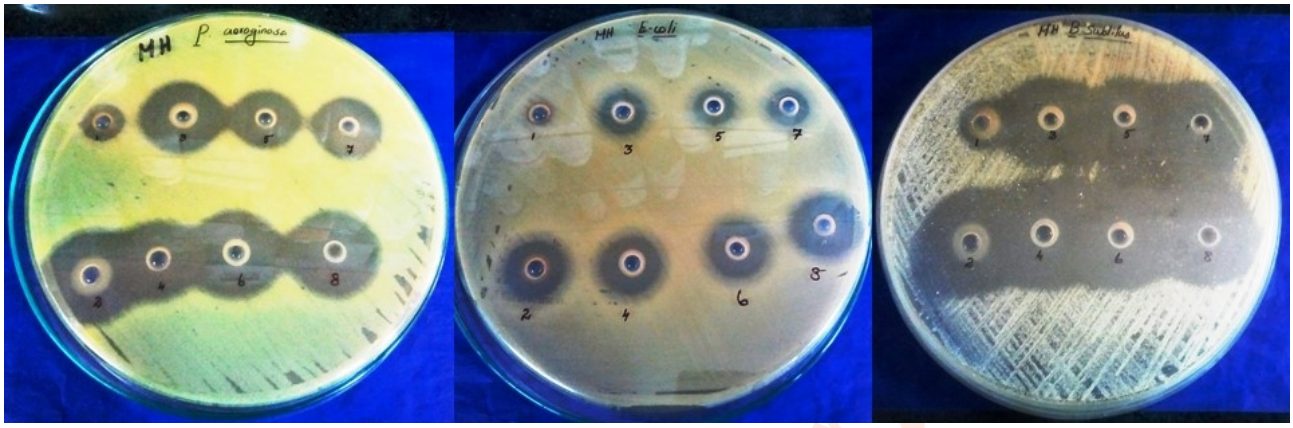

Fig. 3: Antibiogram for different molecular weight chitosan and its ZnO-NP combination.

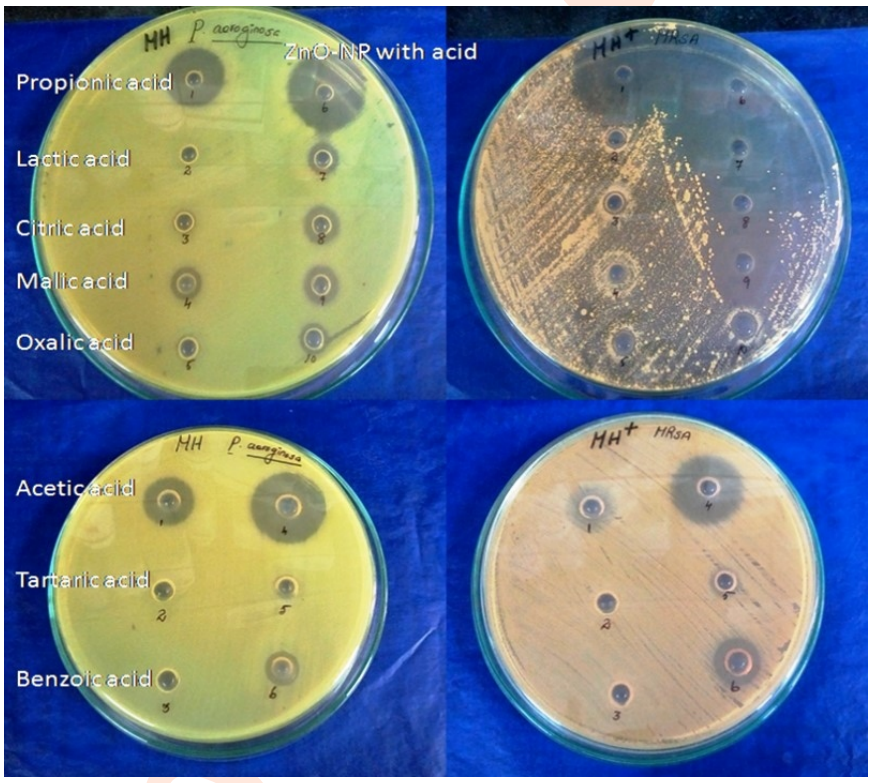

Fig. 4: Zone of inhibition of Chitosan and ZnO-NP-CH prepared by different organic acids.

