Antimicrobial Chitosan-Zinc Oxide Hybrid Nanoparticles Incorporated Polymer Membranes for Water Disinfection

In

Biomedical Sciences and Engineering

By

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H-12 Islamabad, Pakistan

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A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

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MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by:

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Titled: Antimicrobial Chitosan-Zno Hybrid Nanoparticles Incorporated Polymer Membrane for Water Disinfection

be accepted by the School of Mechanical and Manufacturing Engineering, Department of Biomedical Engineering and Sciences, National University of Sciences and Technology, Islamabad in partial fulfillment of the requirements for the award of MS in Biomedical Sciences degree with A Grade.

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DECLARATION

It is hereby declared that this research study has been done for partial fulfillment of requirements for the degree of Masters of Sciences in Biomedical Sciences. This work has not been taken from any publication. I hereby also declare that no portion of the work referred in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

_____

Iqra Munnawar

I dedicate my thesis to my parents for their immense support, motivation & love.

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First and foremost I would like to thank **Allah Almighty**, the most beneficent and the compassionate, the most generous who has always helped me in every step of my life and bestowed me with his blessings Alhumdulillah. All respect and reverence for his **Holy Prophet (SAW)** who has enabled us to shape our lives according to the teachings of Islam.

I am grateful to my father and mother for their love, affection and moral support. They have been with me through every thick and thin. They always encouraged me and prayed for me, I am just because of them. I can never pay back the love and warmth that they have given to me throughout the 25 years of my life. I am also thankful to my husband for his moral support.

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TABLE OF CONTENTS

LIST OF FIGURES

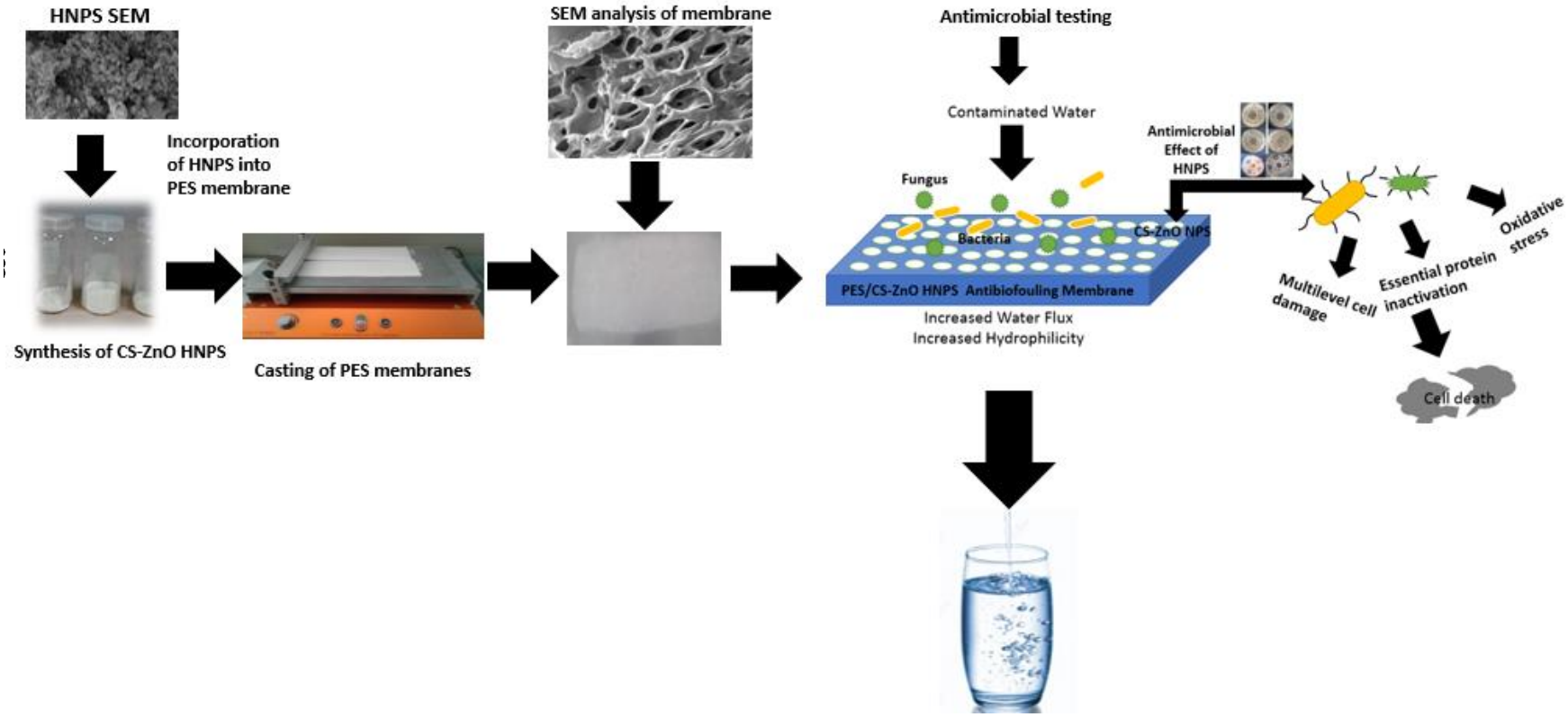
LIST OF TABLES

LIST OF ABBREVATIONS

CS-ZnO HNPS	Chitosan zinc oxide hybrid nanoparticles
E.coli	Escherichia coli
B.cereus	Bacillus cereus
S.typhi	Salmonella typhi
S.aureus	Staphylococcus aureus
A.fumigatus	Aspergillus fumigatus
F.solani	Fusarium solani
PES	Polyethersufone
WHO	World health organization
SEM	Scanning electron microscopy
EDS	Energy dispersive x ray spectroscopy
XRD	X ray diffraction
FTIR	Fourier transform infrared
XRF	X ray fluorescence
DBPS	Disinfection by products
Nm	Nanometer
C^0	Celsius
ml	Milli litre
KBr	Potassium bromide
CNTS	Carbon nanotubes
H/hr	Hour
MH0	Membranes code for polyethersufone
MH1	Membranes code for polyethersulfone having 0.15g HNPS
MH2	Membranes code for polyethersulfone having

	0.3g HNPS
MH3	Membranes code for polyethersulfone having 0.45g HNPS

ANTI-MICROBIAL CS-ZNO HNPS INCORPORATED POLYMERIC MEMBRANE FOR WATER DISINFECTION



ABSTRACT

The present work is focused on the synthesis of Chitosan-Zinc oxide hybrid nanoparticles (CS-ZnO HNPS) and incorporation of these nanoparticles in the polyethersulphone (PES) membranes for enhanced antibiofouling properties. CS-ZnO HNPS were characterized through SEM, XRD and FTIR. The membranes were then fabricated by dispersing, three different concentration of CS-ZnO HNPS (58-60nm) in the casting solution of PES through phase inversion method. The influence of nano-sized CS-ZnO particles on the morphology and the properties of PES was characterized through SEM, XRF, contact angle, water retention, surface roughness and permeability flux. The membrane with the maximum concentration of NPS resulted larger mean pore sizes than the pure PES membrane and the contact angle reduced from 60.73^{o} to 40.33^{o}.The resultant membranes showed incredible water permeability, hydrophilicity and prevention against microbial fouling. The membranes were observed to have significant antibacterial and antifungal properties against *S.Aureus, B.Cereus, E.coli, S.typhi, A.fumigatus* and *F.solani.*

1. INTRODUCTION

1.1 Background

Water is the most vital element of our earth and the basic human right is access to clean and safe drinking water (Khan et al., 2013). Our earth comprises only 3 % of fresh water out of which only 0.01% water is available for human use. Unfortunately this left over minor portion of water is under colossal stress that is due to the fact of increasing urbanization with growing population and untenable consumption of water in agriculture and industries. According to a survey report of United Nation Organization (UNO) world population is rapidly growing with a decline of consumable water (Azizullah, Khattak, Richter, & Häder, 2011). The contamination of drinking water with pathogenic microorganisms is the major threat to human health. Well known water contaminants are various bacteria, viruses, pathogenic fungi and parasites, which may lead to waterborne disease and epidemic (Shannon et al., 2008). According to World Health Organization almost 1.1 billion don't have an access to people clean and safe drinking water. A Lot of people are forced to depend on polluted water for potable use, this increases the vulnerability towards death causing waterborne disease, e.g. hepatitis A, cholera and typhoid etc. Almost 50% of the population in developing country are exposed to contaminated water sources due to lacking supplies of water for personal hygiene and poor sanitation, this leads to 4 billion cases of diarrheal disease per year. This has resulted in 2.2 million deaths, of which children under five are the most (UN 2003).

1.2 Water Pollution in Pakistan

In Pakistan water pollution the major challenge to public health. Drinking water quality in Pakistan falls below acceptable levels. According to a survey the ranking of Pakistan regarding drinking water quality is 80 among 122 nations. Both surface water sources and ground water sources are infected with pathogenic microbes, pesticides, coliforms and toxic metals throughout the country. Parameters that are set by WHO for safe drinking water are frequently violated. Human activities like inappropriate removal of municipal and industrial effluents and indiscriminate applications of agrochemicals in agriculture are the chief elements that are

continuously contributing towards the deterioration of water quality. Chemical pollutants and pathogenic microbes are the main causes that are responsible for various public health problems (Aziz, 2005).

According to a survey in Pakistan contamination of water with bacteria has been regarded as the most potential crisis of drinking water (Azizullah et al., 2011). The Water sources were taken from rivers, lakes and ground aquifers of provinces of Pakistan were tested that showed highly polluted water with bacteriological contamination (Aziz, 2005).

In rural areas low water table and open dug wells make water more susceptible to microbial contamination. It is generally considered that from the sources are free from bacterial contamination and it further becomes contaminated through the leakage in pipes and unauthorized connection. But this is not true because in reality the situation is different in the country as water was found to contain bactericidal contamination in treatment plants and point of source (Hashmi, Farooq, & Qaiser, 2009)

1.3 Bacteria and Fungus in Drinking Water

Waterborne diseases are caused by the pathogenic fungus and bacteria in the drinking water. These infections are transmitted to humans being during washing, bathing and drinking water contaminated with fecal matter. Escherichia coli and salmonella typhi are some important waterborne pathogens.

In the past few period of time fungi has received increased attention and they are generally accepted as drinking water contaminants. A wide variety of allergic and toxigenic mold species has been isolated from drinking water and mostly filamentous fungi (molds) was investigated (Hageskal, Lima, & Skaar, 2009). Aspergillus fumigatus has is one of the most significant infectious pathogen which causes infection in immunocompromised patients. Sensoric changes have been associated with the presence of fungi in drinking water when introduced to immunocompromised patients (Hageskal et al., 2009).

1.4 Emergence of Polymer and Nanomaterials

The combination of polymer and nanomaterial are playing a vigorous role in membranes technology. Currently it is one of most the emerging field of 21st century. Various polymers are being used extensively for the fabrication of membranes because of it multipurpose nature, as they are inexpensive and very easy to handle. The major challenge is provide clean and safe water that meets the human needs. Membrane technology with nanoparticles is performing a great role in water disinfection processes. As nanomaterials holds a great potential as it has antimicrobial mechanism leading towards disinfection of pathogenic microorganisms from water. Numerous nanomaterial such as gold, silver, zinc oxide have been used for disinfecting agents in water.(Qu, Alvarez, & Li, 2013)

1.5 Research Framework

PHASE-I

In the first phase, chitosan Zinc Oxide hybrid nanoparticles (HNPS) were synthesized using chemical precipitation method. The HNPS were characterized by SEM, XRD and FTIR. The effect of nanoparticles on bacterial and fungal strains were investigated by antimicrobial testing of nanoparticles

PHASE –II

After successful preparation of HNPS, polymeric membranes were fabricated using phase inversion method by incorporation of HNPS at three different concentration. The fabricated membranes were then characterized by SEM, EDS optical profilometry, water contact angle, eater retention, permeability flux.

