Journal of Microbiological Methods 159 (2019) 42–50

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Bio-functionalization of phytogenic Ag and ZnO nanobactericides onto cellulose films for bactericidal activity against multiple drug resistant pathogens

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ARTICLE INFO

Keywords:
Bupleurum aureum
Bio-functionalization
Silver nanobactericides
Zinc oxide nanobactericides
Multi-drug resistance pathogens
Bacterial cellulose

ABSTRACT

The present study describes the synthesis of silver and zinc oxide nanobactericides from the phytogenic source *Bupleurum aureum*. The synthesized nanobactericides were characterized and evaluated for bio-functionalization onto bacterial cellulose membrane which was synthesized by *Komagataeibacterxylinus* B-12068 culture strain. The synthesis of nanobacterides were initially confirmed using UV–Visible spectroscopy which indicated localized surface resonance (LSPR) peaks at 415 nm for silver nanobactericides and 280 nm for zinc nanobactericides. The nature of the capping agent for synthesized nanobactericides was predicted using FTIR which confirmed the presence of functional moieties. XRD analysis revealed their crystalline nature while morphological characteristics were studied using TEM which confirmed the polydispersity of nanobactericides with the average size in the range of 20–25 nm. The nanobactericides were tested for their antimicrobial activity against seven multi-drug resistant pathogens which were clinically isolated from patients suffering from a myriad of microbial infections. The tested pathogens had antimicrobial resistance to ten different antibiotics and have been reported to be the major cause of nosocomial infections. The nanobactericides displayed significant activity against the test pathogens. Silver nanobactericides showed the highest activity against *Escherichia coli* strain 55 with a 24 mm zone of inhibition while zinc oxide nanobactericides displayed the highest activity against *methicillin-resistant Staphylococcus aureus* (MRSA) with a 20 mm inhibition zone. The bio- functionalized cellulose films (BCF) were characterized using SEM along with physicochemical analysis. The BCF's were evaluated for antibacterial activity against test pathogens which resulted in marked antimicrobial potential against multi-drug resistant bacteria and therefore has the potential to be utilized as an efficient alternative to counter drug resistant pathogens.

1. Introduction

Nanotechnology is a branch of applied science which deals with manipulating materials at the nanoscale (Syed et al., 2016a). In recent years, the use of nanomaterials in the biomedical sector has rapidly flourished especially in the areas of drug delivery, bio-diagnostics and as potent antimicrobial agents. The rapid expansion of multi-drug resistant pathogens has influenced the global economy with the depletion of effective antibiotics to counter infections from drug-resistant pathogens (Ventola, 2015; Khan et al., 2016; Lobanovska and Pilla, 2017). The magnitude of resistance has created considerable impact on both terrestrial and aquatic living organisms (Fair and Tor, 2014). Ever since the first antibiotic was discovered, there has been a steady rise in the rate of antibiotic resistance against antimicrobial agents (Ventola, 2015). According to WHO, developing new and safe antimicrobial agents is one of the top priorities by 2020 (Baker et al., 2013; Syed et al., 2016b; Reinhardt and Neundorf, 2016; Navya and Daima, 2015). Use of functionalized nanomaterials against the treatment of microbial

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https://doi.org/10.1016/j.mimet.2019.02.009
Received 20 December 2018; Received in revised form 18 February 2019; Accepted 19 February 2019
Available online 20 February 2019
0167-7012/

pathogens has generated a huge potential owing to their significant antimicrobial potential (Dakal et al., 2016). Nanobactericides are nano-sized materials bearing bactericidal properties with multiple modes of action against targeted pathogens (Syed et al., 2016a; King et al., 2018). There are different types of nanobactericides based on their core composition such as silver, gold, zinc, copper and platinum (Wang et al., 2017). Since ancient times, usage of silver is well documented and recent implementation of nano-sized silver has uplifted its applicative potential in different sectors (Yamada et al., 2014). Further, zinc is one of the essential elements required by living organisms and generally regarded as safe according to GRAS (Osredkar, 2011). Based on these considerations, silver and zinc nanobactericides were synthesized using the phytogenic source *Bupleurum aureum*. Use of phytogenic sources to synthesize nanomaterials is considered to be one of the facile routes without use of any toxic element (Iravani, 2014; Khan et al., 2017; Patra et al., 2017). Bio-functionalization is the process of tailoring or conjugating desired materials with biological agents. The use of the bio-functionalization principle is well demonstrated in the medical sector to deliver drugs, develop biosensors, controlled release of drugs and development of dressing materials (Baker et al., 2018). In recent years, bio-functionalized nanomaterial based dressing materials have gained considerable interest to achieve efficient antimicrobial properties. Development of bio-functionalized membranes can uplift their physicochemical characteristics by providing additional stability, strength, biocompatibility and being biodegradable (Torres et al., 2012; Moniri et al., 2017; Volova et al., 2018). The scientific studies have already reported bacterial cellulose as one of the best suited polymers for medical usage. Some of the salient features of bacterial cellulose are ease in large production via fermentation, inexpensive, generation in different shapes, absorptive, elasticity, biocompatible, inert, hypoallergenic and mechanical properties (Faria-Tischer et al., 2016; Tsai et al., 2017). In the present study, bacterial cellulose membrane was produced by *Komagataeibacter xylinus* B-12068 culture strain. Further, nanobactericides were embedded on bacterial cellulose membranes and tested for antibacterial activity against multi drug resistant pathogens. These bio-functionalized membranes can act efficiently, prevent microbial infections and can be of great interest to combat drug resistance pathogens (Hu and Hsieh, 2015; Heli et al., 2016).