well diffusion assay (Fig. 4), variations were observed in the antibacterial activity on the account of different acids used for chitosan solution preparation. Some of the bacteria were susceptible to a particular organic acid (Table 2a,b). *S. aureus was* susceptible to chitosan in all acids, except for lactic and benzoic acid. However, *S. aureus* was susceptible to lactic and benzoic acid after ZnO-NP incorporation. Similarly, *B. subtilis* was resistant to chitosan in lactic, malic, oxalic and benzoic acid, however, on incorporating ZnO-NP, it became susceptible to all acids. *B. cereus* was susceptible to all acids, except benzoic acid, however, it became susceptible to benzoic acid after incorporation of ZnO-NP. *Salmonella* sp. was resistant to chitosan in lactic, citric, oxalic, tartaric and benzoic acid. It became susceptible to lactic and citric acid after ZnO-NP incorporation. *E.*

Table 2a: Zone of Inhibition (nm) of chitosan and Zn-NP-CH prepared with various organic acids

Acids	*P. aeruginosa*		MRSA		*E. coli*		*L. monocytogenes*	
	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH
Propionic acid	31.50±0.71	40.50±0.71**	42.00±1.41	41.50±0.71	25.00±1.41	30.50±0.71*	35.50±0.71	41.00±1.41*
Lactic acid	0.00±0.00	16.50±0.71**	11.50±0.71	41.00±1.41**	0.00±0.00	14.00±1.41**	0.00±0.00	0.00±0.00
Citric acid	0.00±0.00	19.50±0.71**	13.50±0.71	42.00±1.41**	0.00±0.00	0.00±0.00	0.00±0.00	16.50±0.71**
Malic acid	17.00±1.41	17.50±0.71	0.00±0.00	34.50±0.71**	0.00±0.00	17.50±0.71**	14.00±1.41	22.50±0.71*
Oxalic acid	0.00±0.00	19.25±6.01**	12.50±0.71	13.00±1.41	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Acetic acid	24.50±0.71	36.00±1.41**	20.00±1.41	29.00±1.41*	24.50±6.36	26.50±0.71	31.00±1.41	37.00±1.41
Tartaric acid	0.00±0.00	0.00±0.00	0.00±0.00	12.50±0.71**	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Benzoic acid	0.00±0.00	16.00±1.41**	0.00±0.00	18.50±0.71**	0.00±0.00	16.00±1.41**	0.00±0.00	18.50±0.71**

Values are given as Mean±SE. Means with different superscripts indicate significant differences at a 5% probability level. Comparison between the with each bacterial inhibition zone

Table 2b: Zone of Inhibition (nm) of chitosan and Zn-NP-CH prepared with various organic acids

Acids	*S. aureus*		*Salmonella sp.*		*B. cereus*		*B. subtilis*	
	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH	Chitosan	ZnO-NP-CH
Propionic acid	42.00±1.41	42.00±1.41	42.00±1.41	32.50±0.71*	34.50±0.71	42.00±2.83	37.00±0.00	42.00±1.41*
Lactic acid	0.00±0.00	37.50±0.71**	0.00±0.00	16.00±1.41**	11.50±0.71	24.50±0.71**	0.00±0.00	24.50±0.71**
Citric acid	21.50±0.71	34.50±0.71**	21.50±0.71	17.50±0.71*	19.00±1.41	24.00±1.41	11.50±0.71	18.00±1.41*
Malic acid	23.50±0.71	37.00±1.41**	23.50±0.71	19.00±1.41	21.00±1.41	23.50±2.12	0.00±0.00	22.00±1.41**
Oxalic acid	21.00±1.41	30.50±0.71*	21.00±1.41	0.00±0.00**	18.50±0.71	40.50±0.71**	0.00±0.00	16.50±0.71**
Acetic acid	37.50±0.71	41.50±2.12	37.50±0.71	30.50±0.71**	32.50±0.71	42.00±1.41*	33.00±0.00	39.50±0.71
Tartaric acid	18.50±0.71	24.00±1.41*	18.50±0.71	0.00±0.00**	15.50±2.12	25.00±1.41*	13.00±1.41	25.00±1.41*
Benzoic acid	0.00±0.00	34.00±1.41	0.00±0.00	22.50±2.12**	0.00±0.00	28.50±0.71**	0.00±0.00	25.50±2.12**

Values are given as Mean±SE. Means with different superscripts indicate significant differences at a 5% probability level. Comparison between the with each bacterial inhibition zone

coli was susceptible to chitosan in propionic and acetic acids, however, after ZnO-NP incorporation, it became susceptible to lactic, malic and benzoic acid. *L. monocytogenes* was susceptible only to chitosan in propionic, malic and acetic acids, while on ZnO-NP addition it became susceptible to citric and benzoic acids also.