Phase-III

Antibacterial activity of fabricated membranes was carried out against *B.cereus, E.coli, S.aureus* and *S.typhi* by preparing bacterial dilutions. Antifungal activity of membranes were investigated against *Aspergillus fumigatus* and *Fusarium solani.*

1.6 AIM AND OBJECVTIVES

- Fabrication of antimicrobial polyethersulfone membrane incorporated with CS-ZnO (HNPS) that not only inhibit the passage of pathogens but also kill them.
- Prepare a cost effective membrane
- Membrane with increased
    1. Hydrophilicity
    2. Permeability flux

2. LITERATURE REVIEW

2.1 Current Ways of Water Disinfection

In the past few decades different approaches has been established to disinfect water from pathogenic contaminants but the disinfectants commonly used such as free chlorine, chloramines and ozone react with different constituents in natural water which unfortunately results in the formation of toxic disinfectants by products (DBPs) that are cancer initiating agents (Krasner et al., 2006). To inhibit the maximum bacterial growth chlorine is used in higher amount , but according to US Environmental Protection Agency (USEPA) the maximum levels of residual disinfectants for chlorine, chlorine dioxide and chloramine are 4.0,0.8, 4.0 mg/L (USEPA , 2011).If these limits of chlorine residuals cross a threshold level then this might have severe health effects including irritation in eyes, and nose, stomach cramps, and effect on nervous system of young children (Krasner et al., 2006). At the present , another common way of disinfecting water is through aqua filters but the major drawback of these filter membranes is the accumulation of pathogenic microorganism like fungi and bacteria that block the membrane pores resulting in fouling (Kim, Kwak, Sohn, & Park, 2003).

2.2 Significance of Nanotechnology in Water Disinfection

Currently nanotechnology is rapidly growing and its potential for water treatment has been articulated. Waste water treatment through nanotechnology promises not only encounters the current ways of treatment technology but also offer new treatment methods that promotes effective and low cost treatments (Qu, Brame, Li, & Alvarez, 2013).

Nanomaterials are incredibly small particles that range between 1-100nm. At this scale nanomaterials generally possess properties dependent on their size and many of which have been discovered for water disinfection treatment. The multifunctional, efficiently higher and flexible processes of nanotechnology has lead towards the better and cost effective treatments for the elimination of pathogens from polluted water (Qu, Brame, et al., 2013). Presently Various nanomaterials including TiO^2, ZnO , Ag nanoparticles , CNTS and fullerences has been proved to show a broad spectrum antimicrobial properties by lowering the possibilities of forming DPS (Qu, Brame, et al., 2013).

Nanoparticles have strong antimicrobial properties and due to their large surface area, high reactivity they execute as an outstanding sensors, absorbents and effective catalysts (Qi, Xu, Jiang, Hu, & Zou, 2004).

2.3 Chitosan

Chitosan is an organic, high molecular weight and cationic in nature polysaccharide that is produced by partial deacetylation of chitin that is extracted from crab shells and shrimps. Chitosan is only soluble in diluted acids and owing to the fact that it is a very weak base it is in soluble in water. In aqueous diluted acids chitosan can convert its glucosamine unit (-NH2) into highly protonated form (-NH3). The Solubility of chitosan is dependent on its molecular weight, biological origin and the rate at which it deacetyl. In Addition chitosan is readily soluble in diluted acids from which films can be prepared effectively by using c casting or dipping technique which forms a porous and dense structure (Dutta, Duta, & Tripathi, 2004).

2.3.1 Chitosan Properties and Applications

Chemical properties of chitosan encompasses of its linear polyamine structure, amino and hydroxyl reactive groups and its ability of chelating metal ions. Biocompatibility, nontoxicity antitumor, fungistatic ,bacteriostatic and low immunogenic characteristics are the biological properties of the chitosan (Dutta et al., 2004).

Chitosan has ubiquitous applications because of its physiochemical and biologically versatility (Raafat, Von Bargen, Haas, & Sahl, 2008). Moreover due to its outstanding properties it has been widely used in many applications ranging from pharmaceutical to cosmetics and also it's prevailing application in water treatment. Cationic nature and high molecular weight are the two feature that make chitosan a good applicant for skin. Chitosan is cost effective and it can participate with hyaluronic acid in the field of cosmetics. Chitosan is found in nail enamels, cleansing material, foundations, creams, eye shadows and bath agents (Dutta et al., 2004). In water treatment chitosan acts as a flocculating agent and also act as a heavy metal trapper. Chitin/chitosan composite was found to remove arsenic from water. It was investigated that chitosan eliminate petroleum and petroleum products effectively from water (Dutta et al., 2004). Chitosan has played an important role in biomedical application because of its biocompatibility

with the human body it has contributed its role in sutures, wound dressing, space filling implants and scaffolds. Therefore in tissue engineering chitosan has an accelerating effect due its positive surface charge. (Ravi Kumar, 2000). Chitosan films can treat the burnt skin as it allows excellent permeation of oxygen and constrain the injured skin from deficiency of oxygen. This means that it's not essential to remove chitosan film (Dutta et al., 2004).

2.4 Zinc Oxide

Among the metals oxide, zinc oxide is most frequently studied due to its unique properties. Zinc Oxide is a white inorganic powder whereas in nature is appear as rare zinc mineral zincite. Pyrometallurgical methods are most common methods adopted by industries for the production of zinc oxide powder which involves direct processes, indirect processes or spray pyrolysis. Moreover zinc oxide can be also produced as a byproduct of some chemical reactions (Moezzi, McDonagh, & Cortie, 2012). Zinc oxide is a notorious semiconductor 3.36eV has extensively received attention in in electronic application due to its outstanding electrical, optical and chemical properties. Recently in nanotechnology nanosized zinc oxide is playing a significant role in many applications.

2.4.1 Zinc Oxide Properties and Applications

Zinc oxide has been used for centuries because of its miscellaneous properties .The physical properties of zinc oxide comprises of high electrical and thermal conductivity, also its optical property permits it to absorb detrimental ultraviolet rays. Chemical properties includes stability such as at neutral PH's and slight antimicrobial activity (Moezzi et al., 2012). The cost effectiveness and availability of the zinc oxide has ensured its widespread consumption in industries. Zinc oxide is considered as a nontoxic material since it does not cause any eye or skin irritation besides this, research has showed that it has no evidence of genotoxicity and carcinogenicity in humans. Now days the foremost use of zinc oxide is in rubber industry and ceramics. Zinc oxide is playing a significant role in transparent transistors, memory devices, solar cells and LED,s (Moezzi et al., 2012). Owing to the exceptional properties zinc oxide has

broad range of applications in cosmetics e.g. lip products, mineral makeup bases, face powder, ointments, hand creams, lip products and lotions. Zinc oxide help the cosmetics products to adhere onto the skin but the most important role of zinc oxide is that it acts as a broad spectrum UV absorber which resists the penetration of hazardous ultraviolet rays into skin. Zinc oxide has also vast range of applications in the field of biomedical as it promotes wound healing by keeping the wound clean and moist. Zinc oxide is very effective in inhibiting the growth of acne causing bacteria Propionibacterium and fungal infection (Tinea pedis) that's why it is used in different acne and fungal treatments creams (Z. L. Wang, 2004). Owing to the strong antimicrobial activity of zinc oxide nanoparticles its play a major role in disinfecting water from various disease causing pathogenic microorganism. Moreover it has been investigated that zinc oxide cost effective mixed matrix membranes have been fabricated with improved antifouling properties (Balta et al., 2012).

2.5 Chitosan Zinc Oxide Hybrid Nanoparticles

From the recent studies it has been summarized that organic antimicrobial agents have less stability, the combination of organic materials with inorganic metal oxides intensifies the antibacterial activity and stability of the composite (Qi et al., 2004). As already mentioned above that chitosan that it is a chelating agent therefore the binding mechanism of chitosan with metals ions occur between the interaction of numerous amine and hydroxyl group present on chitosan (Juang & Shao, 2002). Chemical precipitation is the most commonly used method for the synthesis of chitosan/zinc oxide HNPS. Polymer based method is another proposed method for synthesis of CS-ZnO. SEM images of HNPS exhibited particle size between the range of 58-60 nm with porous globular morphology. Previously XRD results summarized showed that the polymer changed to crystalline form. From various survey it has been concluded that chitosan zinc oxide hybrid nanocomposite has been utilized in many application such as for enhanced dye degradation, coating on textile fabric which has exhibited excellent antimicrobial and anti-biofilm properties. Moreover in water treatment it has been used for removal of permethrin pesticide from water. (Dhillon, Kaur, & Brar, 2014; Haldorai & Shim, 2013; Petkova et al., 2014).

2.5.1 In Sight Into Mode Of Mechanism Of Chitosan And Zinc Oxide

From the literature it has been summarized that Chitosan and zinc oxide has strong antibacterial and antifungal activity. Chitosan antimicrobial mode of mechanism is expected due to robust binding between the cationic molecule and anionic surface of microbial cell membrane. The electrostatic interaction between the positively and protonated NH^{+3} and negatively charged membrane promotes many alterations in the cell membrane stimulating leakage of intracellular proteinaceous constituents and osmotic imbalances (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Another proposed mechanism of antimicrobial activity of chitosan is the interaction of chitosan with the microbial DNA. The penetration of chitosan promotes inhibition of mRNA which further leads resists the protein synthesis. In this mechanism chitosan is thought to pass through the microbial cell wall which is composed of multilayer cross linked murein and eventually approach to the plasma membrane (Liu, Guan, Yang, Li, & Yao, 2000). Zinc oxide has broad spectrum antibacterial activity. The proposed primary mechanism for this activity is due to the photocatalytic generation of reactive oxygen species e.g hydrogen peroxide. In addition when ZnO comes in contact with the bacterial cell membrane it disrupts the membrane and inhibit bacterial growth. It has been investigated that when Zn+ ions bind with the cell it prolong the lag phase of the microorganism. Size of the nanoparticles also effect the antimicrobial activity as in ranges of small size the zinc oxide nanoparticles are more toxic (Q. Li et al., 2008). As summarized from the above situation that chitosan and zinc oxide has antimicrobial activity therefore by combining both into a hybrid or composite will eventually results in enhanced antimicrobial activity.

2.6 Membrane Technology

At the present membrane technology is extensively recognized globally as a mean of disinfecting contaminated water. Membranes technology is utilized not only in industrial processes and industrial waste water treatment but it has also moved into the field of treating municipal waste water and oil field producing water. Membranes act as a physical barrier as it eliminates

pathogenic microorganism like bacteria, fungi and also removes other additives like solid particles.

2.6.1 Fouling of Membrane a Drawback

At present membrane technology is getting more and more attention due to their ability to remove pollutants from water but the most common flaw of this technology is the fouling of the membrane which ultimately results in the failure of the flux (Y. Wang, Kim, Choo, Lee, & Lee, 2000). Fouling of the membranes is most common problem which diminishes the productivity of the membranes by blocking the pores of the membrane and reducing the flux either permanently or temporarily. Fouling is somewhere directly correlated with hydrophobicity, reduced flux and reduced contact angle. Fouling generally originates from various sources which involves blockage of the membranes pores with the particles in the feed water, accumulation of sparsely soluble minerals and growth of microorganism on the surface of the membrane which blocks the membrane pores resisting the passing of water. Biofouling of membrane is initiated by the attachment of bacterial and fungal cells onto the membrane surface which is followed by growth and replication of the microorganism. The biofilm formed by the microorganisms produce extracellular substances (EPS) which strongly bind to the membranes leading towards more colonization (Kim et al., 2003).