2. Materials and methods

2.1. Plant processing

The plant materials (Stem and leaves) were collected from the abundant growing area of Krasnoyarsk region, Siberia, Russia. The plant materials were washed and chopped into small segments. Further, 20 g of finely cut materials was added to a one liter beaker containing 500 ml of sterile distilled water. The mixture was boiled for 30 min to obtain aqueous extract which was stored at 4 °C until further use.

2.2. Synthesis of silver and zinc oxide nanobactericides

The aqueous extract was used as reducing agent to synthesize nanobactericides. The purified aqueous extract was treated with 1 mM silver nitrate at a ratio of 7:3. The conversion of Ag^{+} to Ag^{o} was initially confirmed with a color change measured by UV–Visible spectroscopy. Similarly, zinc oxide nanobactericides were synthesized by constantly stirring the aqueous extract with 100 mM zinc sulphate heptahydrate solution. Samples were drawn at regular intervals as mentioned above and monitored using UV–Visible spectroscopy at the spectral range of 100 to 800 nm.

2.3. Characterization of nanobactericides

The synthesized nanobactericides were characterized using analytical techniques. The morphological characteristics of nanobactericides were determined via a high-resolution transmission electron microscope (HRTEM) JEOL JEM-2100 operating at an acceleration voltage of 200 kV. The role of the aqueous extract as a reducing agent was studied using FTIR spectroscopy. The crystalline nature of nanobactericides was studied using an X-ray diffractometer instrument operating at a voltage of 30 kV.

2.4. Production of bacterial cellulose films

The production of bacterial cellulose films was carried out according to the protocol described by Shidlovskiy et al., 2017. In brief, the *Komagataeibacter xylinus* B-12068 culture strain was previously isolated from fermented tea (kombucha). The actively growing isolate was cultured in Hestrin-Schramm (HS) liquid medium for 7 days at a temperature of 30 °C under static conditions. The bacterial cellulose films were down streamed with the treatment of 1.0 M NaOH at 70 °C to remove the media components and washed with distilled water. The separated films were incubated for 24 h in 0.5% solution of hydrochloric acid solution to neutralize alkaline condition. Later, the films were washed with distilled water and preserved in sterile deionized water until further use.

2.5. Bio-functionalization of nanobactericides onto bacterial cellulose film and their characterization

The synthesized nanobactericides were centrifuged and the pellet was dissolved into solution. The cellulose membranes were immersed in nanobactericides solution and incubated at 37 °C for one hour on water bath. Later, BCF were dried to remove excess moisture content. The films were excised into small blocks (5 × 5 mm) for characterization. The morphological characteristics were studied using scanning electron microscopy of ultrahigh resolution S-5500 (Baker et al., 2018). The sample was processed by placing onto the sample stage and sputter-coated with gold, using an Emitech K575X sputter coater (10 mA, 2 × 40 s). The sample was examined and recorded with image analysis program (Image Processing and Data Analysis) in Java. The mechanical properties of the BCF's were investigated using an electromechanical tensile testing machine Instron 5565 (U.K.). Samples 75 mm long and 12 mm wide were prepared for studying physical and mechanical properties of the films.

2.6. Multi-drug resistant pathogens

The test pathogens employed for antibacterial activity are *Acinetobacter baumannii* strain 210, *Acinetobacter baumannii* strain 211, *Pseudomonas aeruginosa* strain 215, *Pseudomonas aeruginosa* strain 40, *Klebsiella pneumoniae* strain 104, *Methicillin-resistant Staphylococcus aureus* and *Escherichia coli* strain 55.

2.7. Preparation of test bacterial suspension

The inoculum of test bacteria was prepared according to the protocol described by Teh et al., 2017 with slight modification. In brief, the actively growing test bacterial strains were inoculated into 10 ml sterile Mueller Hinton broth (MHB) and incubated overnight at 37 °C. The overnight test bacterial suspensions were adjusted to 0.5 McFarland Standard with sterile Mueller Hinton broth under aseptic conditions. The preparation of inoculum was carried out according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Balouiri et al., 2016).

2.8. Antimicrobial activity of Ag and ZnO nanobactericides

The synthesized nanobactericides were centrifuged at 15,000 rpm for 20 min. The obtained pellet was washed thrice with double distilled water and 5 mg/ml concentration was evaluated for antimicrobial

activity via well diffusion assay and micro-broth dilution assay. The concentrations of the nanobactericides were prepared in accordance with the protocol described by Syed et al. (2017). In brief pre-warmed MHA (Mueller-Hinton agar) plates were seeded with 50 µl test bacterial suspension (1.5×10^6 CFU/ml) and swabbed uniformly, later by using sterile cork borer the agar was punched to make wells. The test material of 100 µl nanobactericides was added into each well and incubated at 37 °C for 24 h. The activity was measured as a zone of inhibition across the well.