The above antibiogram results revealed that the addition of ZnO-NP elevates antibacterial activity irrespective of different organic acids. Most bacteria like *Salmonella, B. cereus, S. aureus, L. monocytogenes* and *E. coli* are responsible for food poisoning. Improved control of foodborne pathogens was achieved in ZnO-NP-CH dissolved in different organic acids compared to the chitosan alone. Report on food pathogens in the retail market was periodically observed (Visnuvinayagam *et al.*, 2015, 2016, 2017; 2020; Sivaraman *et al.*, 2016). So, the application of ZnO-NP-COS would be a possible solution to reduce the food pathogen transmitted via fish. Besides these ZnO-NP would be a better option to control foodborne pathogens. When used in food, the palatability of the food depends on the bitterness of the organic acid. Here, Propionic acid has more bitterness than chitosan; malic acid is lesser than acetic acid. Propionic acid has more bitterness than chitosan; malic acid is even less than acetic acid. So, malic acid would also be a suitable material for food application because in the hierarchy next to acetic acid, malic acid has a potent antibacterial activity. So, ZnO-NP-CH made from malic acid would be a better choice for application in food industry.

Similarly, better antimicrobial activity was observed over multi-drug-resistant pathogens viz. MRSA and *P. aeruginosa*. Native chitosan with most organic acids was not able to control multidrug-resistant MRSA and *P. aeruginosa*. These two pathogens are responsible for cutaneous infection. Based on the results of well diffusion assay, MRSA was resistant to malic, tartaric and benzoic acid, but after the incorporation of ZnO-NP, it became susceptible to all acids. Similarly, *P. aeruginosa* was resistant to chitosan in most organic acids, *i.e.,* lactic, citric, oxalic, tartaric and benzoic acid, however, ZnO-NP addition made *P. aeruginosa* susceptible to all acids, except tartaric acid. So, based on the suitability, the composite can be used as an ointment to treat wound infection. Already, Chitosan-ZnO-NP composite prepared from acetic acid has been tried as an ointment to treat MRSA and *P. aeruginosa* (Visnuvinayagam *et*

al., 2019). It was also found that other organic acids were also equally effective for ointment preparation. Still, the present *in-vitro* experiment needs to be confirmed through animal experiments.

The present study screened food-grade organic acids for chitosan preparation, and ZnO-NP incorporation to help enhance the chitosan application. Among eight acids, only oxalic acid was a non-food grade. Similarly, Romanazzi *et al.* (2009) dissolved chitosan in different organic and inorganic acids, and found that chitosan dissolved in low carbon numbers, such as formic, acetic and lactic acids were better solvents to control moulds on grapes in comparison to higher carbon numbers (malic, malic, succinic, L-ascorbic, and L-glutamic acids) or inorganic acids such as hydrochloric or phosphorous acids. Sogvar *et al.* (2016) used the ZnO-NP incorporated chitosan to effectively delay ripening in strawberries and also found a significant reduction in aerobic plate count. Goni *et al.* (2017) found that chitosan dissolved with lactic acid significantly reduced microbial count and they suggested that lactic acid can be a suitable substitute for acetic acid.

Among eight organic acids, Propionic acid incorporated chitosan showed better antibacterial activity. Moreover, the ZnO-NP-CH dissolved in Propionic acid was superior to other acids and ZnO combinations. The agar well diffusion showed that ZnO-NP-CH had around 10 -15 mm wider zone of inhibition than chitosan alone. The hierarchy of different organic acids dissolved in chitosan was in the order: Propionic > Acetic > Malic > Citric > Oxalic > Lactic > Tartaric > Benzoic acids. Even though propionic acid possesses higher antibacterial activity than other eight organic acids, it may not be suitable for food sensory evaluation. So, acetic and malic acid would be suitable in food industry.

Hence, it can be concluded that the antibacterial activity of various molecular weight chitosan was significantly improved by ZnO-NP, addition consequently, many resistant bacteria became susceptible following the addition of ZnO-NP to chitosan. The COS antibacterial activity was improved at par with the low molecular weight chitosan. Since COS-ZnO-NP composite possess a high antibacterial property with water-soluble, it can be used in various biomedical applications.

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References

Abd El-Hack, M.E., M.T. El-Saadony, M.E. Shafi, N.M. Zabermawi, M. Arif, G.E. Batiha, A.F. Khafaga, Y.M. Abd El-Hakim and A.A. Al-Sagheer: Antimicrobial and antioxidant properties of chitosan and its derivatives and their applications: A review. *Int. J. Biol. Macromolec.,* **164**, 2726-2744 (2020).