The above circumstances require proper cleaning of membranes, which is costly and leads to shorter shelf life of the membrane. The replacement of the membrane is the only solution if any of these conditions prevails. Membrane fouling is a severe condition in microfiltration, ultrafiltration and reverse osmosis membranes. Generally mineral fouling is caused by reverse osmosis membranes because they are sensitive to it. Numerous ways have been investigated to reduce the mineral fouling that consists of various techniques to alter the electrical charge of the membrane that ultimately reject certain species (Nicolaisen, 2003).

2.6.2 Improving Antifouling Properties

It is generally believed that improving the hydrophilicity increases the flux as foulants and the proteins are usually hydrophobic in nature. On the other hand, apart from the surface charge,

surface properties of the membrane are involved in antifouling properties. Recently many modification approaches have been attracted to make the polymer membrane hydrophilic due to their easy processing and mild conditions (Luo, Tang, Zhao, & Pu, 2006). One of the most effective method to improve fouling resistance is blending of inorganic materials especially with nanoparticles by which an increase in hydrophilicity is expects (LUO, WEN, LIU, LIU, & JIA, 2011). However increased hydrophilicity was confirmed by reduced contact angle. Nanoparticles blended membranes has led to many advantages which are as follows.

1) Addition of nanoparticles results an increase in water flux due to larger pore size.
2) Photocatalytic metal oxide nanoparticles like TiO2 ZnO and AL2O3 show antimicrobial activity by generating peroxide radicals (Rana & Matsuura, 2010).

2.7 Polyethersulfone

Among the polymers, poly ether sulfone (PES) is renowned in polymer engineering and it is widely used in separation field. It possesses many good characteristics like high heat-aging resistance, environmental durability, good mechanical properties which make it a distinct polymer in material engineering field. PES membranes are prepared through phase inversion method and always show asymmetric structure. The morphology of the PES membranes are influenced by many factors which involves concentration, additives and solvents, in addition temperature of the casting solution and environment also effects (Zhao, Xue, Ran, & Sun, 2013). The undesirable property of PES is its hydrophobicity which makes it low in flux and exhibit poor antifouling performance many studies have revealed that membrane fouling is directly related to hydrophobic character of the PES. Fouling of the membrane leads to shorter life time, demands higher energy and its performance is unpredictable. Nowadays various approaches are used for modification of PES and PES based membranes (Ahmad, Abdulkarim, Ooi, & Ismail, 2013). Three different methodologies for modification are as follows:

1) Surface grafting of PES membrane
2) Modifying bulk material of PES
3) Blending of PES with nanoparticles

2.7.1 PES Blends/Composite

PES blends have been prepared previously. These blends can be further categorized into homogeneous (miscible) and heterogeneous (immisible). Polymeric blends have been used with a high degree of simplicity and efficiency to make it improve in performance. Hydrophilic polymers like Polyvinylpyrrolidione (PVP) and polyethylene glycol (PEG) are the most commonly used polymers that are blended with the PES for better membrane performance. These additives have the ability to resist microvoids making the membrane pore more interconnected. Moreover native PES blended with sulfonated pes showed outstanding miscibility with increased permeability flux (Wienk, Olde Scholtenhuis, Van Den Boomgaard, & Smolders, 1995). Recently nanofilteration membranes have been fabricated by blending organic/inorganic polymeric materials with enhanced antifouling property. The inorganic additives used are silver, zinc oxide, titanium dioxide and silica (Ahmad et al., 2013).

3. SYNTHESIS AND CHARACTERIZATION OF CS-ZnO HNPS

3.1 Materials and Methods

The chemicals used were of analytical grade and used as received without further purification. Chitosan degree of deacetylation was 94% obtain from (Sigma Aldrich), zinc oxide (Sigma Aldrich), polyether sulfone (PES) (Ultrasone Germany), N-methyl-2-pyrrolidione (NMP) (Sigma Aldrich), polyester support and nutrient agar (Merk). All test strains *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Aspergillus fumigatus* and *Fusarium solani* were taken from clinical isolates

3.1.1 Synthesis of Chitosan-Zinc Oxide Hybrid Nanoparticles

CS-ZnO HNPS were synthesized using chemical precipitation method with modifications (Haldorai & Shim, 2013).1.0 g zinc oxide powder was dissolved in 100 ml of 1% acetic acid solution to obtain zinc cations. Then, 1.0 g of chitosan was added in the solution and the mixture was kept for sonication for 30min. To maintain the PH at 10, 1 mol/L NaOH was added dropwise. The solution was heated in water bath for 3 h at 60C^{o}. After that, the solution was repeatedly centrifuged at 8000 rpm for 10 mins using distilled water so that the particles settle down in the bottom. The solution was filtered and dried in vacuum oven at 50 ^{o}C.

3.2 Characterization of Chitosan-Zinc Oxide Hybrid Nanoparticles

Different characterization techniques were used to characterize the CS-ZnO HNPS as described below.

3.2.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) Joel JSM 6490A was performed to investigate, morphology of NPS. EDS was done for the elemental analysis of the HNPS.

3.2.2 Energy Dispersive Spectroscopy (EDS)

EDS was done for the elemental analysis of the HNPS. To investigate the mass percentage of the elements

3.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) FT-IR Perkin Elmer Spectrometer, spectrum 100) was done to confirm the immobilization of zinc oxide onto chitosan. The spectrum was recorded by Perkin Elmer instrument. By adding very small amount of HNPS KBr pellets were prepared.

3.2.4 X-Ray Diffraction

The X-ray diffraction (XRD) Model Theta STOE Germany was done to determine the phase and purity of the NPS. This technique identifies the crystal phase of nanoparticles and provides the size estimate of crystallite

3.3 RESULTS AND DISCUSSIONS:

3.3.1 Scanning Electron Microscopy (SEM) And Energy Dispersive Analysis of X -Rays (EDAX)

SEM analysis was performed to observe the size and shape of nanoparticles whereas EDAX was carried out for elemental composition of nanoparticles. The NPS exhibited porous globular morphology with an average size of 58 nm presented in Fig 1. Present results are supported by previous work in which approximately same size of CS-ZnO HNPS were prepared by chemical

precipitation method (Moradi Dehaghi, Rahmanifar, Moradi, & Azar, 2014). Some agglomerations were seen in the images this was due to the fact that nanoparticles were used in the powder form. The nanoparticles were in nanoscale which means that these can be used further to embedded in polymer membrane. EDX was carried out to confirm the elemental composition of CS-ZnO HNPS. Fig 2 shows that nanoparticles contain about 21.4wt % carbon, 22.56wt% oxygen and wt% 56.03 zinc.