2.9. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined according to the protocol described by Syed et al., 2016b. In brief, the plates were prepared under aseptic conditions and 100 µl of test material (nanobactericides 1 mg/ml). The test material was added to wells of the first row followed by the addition of 50 µL of nutrient broth to the other wells. The serial dilutions were performed using a multichannel pipette and 10 µl of resazurin as growth indicator was added to each well. The final volume of the broth was adjusted with addition of 30 µl isosensitized broth to each well ensuring a final volume of 190 µl. Finally, 10 µL of bacterial suspension (1.5×10^6 CFU/ml) was added to each well. The plate was incubated at 37 °C for 18 to 24 h. The color change was then assessed visually from purple to pink or colorless. The lowest concentration at which color change occurred was taken as the MIC value as per the protocol described by Sarker et al. (2007).

2.10. Antibacterial activity of silver and zinc oxide nanobactericidal cellulose films

The BCF's were tested for antibacterial activity according to the protocol described by Volova et al. (2018) with slight modification. In brief, small blocks (5 × 5 mm) of BCF's were excised under aseptic condition and tested for activity. The pre-warmed MHA (Mueller-Hinton agar) plates were seeded with 50 µL (1.5×10^6 CFU/ml) of test inoculum which was swabbed uniformly. The prepared blocks of BCF were placed onto the swabbed media and incubated at 37 °C for 24 h. The activity was measured as a zone of inhibition across the block in millimeters. All the experiments were performed in triplicates and data was analyzed statistically. Statistical analysis of the antibacterial results was performed using IBM SPSS version. One way analysis of variance (ANOVA) at a value of $p < 0.001$ followed by Tukey's Post Hoc test with $p \leq 0.05$ was used to determine the significant differences between the results obtained in each experiment.

3. Results and discussion

The plant mediated synthesis of nanobactericides has been reported to be a one pot synthesis process (Kavitha et al., 2013). Use of plants to synthesize nanomaterials has been reported to overcome the limitations posed by most conventional methods (Baker et al., 2013). In the present investigation, *Bupleurum aureum* was selected as medium to obtain aqueous extract to synthesize nanobactericides. The selection of plant species was carried out according to the traditional records and knowledge acquired by local Siberian healers. The *Bupleurum aureum* is reported to be one of the widely used herbal plants in curing various diseases (Nyobe et al., 2012). It is rich in phyto-constituents such as saponins, tannins, essential oils, vitamin C and alkaloids (Naboka et al., 2014).

3.1. Synthesis of nanobactericides

The aqueous extract of *Bupleurum aureum* was used as reducing agent to synthesize silver and zinc nanomaterials which were initially checked for antibacterial activity by primary screening which concluded that synthesized nanomaterials bear bactericidal properties. The

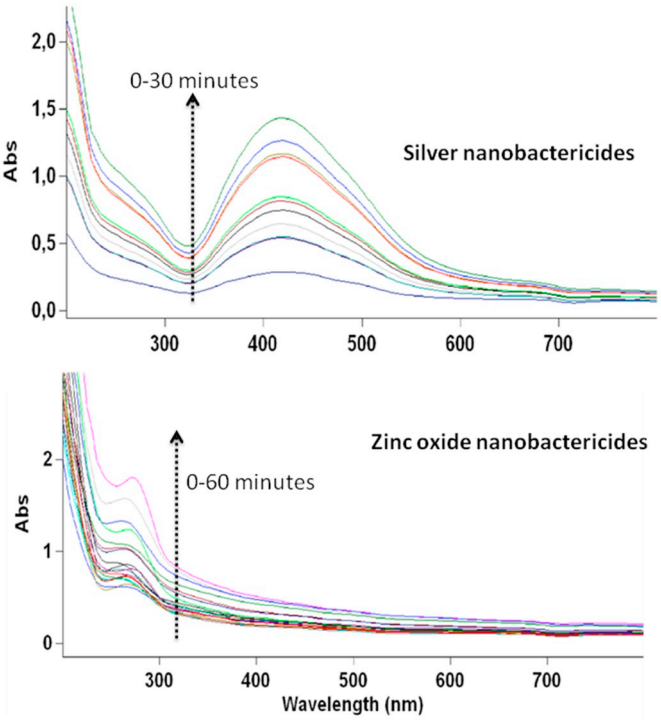

Fig. 1. UV–Visible analysis of silver and zinc oxide nanobactericides.
(Note: the dotted line indicates the time required for the completion of synthesis)

synthesis of nanobacterides were initially confirmed using UV–Visible spectroscopy which indicated a localized surface resonance (LSPR) peak at 415 nm for silver nanobactericides and 280 nm for zinc nanobactericides (Fig. 1). The surface plasmon resonance which describes the collective excitation of electrons in turn increases the absorption at the UV–Visible range (Umadevi et al., 2012). The color change of the reaction mixture is due to the resonating frequency arising from the electromagnetic field which causes strong absorption in the spectral range (Mafuné et al., 2000; Saranya et al., 2017). The UV–Visible spectroscopy is an ideal and sensitive tool for predicting the formation of silver and zinc oxide nanomaterials (Mahmudin et al., 2016).

