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Preparation and antimicrobial activity of CS/PVA membranes containing silver nitrate

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Chitosan/polyvinyl alcohol (CS/PVA) membranes containing silver nitrate have been prepared and investigated for antimicrobial properties of prepared membranes. CS/PVA membranes containing silver nitrate have been successfully prepared by solution casting method. The prepared membranes have been characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and X-ray diffraction analysis (XRD) methods. The elemental composition of the membranes is studied by energy dispersive analysis of SEM-EDX. Correspondingly, the EDX analysis confirms the presence of silver ions in the chitosan matrix. The antimicrobial activities of the membranes have been tested with wide spectrum of bacteria. Efficiency of the antimicrobial effect against the selected twelve bacteria type and two yeasts is measured by disc diffusion method. Effect of the concentration of silver solutions to the developed CS/PVA membranes was investigated. It is noticed that the antimicrobial activity of membranes is improved with the increased silver concentration as a bactericidal agent, as expected. Our observations suggest that Chitosan/polyvinyl alcohol/Ag (CS/PVA/Ag) membranes have an excellent antimicrobial effect. Thus, developed membranes can easily be applied in various fields such as biomedical sector, tissue engineering and water treatment sector.

Keywords: Antimicrobial activity, Chitosan, Membrane, Polyvinyl alcohol, Silver ions

Chitosan (CS) is a linear polysaccharide derived by deacetylation of chitin, which is composed of β-(1-4) linked D-glucosamine and N-acetyl-D-glucosamine. Owing to its high antibacterial and antifungal activities against various microorganisms, chitosan has a number of commercial and possible biomedical uses[1-3]. Chitosan is generally used in food industry, biotechnology, cosmetics, medicine[4-6]. In addition, chitosan-based materials are often preferred in water filtration and as a heavy metal chelating agent[7-9].

The antibacterial activity of chitosan against a wide variety of microorganisms was proposed due to its high percentage of amino groups (6.89%). However, the antibacterial activity of chitosan is influenced by a lot of factors such as *p*H value, the species of bacteria, concentration, molecular weight, and solvent used[10, 11]. Positively charged chitosan binds to the negatively charged bacterial cell wall for antibacterial action in a weak acid environment. Chitosan shows its antibacterial activity only in an acid medium. This interaction can alter membrane permeability of bacterial cell and disrupt their bacterial surface morphology. The binding of chitosan to DNA causes to the inhibition of mRNA synthesis[12]. Chitosan has a strong affinity towards metal ions due to its positively charged groups. To improve the antibacterial capability of chitosan, silver ions can be attached to the amine groups in the chitosan molecules[13]. Silver ions and silver-based compounds are widely used as antibacterial agents in biomedical materials owing to their powerful antibacterial activity[14,15].

Therefore, novel antibacterial materials which combine silver ions with chitosan were prepared for different applications including tissue engineering, wound dressing, drug delivery, and water filtration, recently[16-18]. Ahmed M. Youssef *et al.* (2015) were prepared novel high-performance chitosan-nanocomposite films containing silver and zinc oxide nanoparticles and applied these films against some types of bacteria (*Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Bacillus cereus, and Listeria monocytogenes*) for antibacterial activity[19]. Yunli Ma *et al.* (2008) prepared chitosan-nylon-6 blend membranes containing silver ions by combining solvent

evaporation and a phase inversion technique. These membranes had excellent antibacterial activities against the typical Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*)[20].

In this work, a wide range of bacteria was chosen that have pathogenic properties to evaluate the antimicrobial performance of the prepared membranes. Comprehensive analyses for the proper evaluation of antimicrobial membranes are necessary. However, antimicrobial activity tests are generally difficult to conduct due to their long, exhaustive and detailed procedures. Thus, comprehensive analyses for different bacteria types are rare in related literature. This study tries to fulfil the mentioned gap with a total of twelve bacteria and two yeasts tested, which are listed below.

E. coli is a bacterium that can live independently. More than 700 serotypes of *E. coli* have been identified. Some *E. Coli* are beneficial, while some cause gastrointestinal infections and urinary tract infections. The *E. coli* is responsible for food contamination and produces Shiga toxin[21]. *Salmonella typhimurium* causes gastroenteritis in humans and other mammals[22]. *Staphylococcus* species is not always pathogenic, but it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning[23,24].