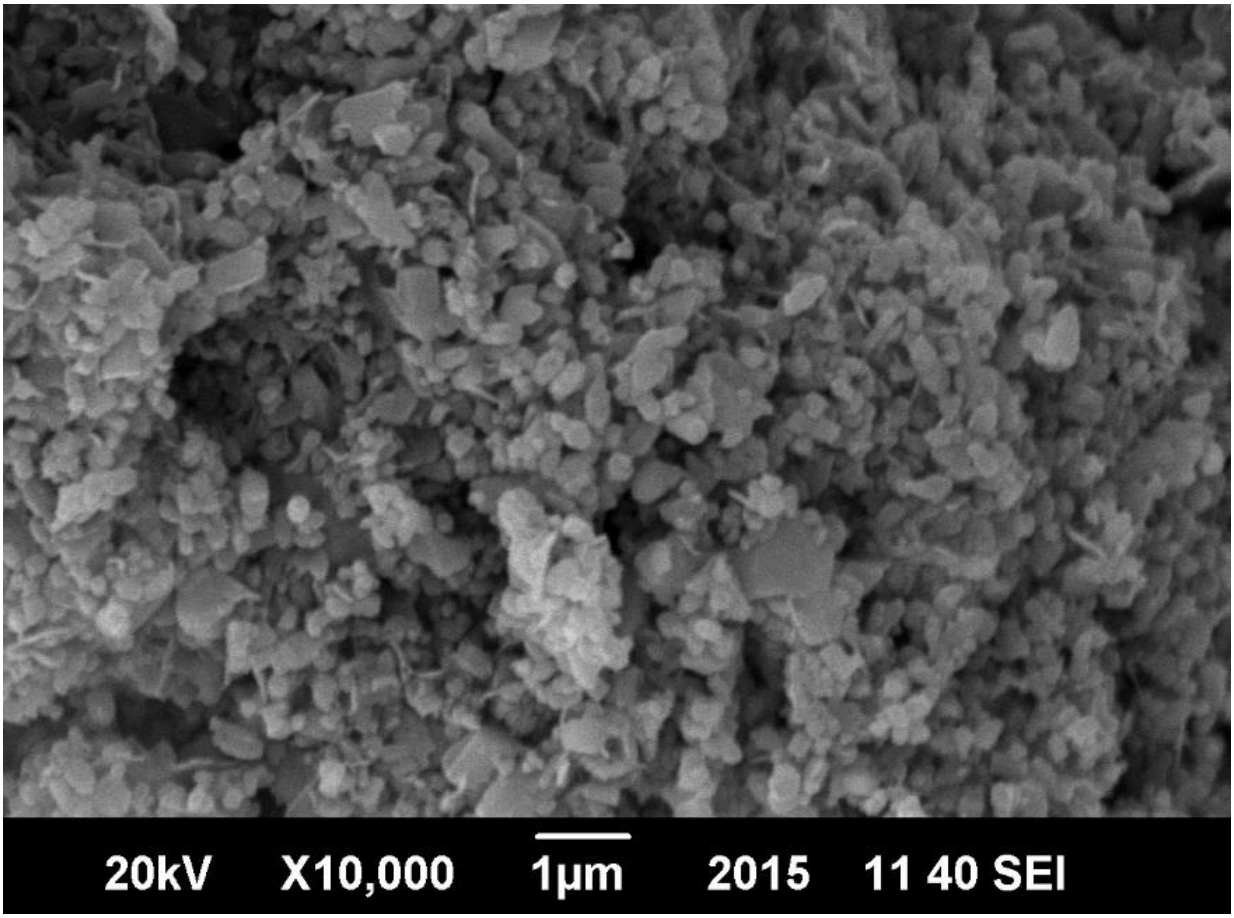


Figure 1: *SEM analysis of CS-ZnO HNPS*

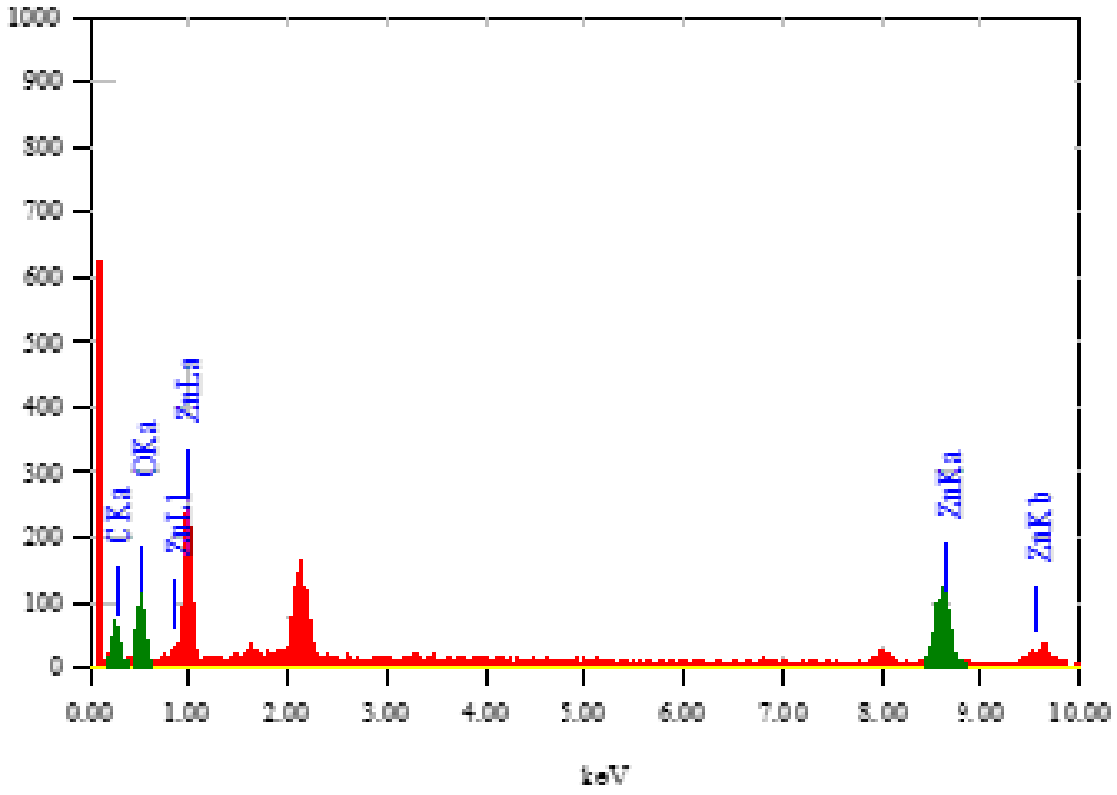


***Figure 2**: EDS analysis of Chitosan-Zinc oxide hybrid nanoparticles*

3.3.2 FTIR Analysis of Chitosan and CS-Zno Hybrid Nanoparticles

The FTIR spectrum of CS and CS-ZnO HNPS show many alterations presented in Fig 3. The small and broad band at 3392cm^{-1} is due to the stretching vibration of O-H and N-H primary amines (asymmetric stretch) is shifted towards lower wave number i.e. 3382.2cm^{-1} reformed to slightly broader stretch due to the immobilization of Zn^{+} onto O-H and N-H functional groups. The band at 2874cm^{-1} exhibited due to asymmetric stretch of C-H group (alkanes). The absorbed frequency at 1601.1cm^{-1} is due to N-H bend of secondary amines (symmetric stretch) transformed to smaller bend at 1589cm^{-1} due to N-H deformation mode (Haldorai & Shim, 2013). The stretch at 1091.38cm^{-1} wavenumber exhibit carboxylic acids (C-O) shifted towards lower wavenumber to 1033 cm^{-1}. A small stretch at 550cm-1 was attributed due to stretching vibrations of N-Zn (Dhanavel, Nivethaa, Narayanan, & Stephen, 2014). In the CS-ZnO HNPS

all frequencies shifted to lower wavenumber which is the confirmation of successful preparation of CS-ZnO HNPS (Haldorai & Shim, 2013).

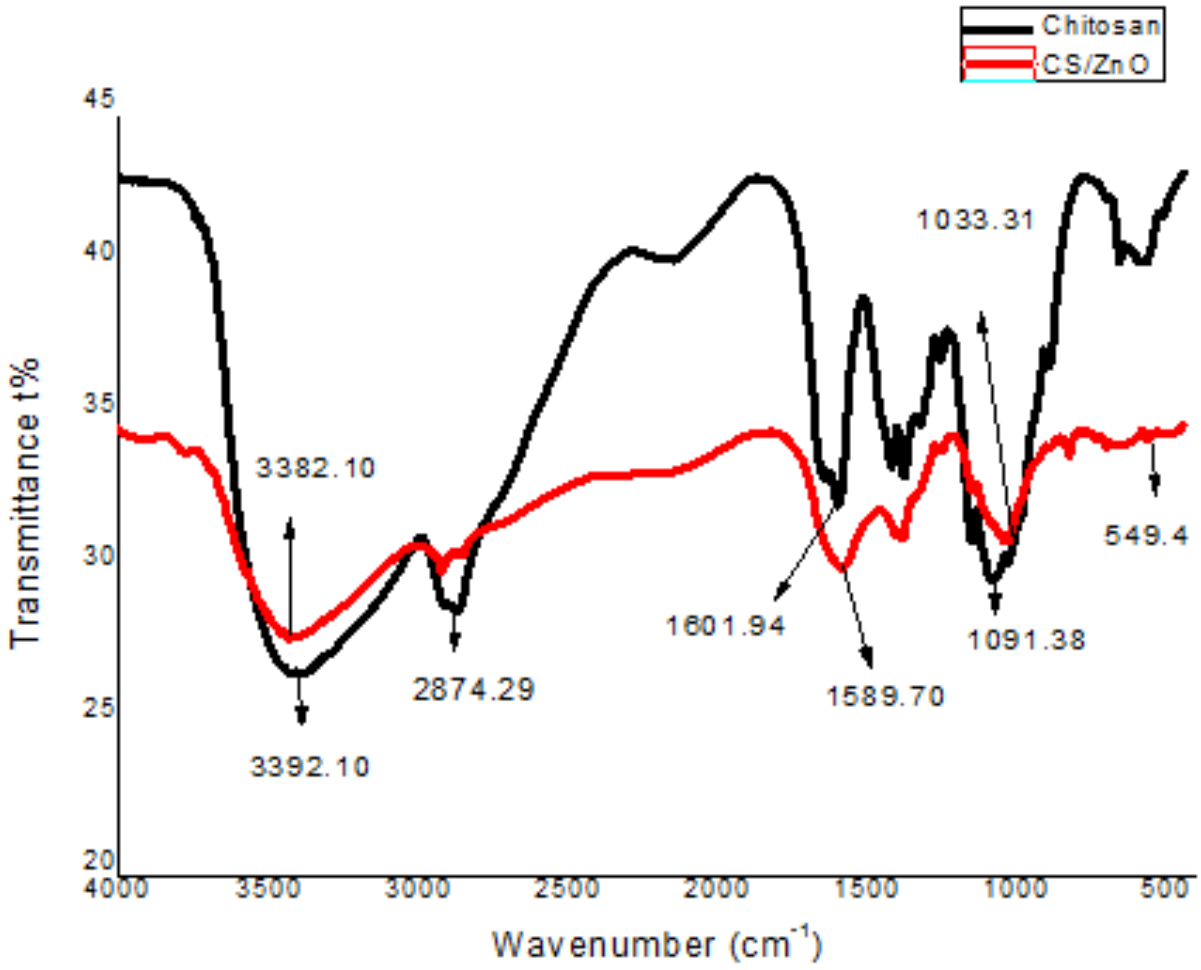


Figure 3: *FTIR analysis of pure chitosan and Chitosan-Zinc oxide Hybrid Nanoparticles*

3.3.3 X-Ray Diffraction

Fig 4 represents the XRD pattern of the CS and CS/ZnO HNPS. The small broad peak exhibited in the Fig 4 (A) at 19.77° shows the amorphous nature of the polymer. In the figure 4 (B) the diffracted peaks in the CS/ZnO HNPS appeared at 31.69°, 34.38°, 36.18°, 47.45°, 56.46°, 62.76°, 66.21°, 67.80°, 68.92°, 72.47°, 76.78°, 81.25° and 89.41° were allocated to <100>, <002>, <101> ,<102> ,<110>, <103> ,<200> ,<112> ,<201>, <004> , <202>, <104>, and <203> planes of the ZnO with hexagonal wurzite crystal system reference to JCPDS card no 01-079-0207 (Salehi,

Arami, Mahmoodi, Bahrami, & Khorramfar, 2010). The results demonstrated both characteristic peaks of the CS and ZnO in the CS-ZnO HNPS which is a conformation of successful preparation of the composite, as the chitosan is transformed from amorphous to crystalline phase.

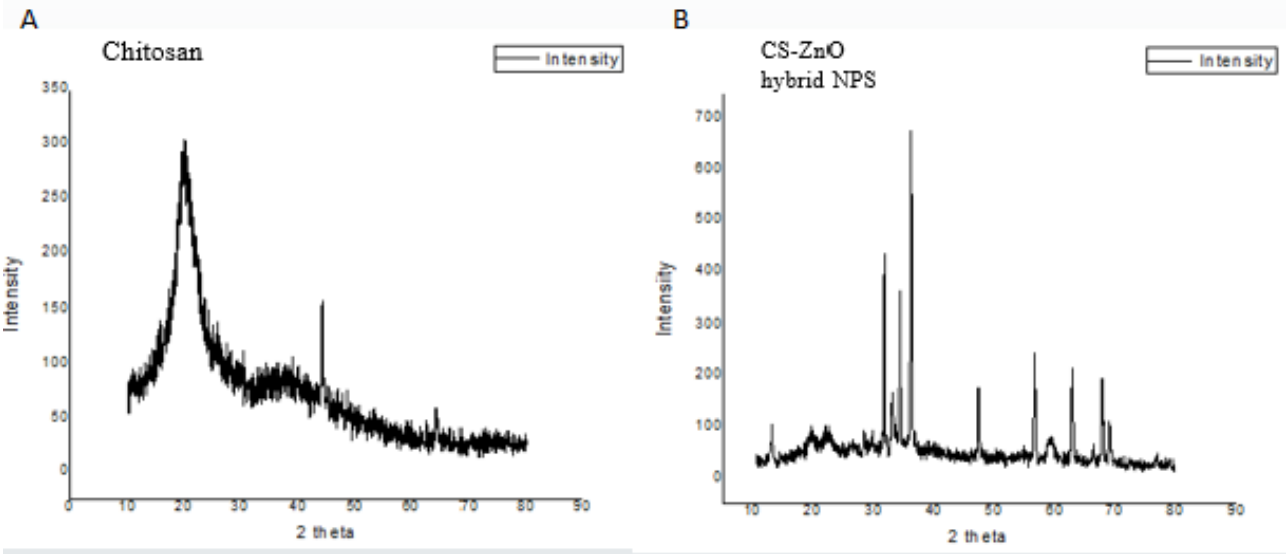


Figure 4: *XRD analysis of pure chitosan (A) and CS-ZnO HNPS*

4. Fabrication and Characterization of Mixed-Matrix Membranes

4.1 Membrane Fabrication

The membranes were fabricated by mixing 20% w/w polyether sulfone in NMP solvent (solution A). Magnetic stirring was done overnight at room temperature until polymer was completely dissolved followed by casting of membranes. For synthesis of nanoparticles entrapped in mixed matrix membranes CS-ZnO HNPS were added in three different concentration (5%, 10% and 15% w/w) in NMP and dissolved through magnetic stirring (solution B). Both solutions were mixed and sonicated for 30 mins for complete dispersion of the HNPS. Phase inversion method was used for membrane fabrication using a thin film applicator and custom design tray. Before spreading the casting solution, the polyester support was wetted with NMP to prevent the polymer solution to penetrate into the support. After spreading the casting solution on the support, the membranes were immersed in distilled water for the removal of any leftover solvent. Finally drying was done under ambient condition to obtain the membranes.