3.2. Optimization of synthesis of nanobactericides

To achieve rapid synthesis, different parameters like temperature and pH were optimized. The rate of synthesis increased at elevated temperatures, studied by varying the temperature from 30 °C to 90 °C and maximum synthesis was observed at 90 °C (Suppl.1). The influence of temperature on the synthesis of nanomaterials using aqueous extract has been well demonstrated (Phanjom and Ahmed, 2017). Similarly, the pH was increased from 5 to 9 and maximum synthesis was observed at pH 9 indicating maximum synthesis at alkaline condition (Suppl.2). The influence of intrinsic factors to mediate the synthesis is well studied which justifies the findings of the present investigation (Nagarajan and Kuppusamy, 2013; Mendis et al., 2016).

3.3. Biophysical characterization of nanobactericides

3.3.1. FTIR analysis of nanobactericides

The possible role of phyto-components present in *Bupleurum aureum* to mediate the synthesis and stabilization of nanobactericides was studied using FTIR analysis (Fig. 2a,b). The absorption range in the FTIR analysis displayed various vibrational stretches which corresponded to different functional groups (Table 1). The nature of capping agent for synthesized nanobactericides was also predicted using FTIR

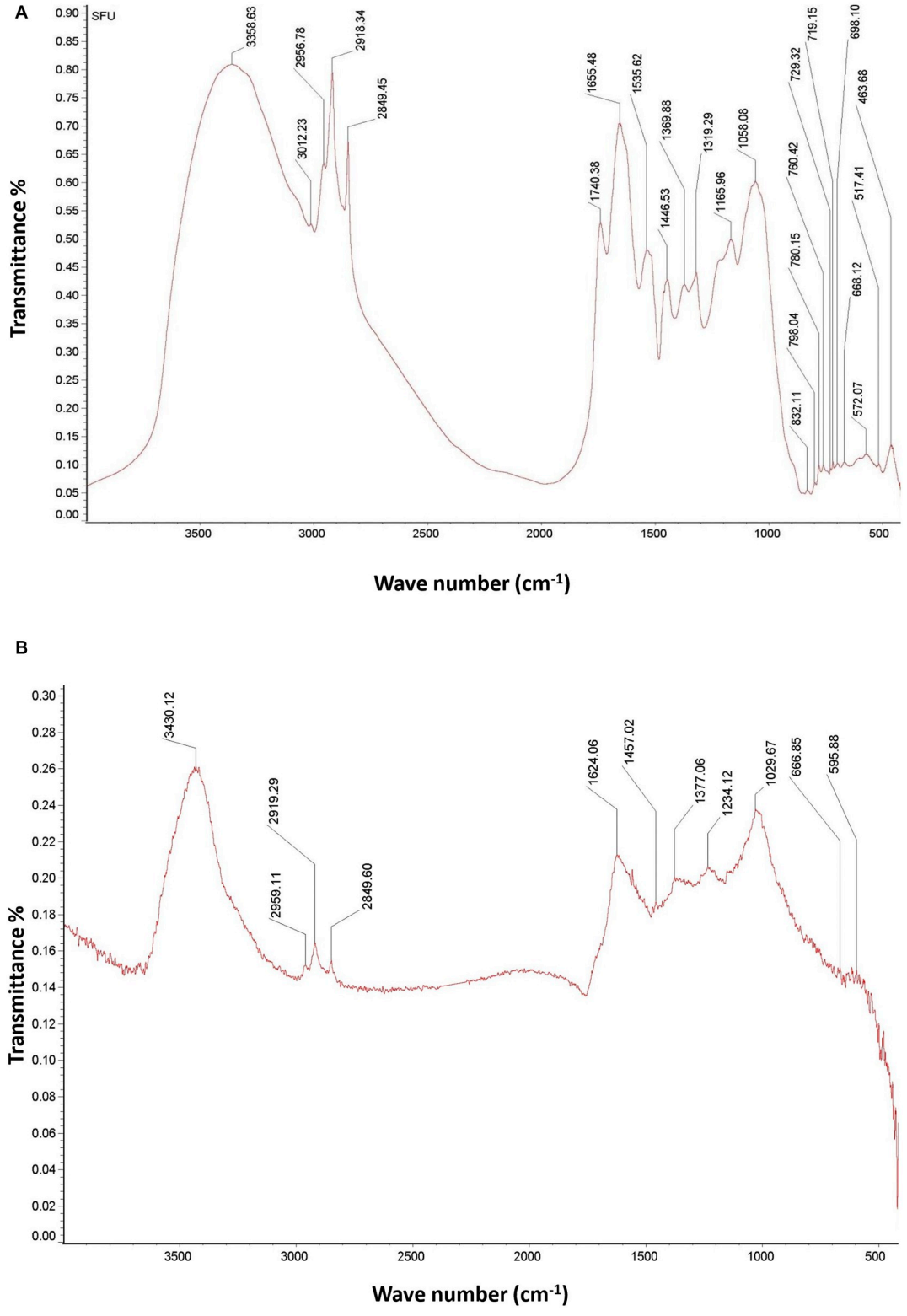

Fig. 2. a) FTIR analysis of Silver nanobactericides. b) FTIR analysis of Zinc oxide nanobactericides.

which acted as a stabilizing agent (Pawlikowska-Pawlęga et al., 2013). Studies have reported the IR absorption at varied frequencies is confined to characteristic functional groups (Parida et al., 2008; Chaitanya et al., 2011; Lin et al., 2014). The obtained FTIR analysis was consistent with previous scientific reports which highlighted the role of phytocomponents in mediating the synthesis process (Kuppusamy et al., 2016).