Although *Micrococcus luteus* is not a pathogenic bacterium, it can be infected at the time the immune system falls such as AIDS patients or new-born infants[25,26]. *Klebsiella pneumonia* can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum although found in the normal flora of the mouth, skin, and intestines[27]. *Pseudomonas aeruginosa* typically infects the airway, urinary tract, burns, wounds, and also causes other blood infections[28]. *Proteus* sp. is widespread in the environment and make up part of the normal flora of the human gastrointestinal tract. *Proteus mirabilis* accounts for approximately 3% of nosocomial infections in the United States[29].

Serratia marcescens are frequently seen in the shower cabins and tiles in bathrooms or toilets. *S. marcescens* can easily develop in wounds and eyes as it causes respiratory and urinary tract infections[30]. *Enterobacter* species cause a wide variety of nosocomial infections, including those affecting the lungs, urinary tract, intra-abdominal cavity and intravascular devices[31]. *Bacillus* organisms are widely distributed in the environment although the primary habitat is the soil. The various species implicated in serious infections include *B.cereus*, *B.subtilis*, *B. licheniformis*, *B.megaterium* and *B. pumilus*[32]. Although Candida is a member of normal flora in the mouth, skin and vagina, it can be infected by candidias is when the immune system falls[33].

The present study aims to prepare CS/PVA membranes containing silver nitrate by solution casting method and investigate antimicrobial properties of the membranes. Furthermore, this study focused to investigate the antimicrobial properties of CS/PVA/Ag membranes against *Escherichia coli, Salmonella typhimurium, Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Serratia marcescens, Enterobacter aerogenes, Bacillus cereus, Bacillus subtilis, Candida albicans, and Saccharomyces cerevisiae.* In addition, the prepared CS/PVA/Ag membranes were characterized by SEM, SEM-EDX, FTIR and XRD.

Experimental Section

Materials and Methods

CS, PVA, silver nitrate, acetic acid, N,N-Dimethyl formamide (DMF) and glutaraldehyde (GA) were purchased from different companies (Sigma-Aldrich, USA; Riedel de Haen and Merck, Germany). Twelve bacterial strains as *Escherichia coli* ATCC 25922*, Salmonella typhimurium* ATCC 14028*, Micrococcus luteus* ATCC 9341*, Staphylococcus aureus* ATCC 25923*, Staphylococcus epidermidis* ATCC 12228*, Klebsiella pneumonia* ATCC 13882*, Pseudomonas aeruginosa*ATCC 35032*, Proteus vulgaris* ATCC 33420*, Serratia marcescens* ATCC 13880*, Enterobacter aerogenes*ATCC 13048*, Bacillus cereus* ATCC 11778*, Bacillus subtilis* ATCC 6633) and two yeast strains as *Candida albicans* ATCC 10231*, Saccharomyces cerevisiae* ATCC 9763 were supplied from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Preparation of membranes

The casting solution contains CS: PVA at a ratio of 2:1. CS and PVA were dissolved in acetic acid solution. First, different ratio $AgNO_3$ ($AgNO_3$ equal to 0.5-20 (w/v) % based on total mass of the polymers) was dissolved in the mixture of water and DMF (volume ratio 1:1) in 2 mL. Then, $AgNO_3$ solution was added dropwise to CS/PVA homogeneous

solution under continues stirring for 3 h. *In situ* crosslinking was performed via adding 0.125 mL GA (0.625 (w/v) %) under continues stirring. Membrane #8 (GA 2.5 (w/v) %) was prepared by the same approach of Membrane #4 but only the amount of glutaraldehyde was different. Afterwards, the resulting solution was casted and then dried at room temperature[34].

Blank CS/PVA membrane (Membrane #9) was prepared by the same procedure without including silver nitrate for antimicrobial assay. The membranes prepared with various compositions were given in Table 1.