4.2. Membrane Characterization

4.2.1 Scanning Electron Microscopy

Polymeric membranes were characterized through different techniques. Scanning electron microscopy (SEM) Joel JSM 6490A was performed to evaluate morphology, topography and pore size. Samples were cut into 0.25 cm^2 pieces, mounted on blocks, gold coated and then analyzed.

4.2.2 Energy Dispersive Spectroscopy (EDS)

EDS was done for the elemental analysis of the membranes.to investigate the presence of nanoparticles in the membranes

4.2.3 Contact Angle Measurements

Contact angles were investigated to measure hydrophilicity and surface wettability of the membranes. The measurements were carried out by dropping 10µl water on the membrane after that the images were captured through camera. Distilled water was used in all measurements and on average 3 different measurements was taken to calculate average contact angle. Experimental error was minimized by taking measurements at different locations and then calculating the average.

4.2.4 Optical Profilometery

Non-contact Optical profilometer NONOVEA PS50 was used to measure the surface roughness Ra was the main parameter was selected according to ISO 4287.

4.2.5 Water Retention

Water retaining capability of membranes was investigated by soaking 1g of membrane in water for 24 hours. The membranes were then oven dried for 12h and dry weight was calculated. Water content was then calculated using this formula

$$[(\text{wet weight}-\text{dry weight})/\text{wet weight}]\times 100.$$

4.2.6 XRF

XRF test was carried out using Joel JSK-3202M Element analyzer Japan to find out if there is any CS-ZnO HNPS present in filtered water.

4.2.7 Water Permeability Flux

Membrane flux test was carried out using filtrate assembly at constant pressure of 60cmhg with an area of membrane 0.025m at room temperature.

4.3 RESULTS AND DISCUSSIONS

4.3.1 Scanning Electron Microscopy

Scanning electron microscopy images were taken to analyze the average pore size of the membrane so that it can further assist in the analytical studies. Cross section images of all membranes are labelled and presented in the Fig 5. The micrographs of the membranes illustrate remarkable porosity. The porosity of the membranes diverges with the addition of NPS. In case of MH0 the aperture size reduced suddenly and was enhanced with an increment of NPS. The membranes with the code MH0, MH1, MH2 and MH3 can be observed in Fig 5 labeled as A, B, C, and D with an average pore size of 0.17µm, 0.351µm, 0.451µm and 0.586µm. An apparent phenomenon is induced that higher concentration of filler yields a significant number of large pores that alters the hydrophobic nature of the MH0 by transforming it to hydrophilic as investigated before that addition of NPS has large influence on membrane morphology so we can conclude that morphology of membrane fluctuated with the increasing content of NPS (Y. Yang, Zhang, Wang, Zheng, & Li, 2007)

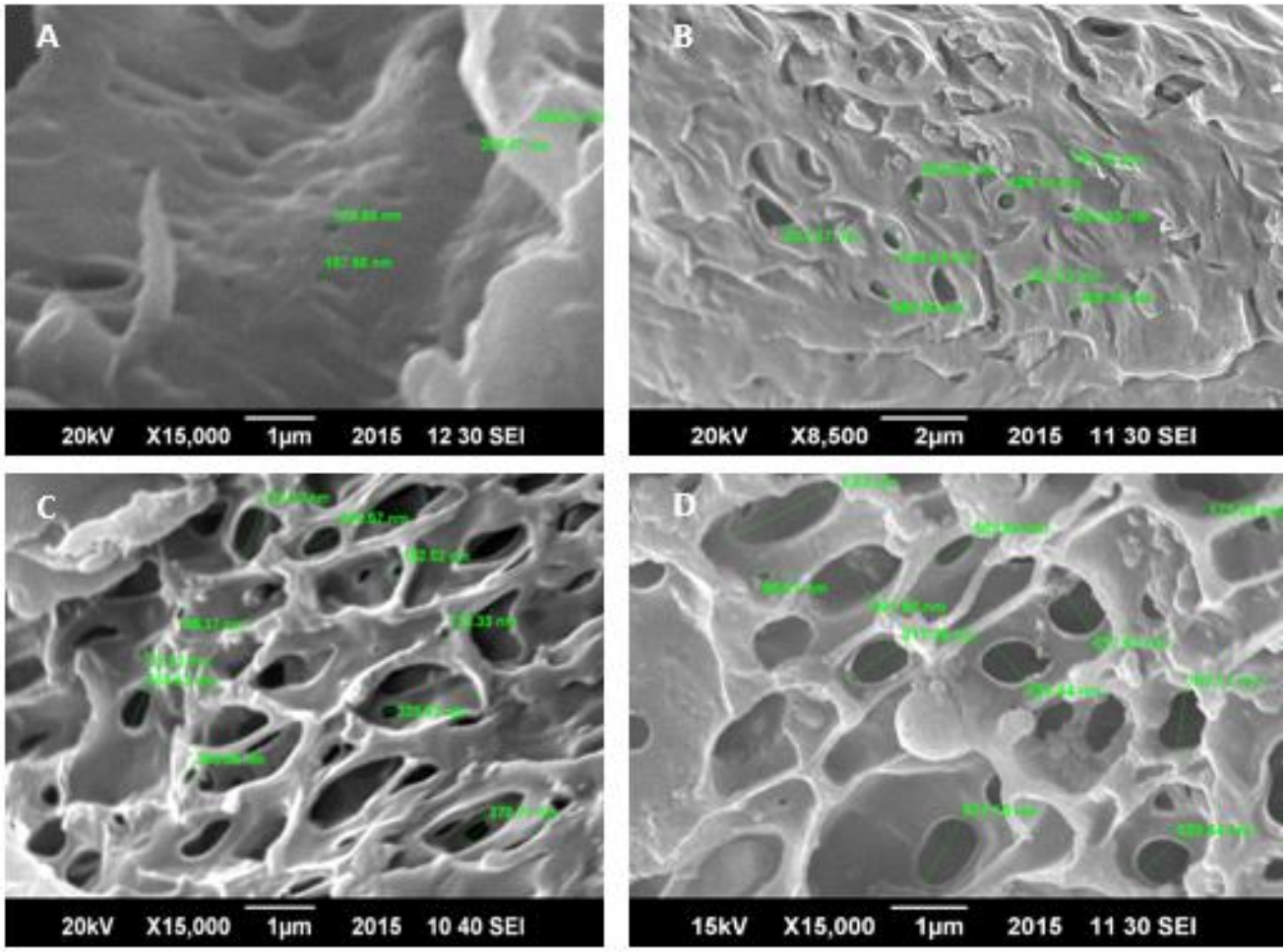


Figure 5: *Micrographs display the pore size of the polymer membranes **A**: PES (MHO), **B**: PES+0.15g NPS (MH1), **C**: PES+0.3g NPS (MH2), **D**: PES+0.45g NPS (MH3).*

4.3.2 Energy Dispersive Spectroscopy (EDS)

Figure 6 represent the elemental analysis of the membranes. The EDS results depicted the presence of Carbon “C”, oxygen “O” and sulfur “S”, in MH0 with no addition of nanoparticles. The membranes with the codes MH1, MH2, MH3 were detected with the Carbon “C”, Oxygen “O”, and Sulfur “S” and “Zn” zinc as chitosan was organic so its elements were mainly covered in carbon and oxygen. So it can be concluded that HNPS were present in rest of the three membranes

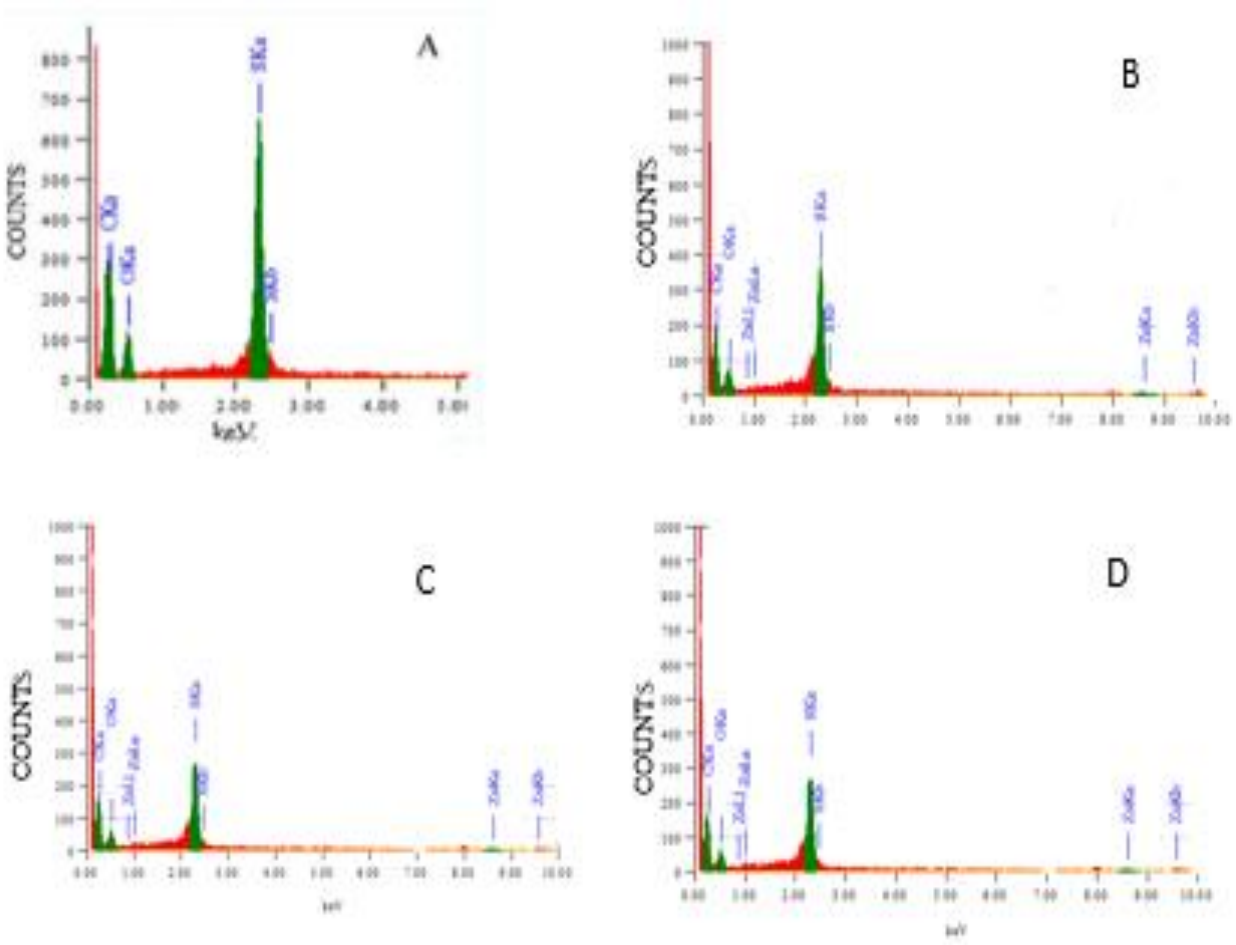


Figure 6: *EDS analysis of MH0 (A), MH1 (B), MH2 (C) and MH 3(D)*

4.3.3 Water Retention, Contact Angle and Surface Roughness Analysis

Table 1 summarizes the outcome of water content percentage, average contact angle and surface roughness of membrane. Water retention has a correspondence with the water contact angle. An upsurge in water content and a decline in contact angle designates to improved wettability of the membranes. Considering that contact angle is associated to the surface property whereas the water retention is as bulky property (Kanagaraj et al., 2015) . The MH0 membrane has the least water retention content 29.43% and the highest contact angle 60.7^0, as this demonstrate the hydrophobic nature of the PES (Leo, Lee, Ahmad, & Mohammad, 2012) .The percentage of water retention content is increasing with the addition of NPS in MH1 and MH2 from 34.22% to 42.99% respectively. In case of MH3 the significant value of the water retention content can be

observed to around 54.26%. Therefore it can be established that an elevation in concentration of NPS has a noticeable and positive effect on membrane hydrophilicity due the enlarged aperture size (Mobarakabad, Moghadassi, & Hosseini, 2015). Maximum reduction in the contact angle was experienced in MH3 having 40.33º contact angle which shows a direct relationship between increasing amount of NPS and decreasing water contact angle . Fig 7 represents the contact angles of all membranes. Surface roughness analysis in the Table 1 reveal that, greater roughness was observed with membrane with no addition of NPS, whereas with an increasing content of NPS had a positive effect on membrane flux and permeability by making its surface smooth. In MH3 the Ra value obtained was 5630 nm .Wei et al., in his research also found that a decrease in surface roughness eventually resulted in improved antifouling property of membrane (Wei et al., 2011)