Alwan, R.M., Q. A. Kadhim, K.M. Sahan, R.A. Ali, R.J. Mahdi, N.A. Kassim and A.N. Jassim: Synthesis of zinc oxide nanoparticles via sol–gel route and their characterization. *Nanosci. Nanotechnol.,* **5**, 1-6 (2015).

Asbell, P.A., C.M. Sanfilippo, and H.H. DeCory. Antibiotic resistance of bacterial pathogens isolated from the conjunctiva in the Antibiotic Resistance Monitoring in Ocular micRoorganisms (ARMOR) surveillance study (2009–2021). *Diagnos. Microbiol. Infect. Diseas.*, **108**, 16069 (2024).

Azuma, K., T. Osaki, S. Minami and Y. Okamoto: Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *J. Functi. Biomateri.,* **6**, 33-49 (2015).

Chen, R.H., W.Y. Chen, S.T. Wang, C.H. Hsu and M.L. Tsai: Changes in the Mark–Houwink hydrodynamic volume of chitosan molecules in solutions of different organic acids, at different temperatures and ionic strengths. *Carbohyd. Polym.,* **78**, 902-907 (2009).

Davidson, P.M., J. N. Sofos and A.L. Branen: Antimicrobials in Food. 3rd Edn., CRC Press, 720 pages (2005)

Divya, K., S. Vijayan, T.K. George and M.S. Jisha: Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. *Fibers Polym.,* **18**, 221-230 (2017).

Dobrucka, R. and J. Długaszewska: iosynthesis and antibacterial activity of ZnO nanoparticles using *Trifolium pratense* flower extract. *Saudi J. Biol. Sci.,* **23**, 517-523 (2016).

Goni, M.G., B. Tomadoni, S.I. Roura and M.R. Moreira: Lactic acid as potential substitute of acetic acid for dissolution of chitosan: preharvest application to Butterhead lettuce. *J. Food Sci. Technol.,* **54**, 620-626 (2017).

Gunalan, S., R. Sivaraj and V. Rajendran: Green synthesized ZnO nanoparticles against bacterial and fungal pathogens. *Progr. Nat. Sci. Materi. Int.,* **22**, 693-700 (2012).

Gupta, A., P. Srivastava, I. Bahadur, D.P. Amalnerkar and R. Chauhan: Comparison of physical and electrochemical properties of ZnO prepared via different surfactant-assisted precipitation routes. *Appl. Nanosci.,* **5**, 787-794 (2015).

Jeyakumari, A., G. Ninan, C.G. Joshy, U. Parvathy, A.A. Zynudheen and L.V. Lalitha: Effect of chitosan on shelf life of restructured fish products from pangasius (*Pangasianodon hypophthalmus*. *J. Food Sci. Technol.,* **53**, 2099-2107 (2016).

Kaczmarek, M.B., L.L. Struszczyk-Swita, X. Li, M. Szczesna-Antczak and M. Daroch: Enzymatic modifications of chitin, chitosan and chitooligosaccharides. *Fronti. Bioenginee. Biotechnol.,* **7**, 243-50 (2019).

Koch, U., A. Fojtik, H. Weller and A. Henglein: Photochemistry of semiconductor colloids. Preparation of extremely small ZnO particles, fluorescence phenomena and size quantization effects. *Chem. Phys. Lett.,* **122**, 507-510 (1985).

Kong, X., M. Yang, H. Abbas, J. Wu, M. Li and W. Dong: Antimicrobial genes from *Allium sativum* and *Pinellia ternata* revealed by a *Bacillus subtilis* expression system. *Scienti. Rep.,* **8**, 14514 (2018).

Kumirska, J., M.X. Weinhold J. Thöming and P. Stepnowski: Biomedical activity of chitin/chitosan based materials—influence of physicochemical properties apart from molecular weight and degree of N-acetylation. *Polymers,* **3**, 1875-1901 (2011).

Lodhi, G., Y.S. Kim, J.W. Hwang, S.K. Kim, Y.J. Jeon, J.Y. Je, C.B. Ahn, S.H. Moon, B.T. Jeon and P.J. Park: Chitooligosaccharide and its derivatives: preparation and biological applications. *BioMed Rese. Int.,* **2014**, 654913 (2014).

Meng, X., R. Xing, S. Liu, H. Yu, K. Li, Y. Qin and P. Li: Molecular weight and pH effects of aminoethyl modified chitosan on antibacterial activity *in vitro*. *Int. J. Biologi. Macromole.,* **50**, 918-924 (2012).

Naveed, M., L. Phil, M. Sohail, M. Hasnat, M.M.F.A. Baig, A.U. Ihsan, A.U., M. Shumzaid M.U. Kakar,T.M. Khan, M.D. Akabar and M.I. Hussain: Chitosan oligosaccharide (COS): An overview. *Int. J. Biol. Macromolec.,* **129**, 827-843 (2019).