Characterization of CS/PVA/Ag membranes

The FT-IR analysis was carried out in the spectral range 400-4000 cm^{-1} by use of a Fourier Transform Infrared spectrophotometer (Schimadzu IR Prestige-21 FT-IR Spectrophotometer). The surface morphologies and elemental analyses of the prepared membranes were examined by a scanning electron microscope (SEM-EDX) (FEI Quanta FEG250). XRD patterns were recorded at 40 kV and 40 mA on a Bruker D8 Advance Twın-Twın diffractometer with the diffraction angle range 2ϕ=10-70º.

Antimicrobial assays

Effect of the concentration of silver solutions to the developed CS/PVA/Ag membranes was investigated for antimicrobial assays. Therefore, the used microorganisms were provided from the American Type Culture Collection (ATCC, Rockville, MD, USA).

While *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumonia* ATCC 13882, *Pseudomonas aeruginosa* ATCC 35032, *Proteus vulgaris* ATCC 33420, *Serratia marcescens* ATCC 13880 and *Enterobacter aerogenes* ATCC 13048 are Gram-negative bacteria *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacilllus cereus* ATCC 11778 and *Bacilllus subtilis* ATCC 6633 are Gram-positive bacteria. *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763 are yeast strains. The microorganisms were tested using disc diffusion method[35, 36].

E. coli ATCC 25922, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *S. typhimurium* ATCC 14028, *K. pneumonia* ATCC 13882, *P. aeruginosa* ATCC 35032, *P. vulgaris* ATCC 33420, *S. marcescens* ATCC 13880, *E. aerogenes* ATCC 13048 were incubated in Nutrient Broth (NB) (Merck, USA) at 37°C for 24h and *M. luteus* ATCC 9341, *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633 were incubated in Nutrient Broth (NB) (Merck, USA) at 30°C for 24h and *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763 were incubated in Malt Extract Broth (MEB) (Merck, USA) at 30°C for 24h.

Disc diffusion method

The prepared membranes were analysed for antimicrobial and antifungal effects using disc diffusion method. Firstly, the used bacteria and yeasts were incubated in broth cultures (24 h). Secondly, concentrations of 1×10^8 bacterial cells and 1×10^6 yeast cells/mL were prepared as 0.5 McFarland standard turbidity tubes. After, broth cultures of bacteria and yeast (0.1 mL) were inoculated into the Mueller Hinton Agar plates and dried at room temperature.

At last, to test the antimicrobial activity, the CS/PVA/Ag membranes (0.5 g) were cut and placed on agar plates.

Some microorganism cultures (*E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *K. pneumonia* ATCC 13882, *P. aeruginosa* ATCC 35032, *P. vulgaris* ATCC 33420, *S. marcescens* ATCC 13880, *Enterobacter aerogenes* ATCC 13048) were incubated at 37°C for 24 h and the other microorganism cultures (*M. luteus* ATCC 9341, *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231 and *S. cerevisiae* ATCC 9763) were incubated at 30°C for 24 h.

Zone diameters were measured at the end of incubation. The commercial disks were used as Chloramphenicol (C30, Oxoid), Gentamycin (GN10 Oxoid), Tetracycline (TE30), Erythromycin (E15), Ampicillin (AMP10) and Nystatine (NS100) for comparison.

Table 1 — Composition of prepared membranes

Membranes	Ratio of components presents in the membrane (%w/v)			
	Chitosan	PVA	$AgNO_3$	GA
1	1.0	0.5	0.5	0.625
2	1.0	0.5	1.0	0.625
3	1.0	0.5	3.0	0.625
4	1.0	0.5	5.0	0.625
5	1.0	0.5	10	0.625
6	1.0	0.5	15	0.625
7	1.0	0.5	20	0.625
8	1.0	0.5	5.0	2.500
9	1.0	0.5	0.0	0.625