Table 3: Membrane composition, %water content, average contact angle and surface roughness

Membrane code	**Membrane composition**	**%Water retention content**	**Average Contact angle**	**Roughness Ra (nm)**
MH0	PES	29.43	60.73 ± 0.37	32775
MH1	PES+0.15g NPS	34.22	44.6 ± 0.33	4663
MH2	PES+0.3g NPS	42.99	43.3 ± 0.33	2933
MH3	PES+0.45g NPS	54.26	40.33 ± 0.33	2006

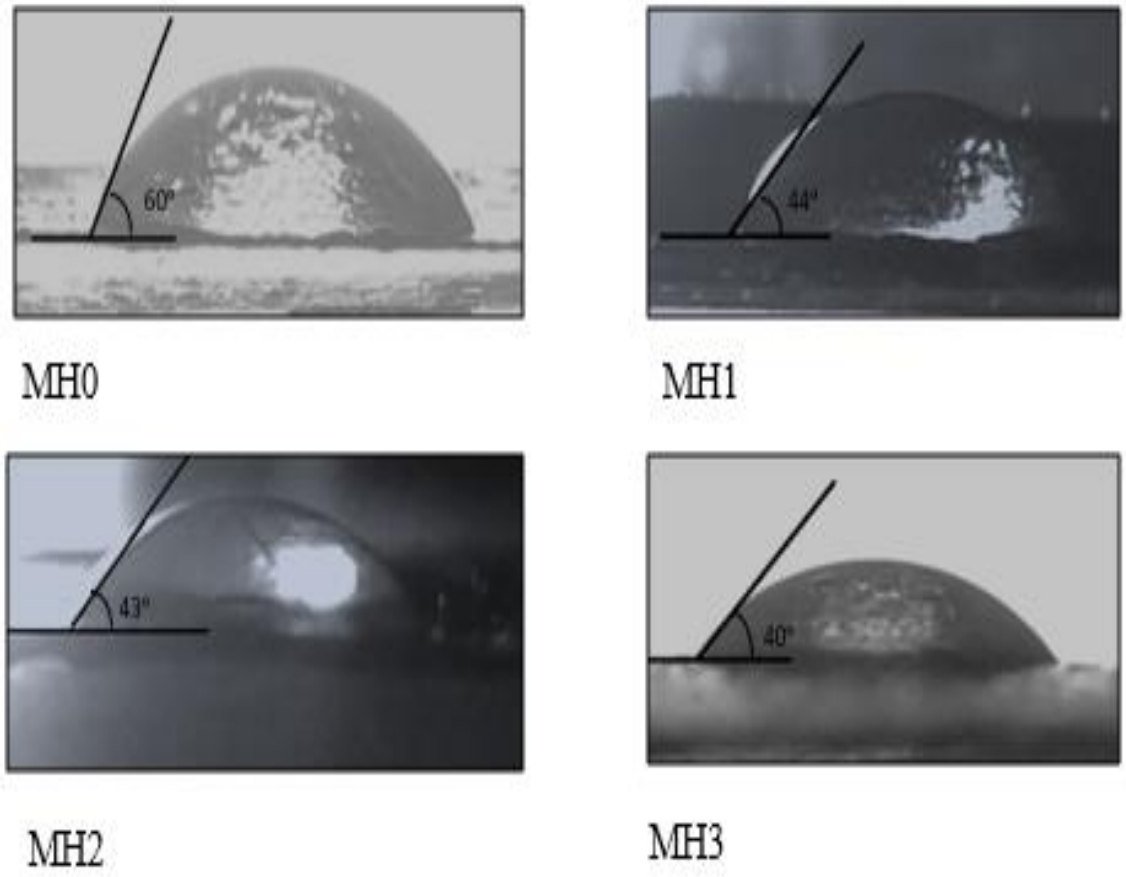


Figure 7: *Contact angle of polymeric membranes presented with codes*

4.3.4 Permeability Flux

Water flux was measured using a custom made filtrate assembly and results are presented in Fig 8. It was noted that increasing the amount of CS-ZnO HNPS initiated a drastic change in the permeability flux. The lowest value for water flux was seen in MH0 which was 1215.8J as this was owing to the fact of hydrophobic nature of the PES. The highest value for water flux was observed in MH3 which was 4135.8J, with maximum content of NPS. The improved pore size made the membrane hydrophilic and because of this hydrophilic character water molecules were attracted by the membrane promoting the passage of water. Similar outcomes were observed when Wei et al ., experienced that an elevation of TiO2 nanowires in PVDF membranes

significantly resulted in greater permeability flux (Wei et al., 2011). Similarly it has been also concluded from the above SEM, contact angle and surface roughness analysis that porosity increases the antifouling property of the membranes. The pore size and the surface hydrophilicity are the two parameters that are responsible for higher water flux as it has been concluded from the general concept that increase in the NPS content results in greater porosity (Ng, Mohammad, Leo, & Hilal, 2013; Q. Yang, Xu, Dai, Wang, & Ulbricht, 2005). The XRF analysis of the filtered water confirmed no leaching of NPS into the water this was a conformation that the HNPS were significantly bonded with the polymer. As reported that acceptable levels of WHO standards for zinc oxide in water is 3-5mg/L (Motshekga, Ray, Onyango, & Momba, 2015).

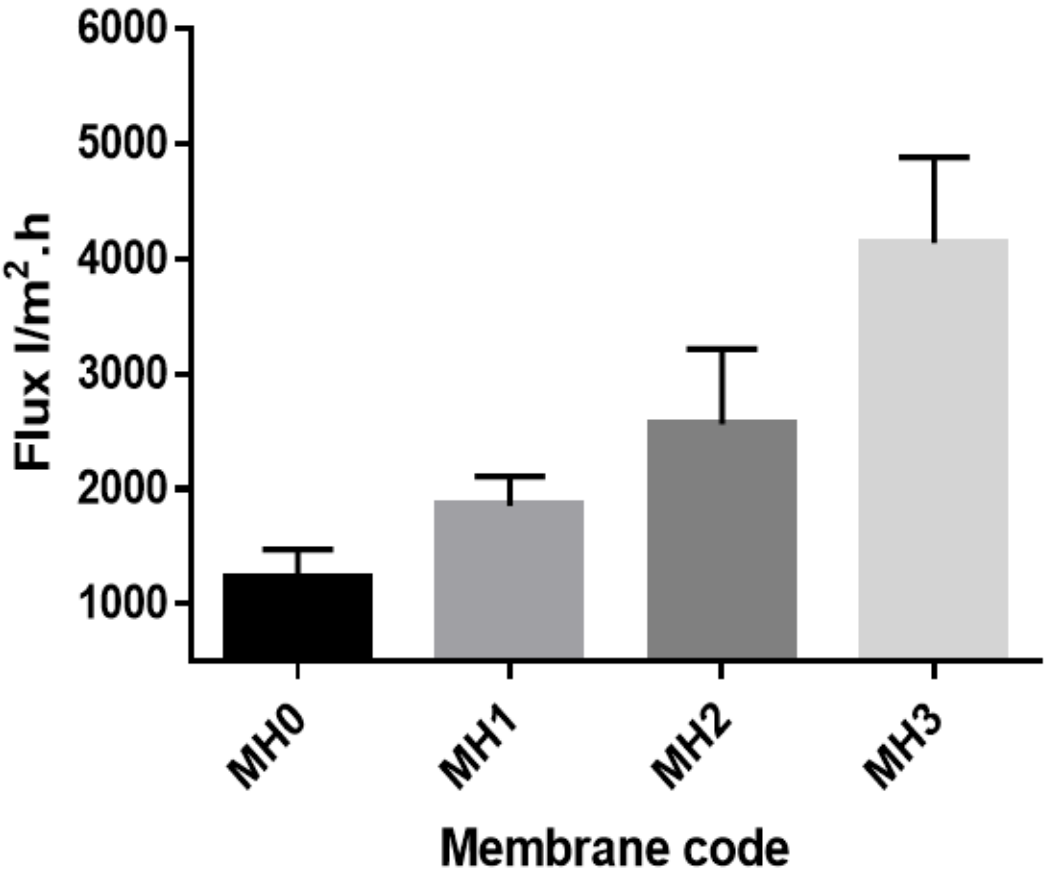


Figure 8: *Water flux measurements of all membranes at 60 mmHg pressure*

5. Evaluation of Antimicrobial Activity

5.1 Anti-Bacterial Testing of Nanoparticles

Antibacterial activity was investigated against *Staphylococcus aureus (ATCC 653), Escherichia coli* (ATCC 8739*), Bacillus cereus, Salmonella typhi,* (ATCC 6539) through well diffusion method. Nutrient agar was poured into the petri plates and allowed for solidification. Wells were made by using 6mm cork borer. 0.1 g of NPS were taken and three different concentrations were prepared 50%,75% and 100% by mixing them in 1% diluted of acetic acid and 10 µl of each concentration was poured into the well. Chloramphenicol was taken as positive control.

5.2 RESULTS AND DISCUSSIONS

Table 2 show results for well diffusion method were positive as all the strains tested display prominent zone of inhibition against the three concentrations 50%, 75% and 100%. The zone of inhibition was increased as the concentration of nanoparticles increased. From the outcomes it can be observed that gram positive bacteria exhibited slightly more significant zones of inhibition than the gram negative bacteria. As zinc oxide produces reactive oxygen species, the membrane of gram negative is more resistant because of the fact that gram negative bacteria cell wall is more complex due to the presence of an outer membrane. This outer membrane acts as a permeability barrier shows less sensitivity against reactive oxygen species (ROS) when compared to gram positive (Russell, 2003). As investigated that the possible reason for antibacterial activity was due to the strong interaction between electronegative microbial surface with the positively charged CS-ZnO HNPS, which penetrate into the bacterial membrane permitting it to leak all the main constituents which are involved in the growth of bacteria (L.-H. Li, Deng, Deng, Liu, & Li, 2010).

Table 4: Antibacterial activity of CS-ZnO HNPS against gram negative and gram positive bacteria through well diffusion method.