3.3.2. XRD analysis of nanobactericides

The crystalline nature of nanobactericides was studied using XRD which displayed diffraction patterns at an angle of 2θ denoting the (111), (200), (220) and (311) planes which suggested the face-centered cubic of silver. The result obtained is in accordance with the scientific findings of Agnihotri et al. (2014). Similarly, for zinc nanobactericides, the most intense peak at 2θ displaying at 40.89° indicated the (102)

Table 1
Prediction of FTIR analysis and their functional groups.

FTIR analysis	Functional group
3358	O\H stretching
2918	OH
2949	CH2
1655	C=O
1535	N-H
1058	C=O
1165	CO-O-C asymmetric stretching

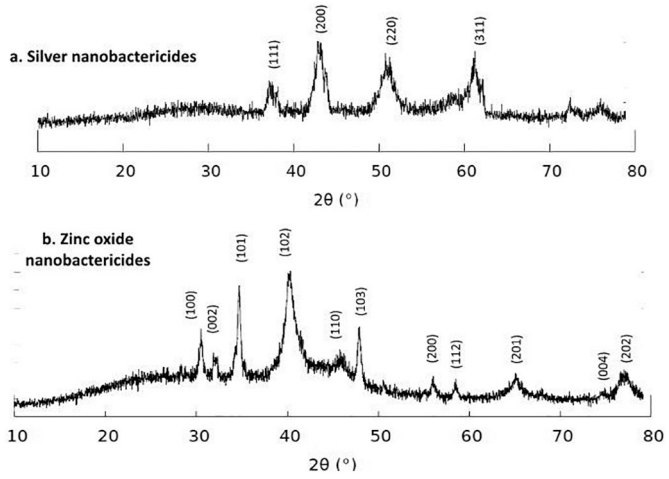

Fig. 3. XRD analysis of nanobactericides.

orientation. The diffraction patterns obtained in the present study are in accordance with the typical zincite structure of ZnO diffraction and all the peaks were well indexed to the hexagonal ZnOwurtzite structure (Fig. 3). The results coincide with the findings of Khalil et al. (2014).

3.3.3. TEM analysis of nanobactericides

The morphological characteristics of nanobactericides were studied using TEM analysis which depicted the polydispersity with different sizes and shapes. The average size of the synthesized nanobactericides was found to be 20 ± 5 nm (Fig. 4). The polydispersity and morphological characteristics obtained in the present investigation coincide with earlier reported studies (Syed et al., 2016b; Al-Shabib et al., 2016).

3.4. Antibacterial activity of nanobactericides

The synthesized nanobactericides were tested for antibacterial activity against multi drug resistant pathogens. The activity was determined via well diffusion assay and measured as a clear zone of inhibition across the well (Fig. 5). Among the test pathogens, *Escherichia coli* strain 55 was found to be the most sensitive to silver nanobactericides with a 24 mm zone of inhibition. Similarly, zinc oxide nanobactericides displayed highest activity against *methicillin-resistant Staphylococcus aureus* with a 20 mm inhibition zone. The least concentration of nanobactericides to suppress the growth of test bacteria was determined using minimum inhibitory concentration. The activity was measured with resazurin as a growth indicator. The growth was measured via optical density at 600 nm and the activity was measured and tabulated. It was found that, nanobactericides displayed activity in the range of 0.625 to 1.25 µg/ml as shown in Table 2. To confirm the bactericidal effects, 10 µl from a well displaying no growth was inoculated into fresh nutrient broth and incubated to show no growth. The obtained results marked the potential of nanobactericides to suppress the growth of multi-drug resistant pathogen which can be utilized as efficient alternatives to counter antimicrobial resistance. The scientific evidence of nanomaterials as antimicrobial agent is well reported owing to their size dependent properties (Beyth et al., 2015). Interestingly, in the present investigation the test pathogens were tested for their drug resistance capacity to more than ten antibiotics viz., ampicillin, cefoperazone, cefepime, chloramphenicol, imipenem, meropenem, gentamicin, tetracycline, tobramycin and vancomycin. According to WHO, the test pathogens evaluated in the present study are reported to be the leading contributors of multi-drug resistance across the globe in comparison to other microbial pathogens (Santajit and Indrawattana, 2016; Syed et al., 2016c). The efficacy of nanobactericides as antimicrobial agents have been studied earlier but to the best of our knowledge, scanty reports are available on the evaluation of nanobactericides against multi drug resistant pathogens which are clinically isolated including both Gram +ve and Gram -ve (Ansari et al., 2011; Sadanand et al., 2016).