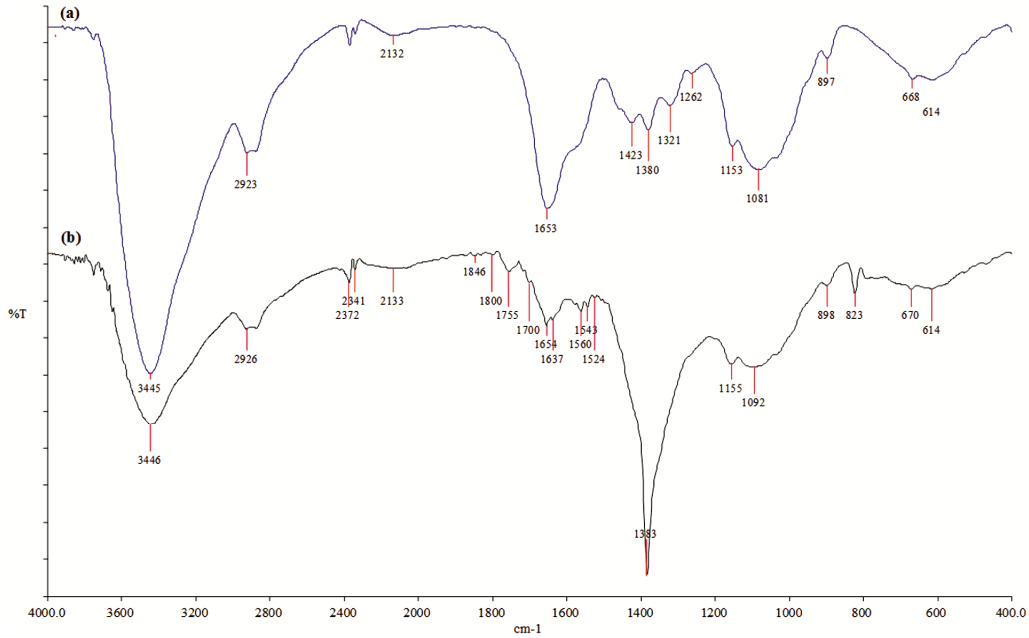

Fig. 1 — FTIR spectra of membranes (a) blank CS/PVA and (b) CS/PVA/Ag (membrane #4)

Results and Discussion

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of CS/PVA (a) and CS/PVA/Ag (b) membranes are shown in Figure 1. In curve a, the broad absorption peak at 3445 cm^{-1}corresponds to N-H and O-H stretching vibrations of CTS, and the peak at 2923$cm^{-1}$is C-H stretching band of CTS. The C=O stretching band (carbonyl group) is shown at 1653 cm^{-1} and the C-O stretching band is observed at 1081 cm^{-1}.

Compared with CS/PVA membrane (a), the CS/PVA/Ag membrane showed a significant decrease in intensity in the band at 3445 cm^{-1}, it also shifts to 3446 cm^{-1}. The C-H stretching band around 2923 cm^{-1} shifted to 2926 cm^{-1} along with a decreasing in intensity. The peak at 1653 cm^{-1}slightly shift to 1654 cm^{-1}, which indicates that the C=O bond is adjacent to an electron-withdrawing group. The spectrum of the CS/PVA/Ag membranes shows a new absorption band at 1382 cm^{-1} which corresponds to C-N bond of the dimethyl formamide solvent remaining in the membranes. In addition, a new absorption band at 823cm^{-1} (curve b), which confirms the existence of NO_3^- as a residual ion from $AgNO_3$[37].

SEM and EDX analysis

The scanning electron microscopy and EDX was used in details to define morphology structure of the prepared CS/PVA and CS/PVA/Ag membranes. The blank CS/PVA membrane surface was demonstrated in Fig. 2. As can be seen in the Figure 2, the blank CS/PVA membrane has a smoother and undulating surface. Figure 3 shows the morphology of the