	Zone of inhibition			
	Concentration mg/ml			
Bacterial strain	50%	75%	100%	Positive control
S. aureus **(gram positive)**	12 ± 0.28	13 ± 0.50	18.5 ± 0.28	23.6 ± 0.33
B. cereus **(gram positive)**	9.23 ± 0.17	13 ± 0.50	18.5 ± 0.28	23.6 ± 0.33
E.coli **(gram negative)**	9.76 ± 0.14	12.27 ± 0.14	13.63 ± 0.31	23.73 ± 0.37
S. typhi **(gram negative)**	9.5 ± 0.28	10.83 ± 0.16	11.50 ± 0.28	22.67± 0.33

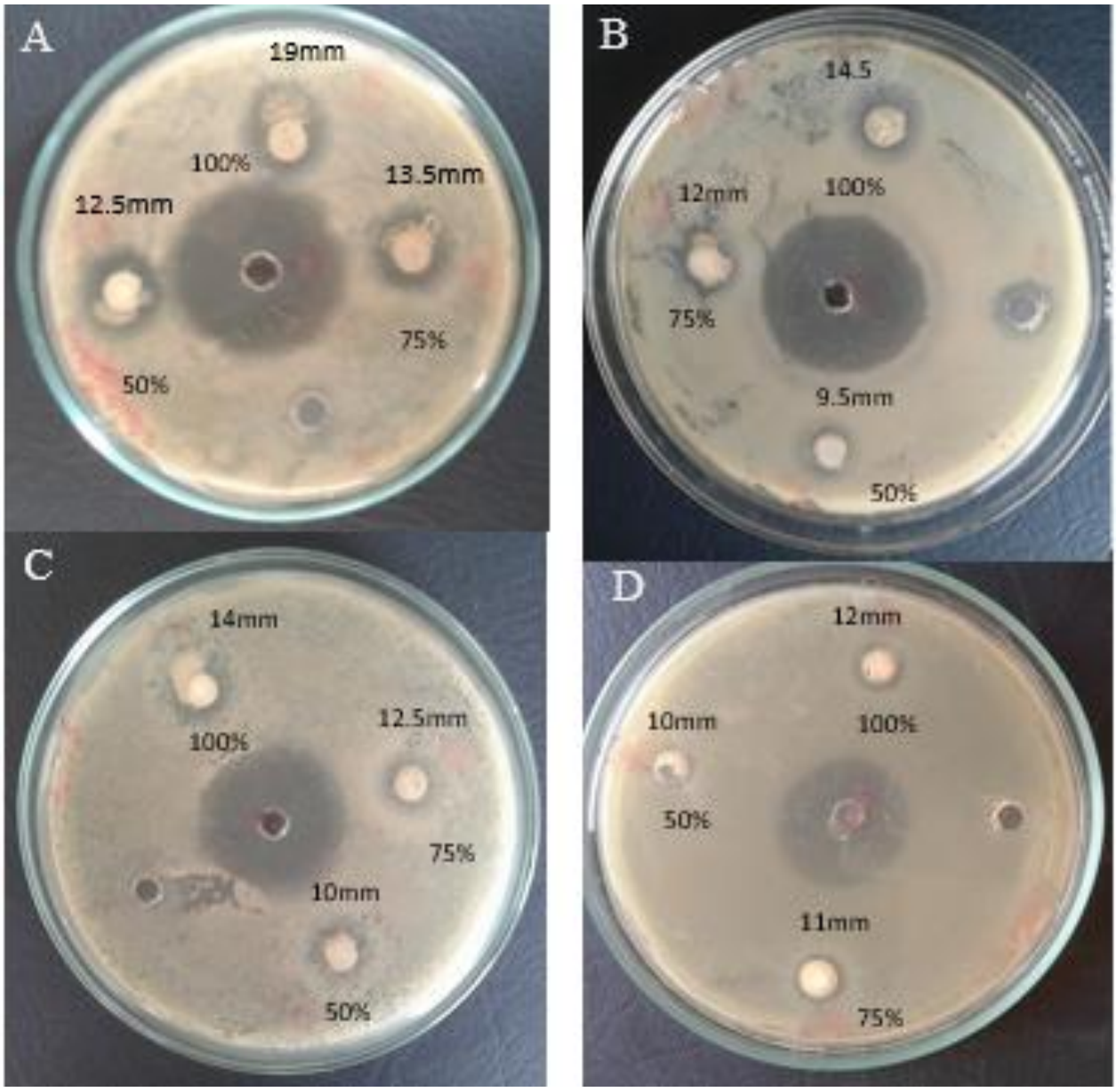


Figure 9: *Antibacterial activity of CS-ZnO HNPS against S.Aureus (A), B.Cereus (B), E.coli (C), and S.typhi (D).*

5.3 Antifungal Testing of Nanoparticles

Antifungal activity was studied against *Aspergillus fumigatus* and *Fusarium solani* through well diffusion method. 0.1 g of NPS were taken and three different concentrations were prepared 50%, 75% and 100% respectively by mixing them in 1% diluted acetic acid. Saboured dextrose

media was prepared and poured into the petri plates. 10 µl of each concentration was poured into the well. Amphotericin A was taken as positive control. Testing was done in triplicates and average readings were calculated.

5.4 RESULTS AND DISCUSSIONS

The table 5 illustrates the results of antifungal activity of nanoparticles through well diffusion method. The zone of inhibition increased as the concentration of nanoparticles increased. *A.fumigatus* exhibited an effective antifungal activity than *F.solani.* In 100% concentration of NPS significant zones of inhibition can be seen in *A.fumigatus* and *F.solani* i.e. 22.3 mm and 15.8 mm respectively. The region of inhibition for both of the strains were remarkably greater than the amphotericin A antifungal drug which was taken as control presented in Fig10. This activity was due to chelation of chitosan with Zn^{+} which makes it more prevailing to interact with the anionic membrane of the fungus and finally causes widespread destruction of the fungal hyphae (Muzzarelli et al., 2001; X. Wang, Du, & Liu, 2004)

Table 5: Antifungal activity of CS-ZnO HNPS against A.fumigatus and F.solani through well diffusion method

	Zone of inhibition (mm)			
	Concentration mg/ml			
Fungal strain	50%	75%	100%	Positive control
A.fumigatus	19.6 ± 0.30	21.1 ± 0.16	22.3 ± 0.66	15.6 ± 0.16
F.solani	10.8 ± 0.44	13.9 ± 0.15	15.8 ± 0.08	14.6 ± 0.30

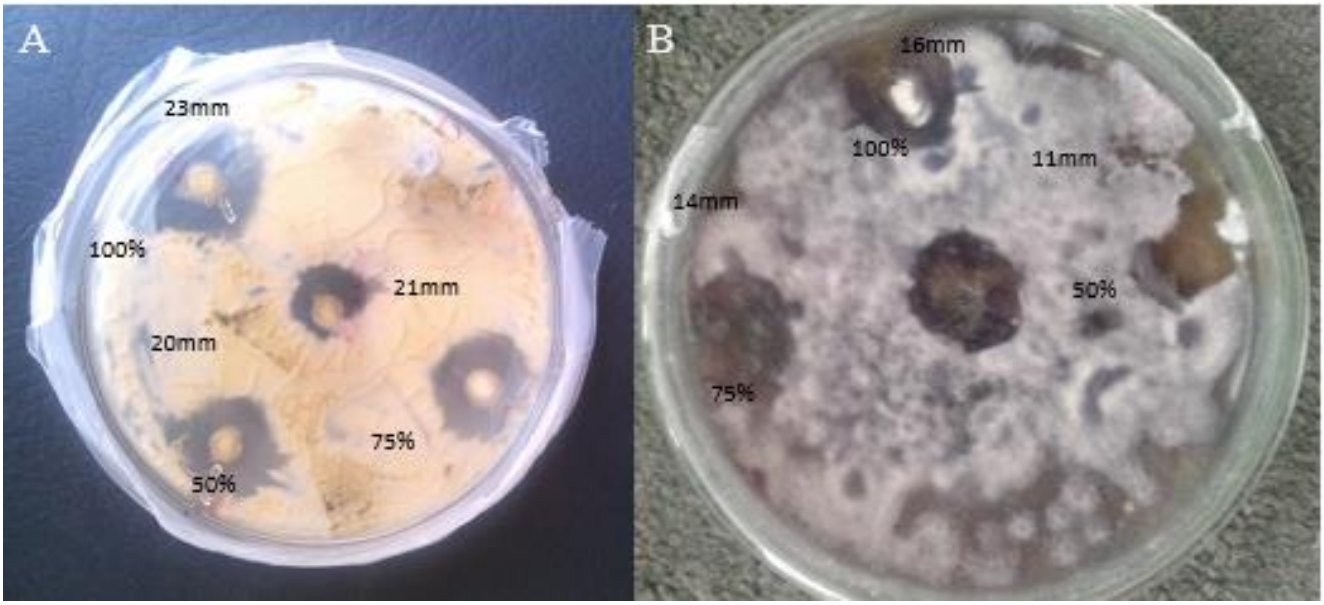


Figure 10: *Antifungal activity of CS-ZnO HNPS against A.fumigatus (A) and F.solani (B)*

5.5 Bactericidal Testing of Membranes

Bactericidal testing of the fabricated membranes were done to investigate the proficiency of membranes to block and eradicate the bacteria in the water. For this purpose 10^{-6} dilutions of *E.coli, S. Aureus,* and *S.Typhi and B.Cereus* solutions were prepared .These dilutions were then passed through the PES/CS-ZnO HNPS membrane fixed in the Millipore filtrate assembly. After that, the resultant filtrate water was finally spread over the prepared nutrient agar plates. These plates were incubated at $37C^0$ for 24 h. After incubation colony forming units per ml was calculated for each membrane. Testing was done triplicates.

5.6 RESULTS AND DISCUSSIONS

Anti-bacterial testing of all polymer membranes were carried out to confirm the bacterial rejection through the membranes. Dilutions were prepared at 10^{-6} for all bacterial strains and then were passed through the filtrate assembly. For gram positive bacteria *S.aureus* and *B.Cereus* the average reading of cfu/ml at 10^{-6} was 3.17×10^9 and 3.5×10^9 which was taken as control. This

was effectively reduced 0.36×10^9 and 0.40×10^9 respectively when the bacterial dilutions were passed through MH3. The control cfu/ml for gram negative bacteria *E.coli* and *S.typhi* were 3.2×10^9 and 3.7×10^9 and reduced to 0.48×10^9 and 0.61×10^9 for MH3. A significant decline for colony count was observed when the content of CS-ZnO HNPS increased in the membrane. When correlating pore size of the membrane with an average size of bacteria it has been reported that these microorganism approximately range from 0.5 to 3 µm in size (Tuomela, Vikman, Hatakka, & Itävaara, 2000).The above SEM images of membrane shows that the highest pore size of the membranes obtained is 0.586µm that is a slightly larger than the bacteria size but due the highest concentration of nanoparticles in MH3 membrane the filtered water from the membrane has the least colony count. So it is concluded that the pore size and concentration of HNPS both are playing a significant role in reduction of bacterial count. The extreme drop for all bacterial colony was observed in MH3, with an overall 85.6 % reduced bacterial colony. The mechanism which was involved in enhanced antibacterial activity was due to the synergistic effect of chitosan and Zinc oxide. As reported earlier that zinc oxide initiate the production of reactive oxygen species (ROS) leading to oxidative stress (Ohira, Yamamoto, Iida, & Nakagawa, 2008). In a similar work carried out to investigate the effect of NPS, polyethersulfone ultrafiltration membranes embedded with silver NPS were fabricated with a high antibacterial activity which caused 99.9% reduction in *E.coli* colony(Sawada et al., 2012). Moreover Kim et al,. in his study fabricated antibiofouling TiO2 nanoparticles self-assembled polyamide thin films. These films exhibited photocatalytic bactericidal activity as TiO2 generated active oxygen species resulted to cell death (Kim et al., 2003).