Ospina, N.M., S.P.O. Alvarez, D.M.E. Sierra, D.F.R. Vahos, P.A.Z. Ocampo and C.P.O. Orozco: Isolation of chitosan from *Ganoderma lucidum* mushroom for biomedical applications. *J. Materi. Sci. Materi. Medi.,* **26**, 135-140 (2015).

Patil, B.N. and T.C. Taranath: Limonia acidissima L. leaf mediated synthesis of zinc oxide nanoparticles: a potent tool against *Mycobacterium tuberculosis*. *Int. J. Mycobact.,* **5**,197-204 (2016)

Romanazzi, G., F.M. Gabler, D. Margosan, B.E. Mackey and J.L. Smilanick: Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology,* **99**,1028-1036 (2009).

Summer, M., S. Ali H.M. Tahir, R. Abaidullah, U. Fiaz, S. Mumtaz, H. Fiaz, A. Hassan, T.A. Mughal, and M. A. Farooq. Mode of action of biogenic silver, zinc, copper, titanium and cobalt nanoparticles against antibiotics resistant pathogens. *J. Inorg. Organomet. Polymer. Material*., **34**, 1417-1451 (2024).

Sivaraman, G.K., S. Visnuvinayagam, J.A. Kumar, V. Renuka, S. Remya and V. Deesha: Assessment of microbial quality of fish processing industrial effluent in bar-mouth at Bhidia landing site, Veraval, Gujarat, India. *J. Environ. Biol.,* **37**, 537-541 (2016).

Sogvar, O.B., M.K. Saba, A. Emamifar and R. Hallaj: Influence of nano-ZnO on microbial growth, bioactive content and postharvest quality of strawberries during storage. *Innov. Food Sci. Emerg. Technolog.,* **35**, 168-176 (2016).

Visnuvinayagam, S., Toms C. Joseph, V. Murugadas, R. Chakrabarti, and K.V. Lalitha: Status on methicillin resistant and multiple drug resistant Staphylococcus aureus in fishes of Cochin and Mumbai coast, India. *J. Environ. Biol.,* **36**, 571 – 575 (2015).

Visnuvinayagam, P., P. Viji, L.N. Murthy, A. Jeyakumari and G.K. Sivaraman: Occurrence of faecal indicators in freshwater fishes of Navi Mumbai in retail outlets. *Fishery Technol.,* **53**, 334–338 (2016)

Visnuvinayagam, S., L. N. Murthy, P. Viji and G. K. Sivaraman:Study on retail fish markets: Possible occurrence and transmission of emerging pathogen from faecal indicators. *J. Environ. Biol.,* **38**, 465- 470 (2017).

Visnuvinayagam, S., L.N. Murthy, A. Jeyakumari, U. Parvathy, R. Anandan, G.K. Sivaraman and C.N. Ravishankar: Combined effect of zinc oxide nano particle incorporated chitosan for better antimicrobial activity towards wound healing. *J. Environ. Biol.,* **40**, 691-697 (2019).

Visnuvinayagam, S., L.N. Murthy, U. Parvathy, A. Jeyakumari, K.P. Rawat, S.A. Khadar and K.S.S. Sarma: Destructive dose determination of electron beam irradiation for pathogenic bacteria in water medium by 96 well plate assay. *J. Environ. Biol.,* **41**, 1013-1017 (2020).

Visnuvinayagam, S., L.N. Murthy, U. Parvathy, A. Jeyakumari, G.K. Sivaraman and D. Karthikeyan: Food grade zinc oxide bulk particle composite can replace the toxic zinc oxide nano composite towards the control of pathogenic and spoilage bacteria. *FEMS Microbiol. Lett.,* **368**, fnaa 210 (2021).

Wang, K., S. Pan, Z. Qi, P. Xia, H. Xu, W. Kong, H. Li, P. Xue, P.X. Yang and C. Fu: Recent advances in chitosan-based metal nanocomposites for wound healing applications. *Adv. Mater. Sci. Engin.,* **2020**, 1-13 (2020).

Yadav, M., P. Goswami, K. Paritosh, M. Kumar, N. Pareek and V. Vivekanand: Seafood waste: a source for preparation of commercially employable chitin/chitosan materials. *Bioresour. Bioproce.,* **6**, 1-20 (2019).

Zhang, Z.Y. and H.M. Xiong: Photoluminescent ZnO nanoparticles and their biological applications. *Materials,* **8**, 3101-3127 (2015).