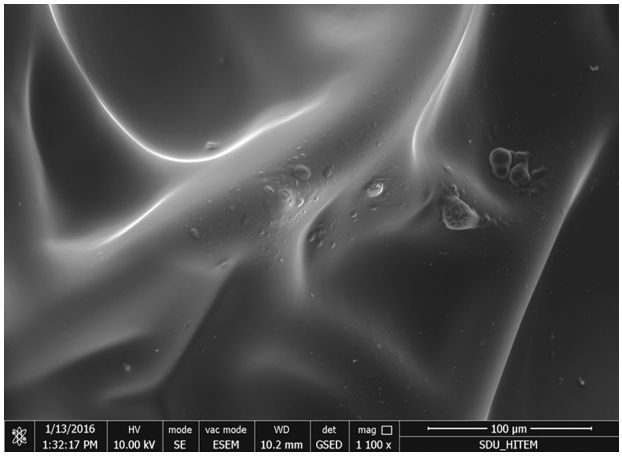

Fig. 2 — SEM of blank CS/PVA membrane

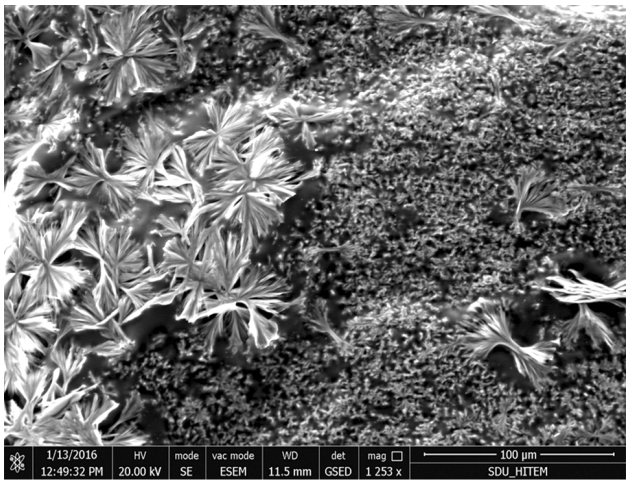

Fig. 3 — SEM of CS/PVA/Ag membrane (membrane #4)

CS/PVA/Ag membranes for 5% $AgNO_3$ loading into the polymer matrix. It can be seen that the shiny silver crystals were obtained with CS and PVA polymer matrix. However, some of the silver particles are non-uniformly mixed in a chitosan matrix.

Elementary analysis of CS/PVA/Ag membrane was carried out by energy dispersive analysis of SEM-EDX. Figure 4 displays the EDX analysis spectrum from a selected area. Also, the EDX analysis confirmed the presence of Ag particles into the chitosan matrix as shown in Fig. 4.

X-ray diffraction analysis

The XRD profile of CS/PVA membrane was presented in Fig. 5. The blank CS/PVA membrane without silver nitrate demonstrates that the characteristic peak at 22.9 (2ϕ) with a broad reflection which are typical peak of semi-crystallinity for chitosan. After the addition of silver nitrate solution to CS/PVA polymer matrix, a typical X-ray spectrum was shown in Fig. 6. The Bragg reflections between 25.51ºC and 70.42ºC are assigned to silver nitrate particles. The results are well consistent with that from the SEM-EDX images.

Antimicrobial activities of CS/PVA/Ag membranes

The antimicrobial activity of CS/PVA/Ag membrane on selected bacteria was studied. It is clearly observed that the membranes loaded with different concentrations of silver ions showed antimicrobial activity against all bacteria and yeasts in Table 2. Among the membranes assayed, membranes