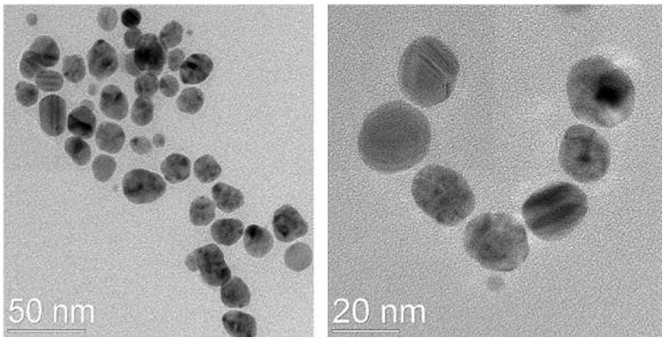

Fig. 4. TEM analysis of nanobactericides.

3.5. Bio-functionalization of nanobactericides with cellulose membrane

The bio-functionalized films were saturated by immersion into nanobactericides which facilitated the binding of an oxygen rich group of cellulose membrane with nanobactericides thus forming a complex. The unbound nanobactericides were removed by drying the films under strict aseptic condition to remove the moisture content.

3.6. Characterization of bio-functionalized cellulose membrane

The BCF's were characterized by studying the mechanical properties. The bio-molecular interaction of nanobactericides with cellulose membrane was confirmed with scanning electron microscopy which displayed the nesting of nanobactericides between the cellulose fibers. The properties of bio-functionalized cellulose films are presented in Table 3. Interestingly, the inclusion of silver nanobactericides significantly changed the mechanical properties of bacterial cellulose which resulted in an increase in the Young's modulus which was found to be 5809,79 ± 899,91 MPa. The tensile strength was increased by two orders in comparison with net bacterial cellulose as mentioned in Table 3. A similar observation was obtained with the inclusion of zinc nanobactericides and Young's modulus was increased to 105,17 ± 26,26 MPa. The obtained results are in accordance with earlier findings which describes the influence of nanomaterials on the physicochemical properties of bacterial cellulose (Pal et al., 2017; Moniri et al., 2017). The mode of embedding and immobilization of nanobactericides directly facilitates the interaction with bacterial cellulose and enhances the mechanical properties (Vivekanandhan et al., 2012; Luan et al., 2012; Liyaskina et al., 2017). The obtained results are in accordance with the study conducted by Benavente et al., 2017 which states, the inclusion of silver nanoparticles on the cellulose membrane for antibacterial activity. In the present investigation, the SEM analysis, displayed the size of nanobactericides which ranged between 20 and 100 nm (Fig. 6). The SEM analysis also reported the agglomeration of particles on the surface of cellulose membrane. The

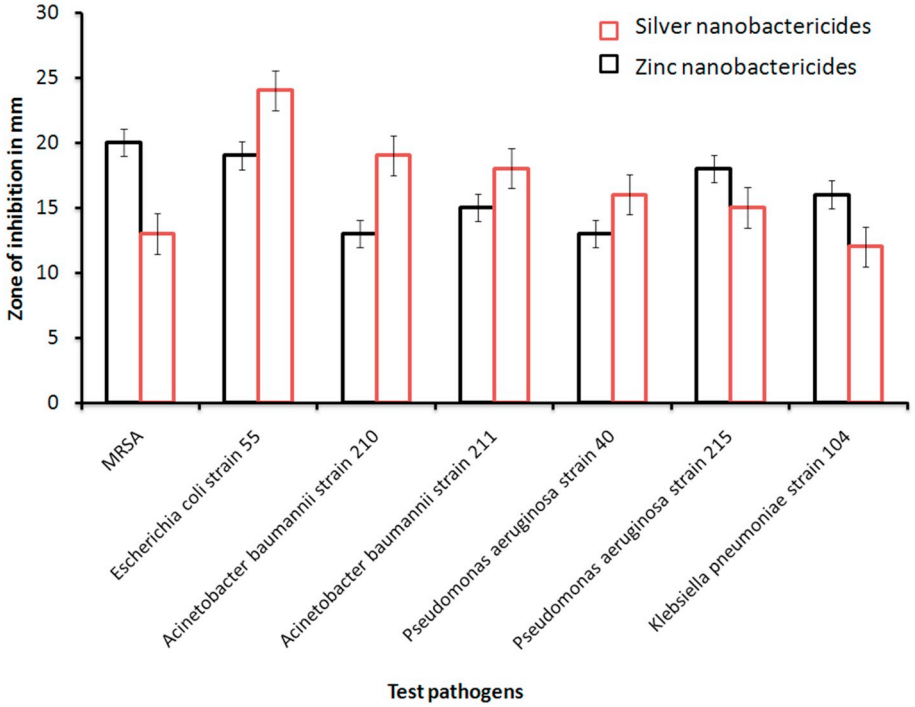

Fig. 5. Bactericidal activity of nanobactericides against multi-drug resistant pathogens measured as zone of inhibition (mm).
(Note: Statistical analysis of antibacterial results was performed using ANOVA (analysis of variance) at value $p < 0.001$. The error bar indicates the percentage of inhibition)

Table 2
Minimal inhibitory concentration of nanobactericides against multi-drug resistant pathogens.