Graphical representation in Fig 11 shows CFU/ml for all bacterial strains. Error bars show the standard deviation and asterisks (*) represent significant p-values. All membranes are compared with a negative control and their significant p-values are represented.

Many polymeric membranes with different inorganic nanoparticles has already been fabricated with improved antifouling properties, chitosan blend films with silver and zinc oxide nanoparticles was previously fabricated with an outstanding antibacterial property (L.-H. Li et al., 2010; Qi et al., 2004). In case of eradication of lethal pathogens from water antimicrobial nanoparticle are playing a significant role in membrane technology (Q. Li et al., 2008).

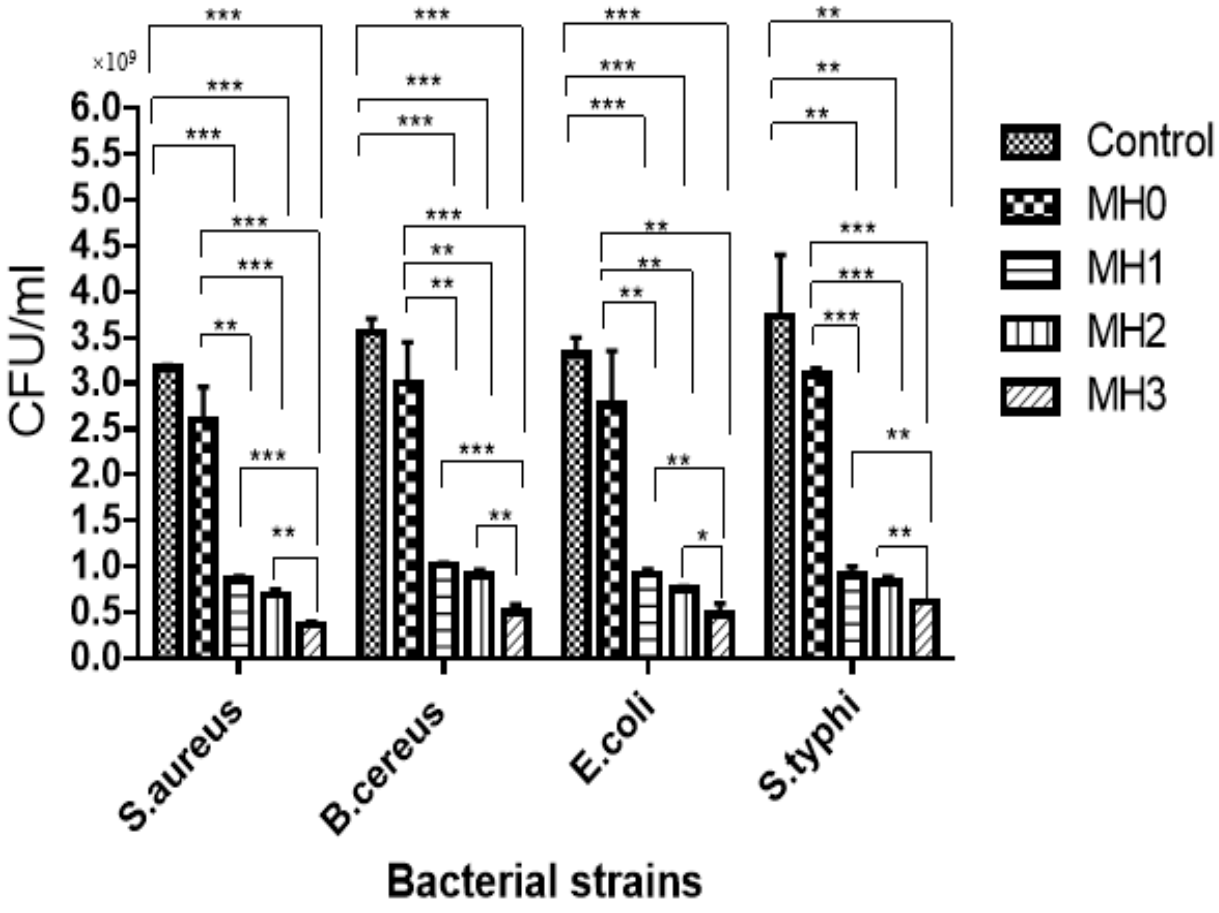


Figure 11: *Represents CFU/ml after passing bacterial dilutions through all membranes. Error bars demonstrate the standard deviation and asterisks (*) represents p value. The asterisks (*), (**), (***) indicate significance difference ($p \leq 0.05$), ($p \leq 0.01$), ($p \leq 0.001$) respectively.*

5.7 Fungicidal Testing of Membranes

Antifungal testing of membranes were carried by preparing a solution of *Aspergillus fumigatus* and *Fusarium solani*. 0.01% tween 20 was added to make a better solution as some fungal species are hydrophobic in nature. The solutions were passed through each membrane fitted in the Millipore filtrate assembly. Spores/ ml were counted using a hemocytometer. Antifungal testing was done in triplicates.

5.8 RESULTS AND DISCUSSIONS

To determine the antifungal activity of membranes the spores dilution was passed from all membranes fitted in the filtrate assembly. For *F.solani* and *A.fumigatus* the spores/ml count for control was 4.5×10^4 and 8×10^4 it was reduced to 3.33×10^4 and 5×10^4 when passed through MH0. The increment of NPS in membranes further declined the spore/ml count. The spore count for *F.solani* and *A.fumigatus* was reduced by 77.7% and 92% in MH3 respectively. This result is an evidence of above antifungal testing of NPS which showed greater zone of inhibition in *A.fumigatus* than *F.solani*. From the literature it has been summarized that zinc oxide nanoparticles and chitosan has shown significant antifungal properties against various fungal species. Which promotes the broad spectrum destruction of the fungal hyphae and suppresses the spore germination (El Ghaouth, Arul, Asselin, & Benhamou, 1992; He, Liu, Mustapha, & Lin, 2011) .Recently polymeric membranes incorporated with nano-silver and copper nanoparticles has been synthesized with an approach to achieve enhanced antifungal properties . (Duan, Zhao, Liu, & Zhang, 2015; Manjumeena, Duraibabu, Sudha, & Kalaichelvan, 2014). Graphical representation in Fig 12 shows conidia/ml for all fungal strains. Error bars show the standard deviation and asterisks (*) represent significant p-values. All membranes are compared with a negative control and their significant p-values are represented.

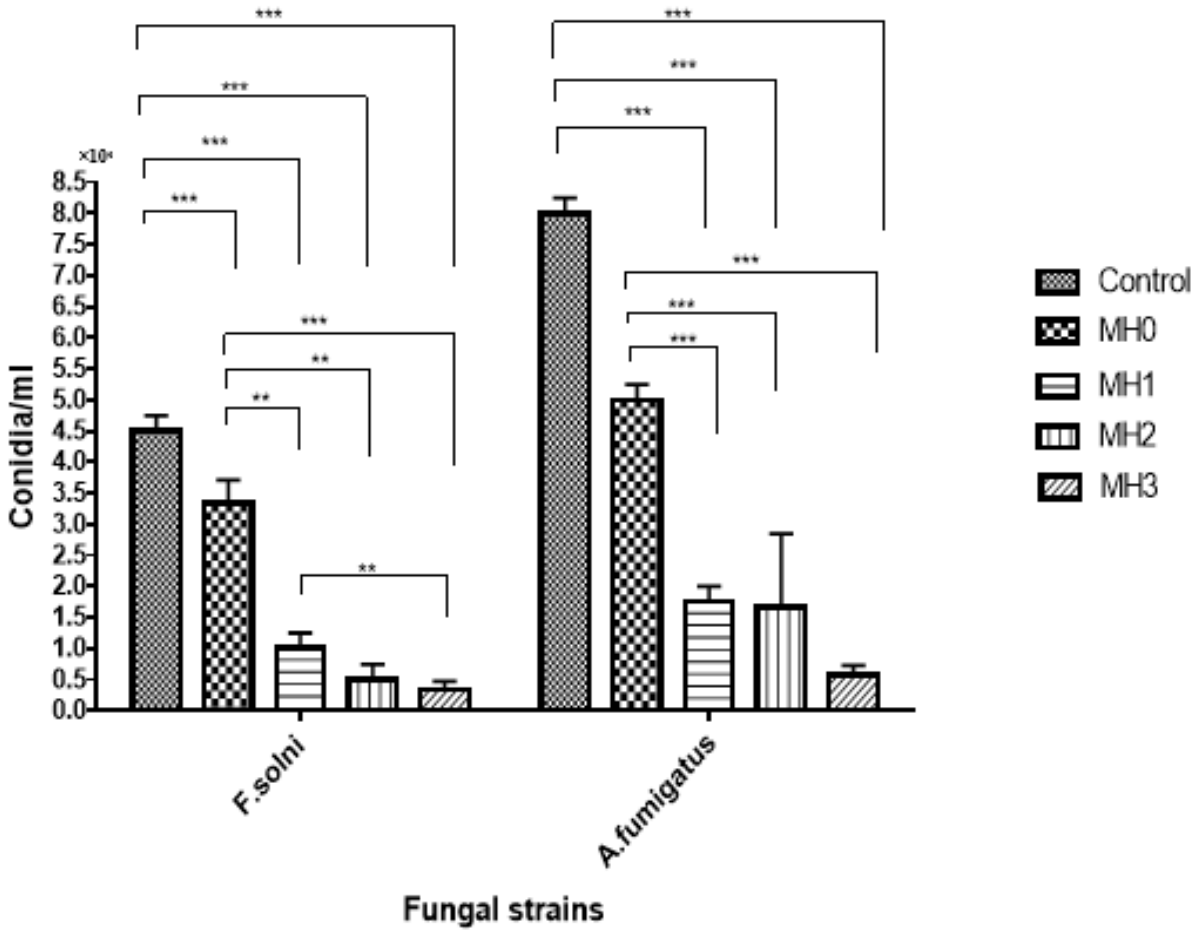


Figure 12: *Represents spores/ml after passing spores dilutions through all membranes. Error bars exhibit the standard deviation and asterisks (*) represents p value. The asterisks (*), (**), (***) indicate significance difference ($p \leq 0.05$), ($p \leq 0.01$), ($p \leq 0.001$) respectively.*

6. CONCLUSION

CS-ZnO HNPS/PES membranes with variation in content of NPS were effectively fabricated and behaviors of membranes for water disinfection were investigated. Several conclusions were subsequently drawn and are as follows

1) The membranes were successfully prepared through phase inversion method. Prior to membrane fabrication CS-ZnO NPS were synthesized and characterized by SEM, XRD and FTIR. The analysis of XRD and FTIR results confirmed the immobilization of Zinc oxide onto the amines and hydroxyl group of chitosan. As chitosan was transformed from amorphous to hexagonal crystalline phase.
2) Membrane characterization outcomes exhibited that increasing the content of HNPS had a remarkable effect on membrane porosity, hydrophilicity, surface roughness and permeability flux .Moreover the morphology of the membrane was also found to be altered with the addition of HNPS.

During the antibiofouling testing of membranes the reduction in bacterial colony and spores/ml count was seen in all membranes with HNPS but the significant inhibition was observed in MH3, with an overall 85.6% and 84.5% in bacterial and colony count

6.1 FUTURE RECOMMENDATIONS

- By adjusting proper ratio of polymer and nanoparticles purification can be enhanced
- Doping with other nanomaterial can also be investigated
- Addition of different drugs and their effect can also be studied
- Blends of different hydrophilic polymers incorporation NP,s can be fabricated that can further improve the hydrophilicity.
- As for biomedical application CS/ZnO particles have shown a very strong antifungal activity particles can be used for preparing antifungal emulsion.

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