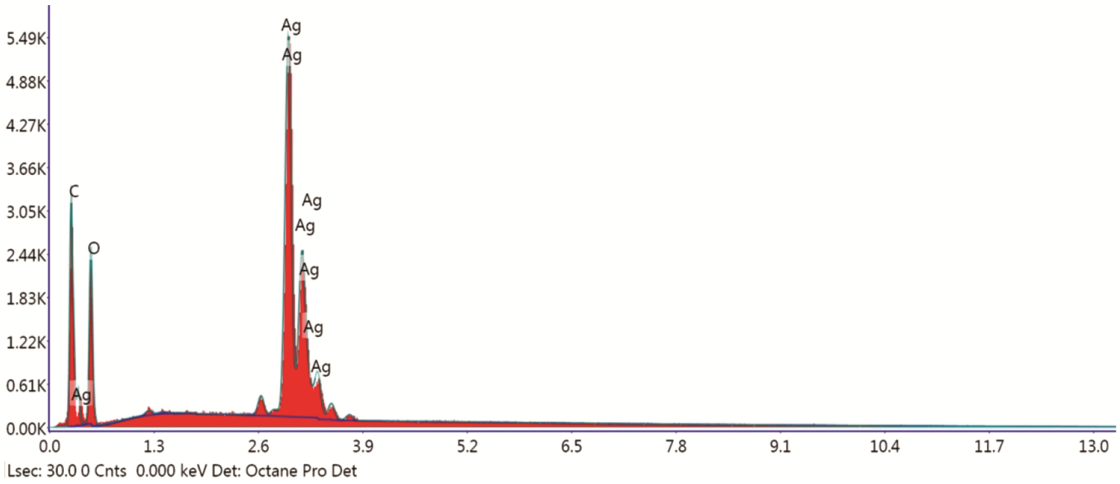

Fig. 4 — EDX profile of CS/PVA/Ag membrane (membrane #4)

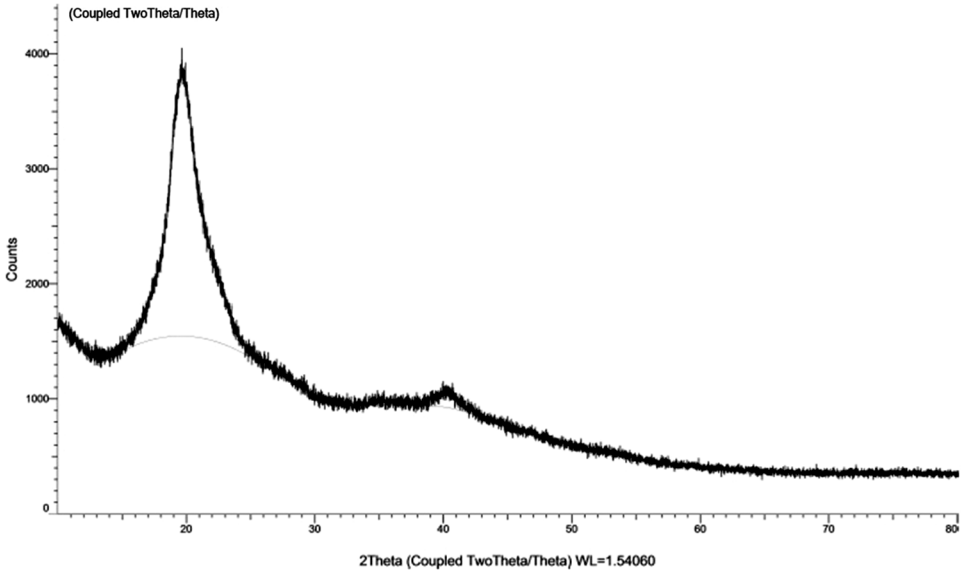

Fig. 5 — XRD pattern of blank CS/PVA membrane

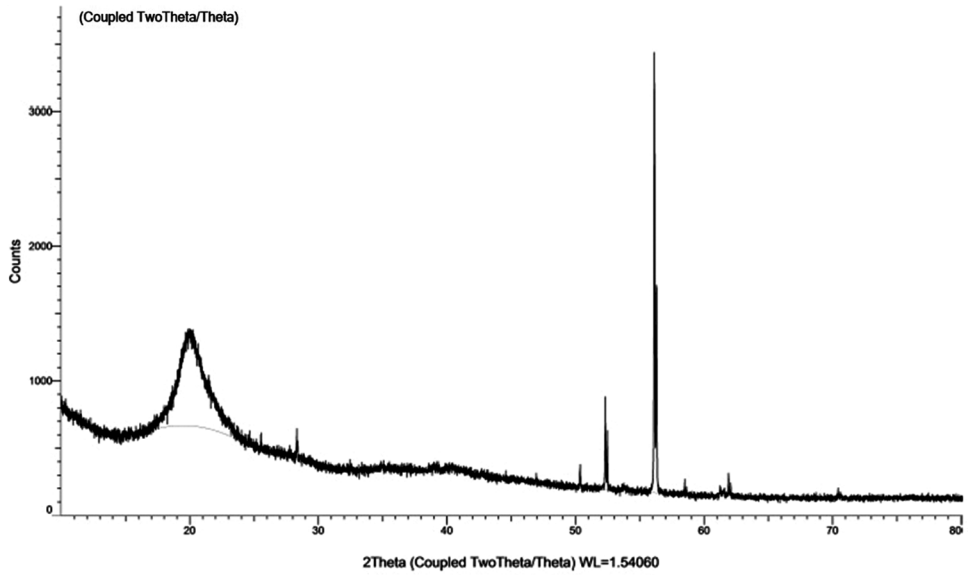

Fig. 6 — XRD pattern of CS/PVA/Ag membrane (membrane #4).