List of pathogens	Silver nanobactericides	Zinc oxide nanobactericides
Acinetobacter baumannii strain 210	0.625 μg/ml	1.25 μg/ml
Acinetobacter baumannii strain 211	0.625 μg/ml	1.25 μg/ml
Escherichia coli strain 55	0.625 μg/ml	1.25 μg/ml
Klebsiella pneumoniae strain 104	1.25 μg/ml	0.625 μg/ml
Methicillin-resistant Staphylococcus aureus	1.25 μg/ml	0.625 μg/ml
Pseudomonas aeruginosa strain 40	1.25 μg/ml	1.25 μg/ml
Pseudomonas aeruginosa strain 215	1.25 μg/ml	1.25 μg/ml

Table 3
Analysis of physicochemical properties of bio-functionalized bacterial cellulose films and their comparison.

Sample	Thickness, μm	The Young'smodulus, MPa	Tensile strength, MPa	Elongation at break, %
Bacterial cellulose with Silver nanobactericides	20	5809,79 ± 899,91	171,39 ± 1,42	3,39 ± 0,34
Bacterial cellulose with Zinc oxide nanobactericides	20	7716,66 ± 772,12	184,24 ± 29,76	1,80 ± 0,61
Control Bacterial cellulose	20	5605,34 ± 60,15	42,87 ± 4,88	1,06 ± 0,25

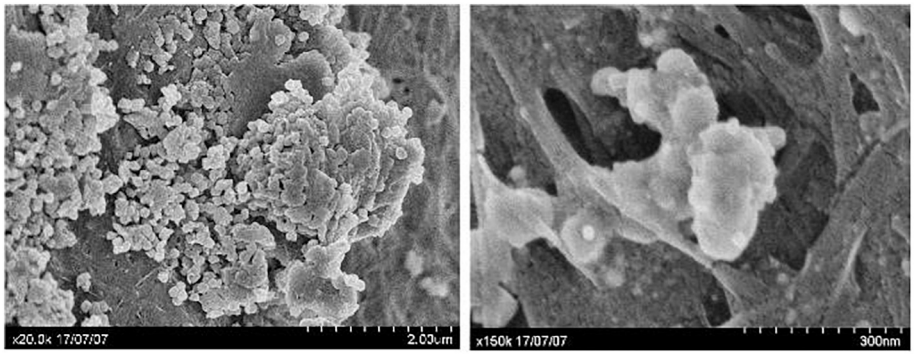

Fig. 6. Scanning electron microscopic analysis of bio-functionalized bacterial cellulose films with nanobactericides.

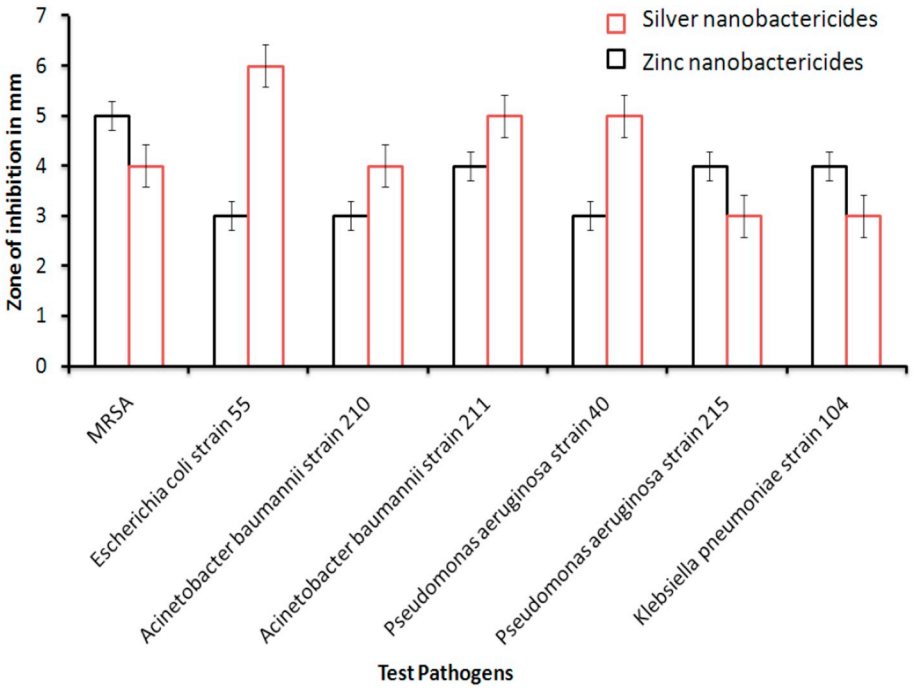

Fig. 7. Bactericidal activity of bio-functionalized bacterial cellulose films with nanobactericides against multi-drug resistant pathogens measured as zone of inhibition (mm).
(Note: Statistical analysis of antibacterial results was performed using ANOVA (analysis of variance) at value $p < 0.001$. The error bar indicates the percentage of inhibition)