Table 2 — Diameter (mm) of zone of inhibition (ZOI) for CS/PVA/Ag membranes produced with different concentrations of $AgNO_3$ (% 0.5, 1, 3, 5, 10, 15, 20, 5*)

Name of Bacteria and Yeast	Diameter (mm) of Inhibitory zone against Bacteria and Yeast Samples CS/PVA/Ag								
	1	2	3	4	5	6	7	8	9
Escherichia coli ATCC 25922	14	14	14	22	_	_	_	17	15
Salmonella typhimirium ATCC 14028	13	13	20	20	_	_	_	16	14
Micrococcus luteus ATCC 9341	21	25	29	30	_	_	_	30	13
Stapylococcus aureus ATCC 25923	14	29	31	27	_	_	_	15	16
Stapylococcus epidermidis ATCC 12228	20	18	30	25	_	_	_	15	15
Klebsiella pneumonia ATCC 13882	16	14	21	20	_	_	_	17	13
Pseudomonas aeruginosa ATCC 35032	20	20	20	23	_	_	_	23	19
Proteus vulgaris ATCC 33420	21	25	18	18	_	_	_	20	_
Serratia marcescens ATCC 13880	25	15	20	26	_	_	_	19	13
Enterobacter aerogenes ATCC 13048	16	15	25	25	_	_	_	17	_
Bacillus cereus ATCC 11778	15	16	27	27	_	_	_	15	12
Bacillus subtilis ATCC 6633	14	24	30	26	17	26	26	28	18
Candida albicans ATCC 10231	12	22	25	27	_	_	_	28	15
Saccharomyces cerevisiae ATCC 9763	-	20	23	25	_	_	_	26	13

1: 0.5% $AgNO_3$, **2:** 1% $AgNO_3$; **3:** 3% $AgNO_3$; **4:** 5% $AgNO_3$; **5:** 10% $AgNO_3$, **6:** 15% $AgNO_3$, **7:** 20% $AgNO_3$ and **8:** 5%* $AgNO_3$, **9:** 0% $AgNO_3$

1, 2, 3, 4 and 8 demonstrated stronger activity against using all bacteria and yeasts (Table 2). According to Table 2, membranes #5 and 6 have only reflected remarkable activity against *Bacillus subtilis* ATCC 6633. On the other hand, membranes #5, 6 and 7 didn't indicate any activity against other microorganisms except *B. subtilis* ATCC 6633(Table 2).

Antimicrobial activity of prepared membranes was given for second assay in Table 3after discs has washed with deionized water. As shown in Table 3, antimicrobial activity has been even continued for membrane #3 and membrane #4 in the second assay. This result was obtained due to content of silver in the membrane. The antimicrobial activity improved for CS/PVA/Ag membrane containing 5 wt. % of silver ions (membrane #4). Thus, the amount of the silver nitrate, which can affect the chelated content of silver ions in the blended CS/PVA membranes, affects the antimicrobial activity. Membrane # 9 showed an antimicrobial activity on the first assay but this membrane did not show the same activity again for second assay as given in Table 2 and 3. This situation clearly indicates the importance of silver for antimicrobial activity. The proposed inhibitory mechanism of CS/PVA/Ag membranes on microorganisms is that the bacteria cell membranes are negatively charged and the positively charged CS can interact to and change the cell wall permeability of bacteria. This action was destroyed the bacteria inherent component and membrane functions which leads to the bacteria death[38].The inhibitory activity was measured based on the diameter of the clear inhibition zone. The antimicrobial activities of all

Table 3 — Diameter (mm) of zone of inhibition (ZOI) for CS/PVA/Ag membranes produced with different concentrations of $AgNO_3$ (% 0.5, 1, 3, 5, 10, 15, 20, 5*) after discs has washed with deionized water

Name of Bacteria and Yeast	Diameter (mm) of Inhibitory Zone Against Bacteria and Yeast Samples CS/PVA/Ag								
	1	2	3	4	5	6	7	8	9
Escherichia coli ATCC 25922	9	10	10	12	_	_	_	10	_
Salmonella typhimirium ATCC 14028	9	7	11	9	_	_	_	7	_
Micrococcus luteus ATCC 9341	9	9	11	11	_	_	_	10	_
Stapylococcus aureus ATCC 25923	9	9	11	12	_	_	_	12	_
Stapylococcus Epidermidis ATCC 12228	9	9	10	10	_	_	_	10	_
Klebsiella pneumoniae ATCC 13882	9	10	11	10	_	_	_	10	_
Pseudomonas aeruginosa ATCC 35032	11	16	13	18	_	_	_	17	_
Proteus vulgaris ATCC 33420	9	12	10	10	_	_	_	10	_
Serratia marcescens ATCC 13880	10	10	10	10	_	_	_	10	_
Enterobacter aerogenes ATCC 13048	10	9	10	12	_	_	_	10	_
Bacillus cereus ATCC 11778	9	9	11	12	_	_	_	9	_
Bacillus subtilis ATCC 6633	9	9	12	10	10	12	_	9	_
Candida albicans ATCC 10231	9	10	12	13	_	_	_	13	_
Saccharomyces cerevisiae ATCC 9763	-	10	10	12	_	_	_	12	_

1: 0.5% $AgNO_3$, **2:** 1% $AgNO_3$; **3:** 3% $AgNO_3$; **4:** 5% $AgNO_3$; **5:** 10% $AgNO_3$, **6:** 15% $AgNO_3$, **7:** 20% $AgNO_3$ and **8:** 5%* $AgNO_3$, **9:** 0% $AgNO_3$

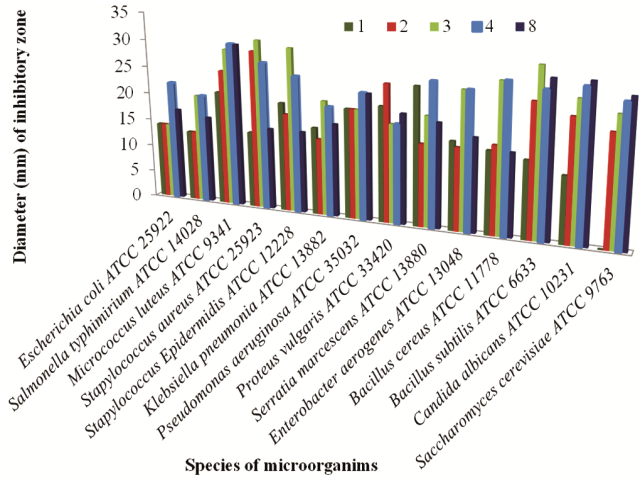

Fig. 7 — Diameters of inhibitory zone of the membranes containing $AgNO_3$ in different concentrations

bacteria and yeasts versus inhibitory zone are shown in Fig. 7. Similar mechanisms and results were reported in previous studies[39,40].

Conclusion

In this paper, CS/PVA membranes containing silver ions were prepared by solution casting method. The morphology of membranes was examined by SEM, SEM-EDX, XRD, and FTIR. The EDX and XRD spectrums of CS/PVA/Ag membranes confirmed the presence of silver ions. The antimicrobial activities of membranes against all bacteria and yeasts were examined by disc diffusion method. The antimicrobial activity increased with the addition of silver ions and increasing with increasing its concentration. Additionally, CS/PVA/Ag membranes containing 5 wt. % of silver ions (membrane #4) showed antimicrobial activity in the second assay after discs has washed with deionized water. Antimicrobial properties of developed membrane were proved with wide-spectrum bacteria tests. The excellent antimicrobial properties suggested that the obtained CS/PVA/Ag membranes could be applied in various areas.

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