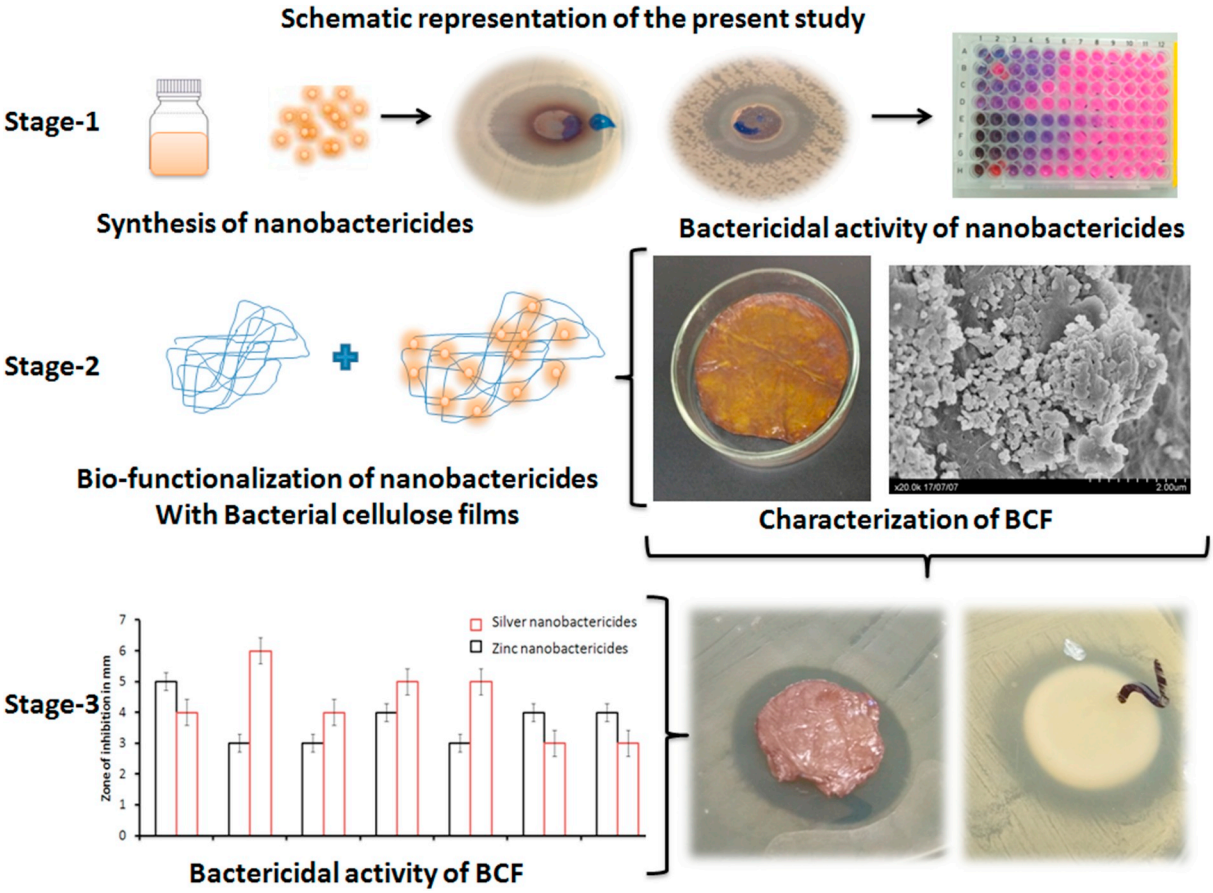

Fig. 8. Schematic representation of synthesis, bio-functionalization and bactericidal activity of nanobactericides against multi-drug resistant pathogens.

interaction of nanobactericides with bacterial cellulose is yet to be completely elucidated, whereas scientific studies provides evidence of electrostatic interaction between nanobactericides and electron rich oxygen atoms present in bacterial cellulose (Maria et al., 2010). The control membrane without nanobactericides is presented as Supplementary File 3.

3.7. Antibacterial activity of bio-functionalized cellulose membrane

The antibacterial activity of BCF's was evaluated against selected

drug resistant pathogens. The activity was measured as a clear zone of inhibition across the BCF blocks (Fig. 7). Interestingly, the binding of nanobactericides with cellulose had no impact on the bactericidal properties. The process of embedding of nanobactericides facilitated the bactericidal potential to the cellulose membrane which aids in controlled release of nanobactericides. The complete schematic representation of the present investigation has been summarized in Fig. 8. The results obtained in the present investigation are promising enough to report the activity of nanobactericides against multi-drug resistant pathogens which can open a new avenue towards developing novel bio-functionalized antimicrobial agents.

4. Conclusion

The present study contributes towards scientific knowledge on development of novel bio-functionalized nanobactericides against drug resistant microbial pathogens. The development of nanobactericides via a phytogenic source expressed as a facile process to synthesize silver and zinc oxide nanobactericides. The efficacy of bio-functionalized bacterial cellulose membrane displayed significant bactericidal activity against the tested multi-drug resistant pathogens.

Acknowledgements

Authors are thankful for facilities provided by Siberian Federal University and Krasnoyarsk State Medical University to carry out the present study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.02.009.

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