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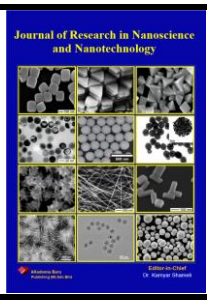
Journal of Research in Nanoscience and Nanotechnology

Journal homepage:
http://akademiabaru.com/submit/index.php/jrnn/index
ISSN: 2773-6180



Microwave Irradiation Assisted Synthesis of Silver Nanoparticles using Pullulan as Reducing Agent and Its Antibacterial Activity

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https://doi.org/10.37934/jrnn.2.1.4250

ABSTRACT

In this studies, synthesis of silver nanoparticles (Ag-NPs) on pullulan-based biofilm was achieved by microwave irradiation technique. Synthesis of Ag-NPs was achieved using pullulan as both a reducing and stabilizing agent. The effect of different microwave irradiation duration on pullulan and silver nitrate in synthesis of silver nanoparticles (Ag-NPs) was investigated. The synthesized Ag-NPs/PL were first screened and identified using surface plasmon peaks of UV–Vis spectroscopy. The research results indicated that the surface plasmon resonance peaks were observed between 400–414 nm wavelengths in UV-VIS spectroscopy studies. From Fourier-transform infrared spectroscopy (FTIR) spectra, stretching vibrations of hydroxyl (OH), carbonyl (C=O) and C=C stretches exhibits the reduction and stabilization of Ag-NPs. Further, five characteristic peaks Ag(111), Ag(200), Ag(210), Ag(220) and Ag(311) confirmed the presence of elemental silver and the crystalline structure of silver nanoparticles from X-ray Diffraction analysis. Biofilms were produced by mixing the synthesized Pulullan-Ag-NPs with polyvinyl alcohol. The AgNP/PL were applied for the antimicrobial activity against *Bacillus subtilis* and found to have high antibacterial activity. In addition, the clear zones of inhibition was found at 11 mm to 16 mm against *Bacillus Subtillis*. The experimental results demonstrated that pullulan could be used as reducing and stabilizing agent for formation of Ag-NPs.

Keywords:
Silver nanoparticles, microwave, pullulan, antibacterial

Received: 10 March 2021 **Revised: 9 April 2021** **Accepted: 21 April 2021** **Published: 12 May 2021**

1. Introduction

Metal-based nanoparticles find tremendous applications in real time biomedical, nutrition and electronic industries. Though several physical and chemical routes have been used for synthesis of metal nanoparticles, the use of non-toxic and benign biological materials like microbes, plant extracts,

actinomycetes were included in the metal nanoparticles preparation. Among these, silver metallic nanoparticles are being produced by common chemical reduction methods with extracellular and intracellular synthesis through eco-friendly, biocompatible methods. In recent times, interest has grown in research groups for using wide variety of chemical and biochemical reducing agents with these metal nanoparticles [1].

Silver nanoparticles (Ag-NPs) are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes due to their unique physical and chemical properties. Nanosized metallic particles are unique and can considerably change physical, chemical, and biological properties due to their surface to volume ratio. Therefore, these nanoparticles have been exploited for various purposes.

Generally, conventional physical and chemical methods seem to be very expensive and hazardous. Interestingly, biologically prepared Ag-NPs show high yield, solubility, and high stability. Among several synthetic methods for Ag-NPs, biological methods seem to be simple, rapid, non-toxic, dependable, and green approaches that can produce well-defined size and morphology under optimized conditions for translational research. In the end, a green chemistry approach for the synthesis of Ag-NPs shows much promise [2].

Chemical methods use water or organic solvents to prepare the silver nanoparticles. This process usually employs three main components, such as metal precursors, reducing agents, and stabilizing or capping agents. In this method the manufactured particles are not of expected purity, as their surfaces were found to be sedimented with chemicals [3]. It is also very difficult to prepare Ag-NPs with a well-defined size, requiring a further step for the prevention of particle aggregation. To overcome the shortcomings of chemical methods, biological methods have emerged as viable options. Recently, biologically mediated synthesis of nanoparticles has been shown to be simple, cost effective, dependable, and environmentally friendly approaches and much attention has been given to the high yield production of Ag-NPs of defined size using various biological systems [4].

This research is proposed to synthesis Ag-NPs on pullulan by microwave irradiation for active biofilm packaging. Ag-NPs antimicrobial was incorporate with polyvinyl alcohol and plasticizer to produce biodegradable biofilm. The potential antimicrobial activity bacteria of the resulted pullulan silver nanoparticles were investigated. Ag-NPs antimicrobial active packaging systems which contain materials within or on packaging that actively inhibit food spoilage microorganisms can be used to extend the shelf life of food products. Incorporation of antimicrobial agent into the packaging is to reduce, inhibit or retard the growth of microorganism that may be present on food surfaces. This extra function will also contribute to extend shelf life or improve safety while maintaining the quality of food. Microwave irradiation has been utilized for synthesis of silver nanoparticles as green method as compared to chemical reduction process. Pullulan based Ag-NPs biofilm with addition of plasticizer produced a biodegradable film for packaging with excellent mechanical properties as compared to conventional method.

2. Materials and Methods

All material and reagents used in this work were analytical grade and used as received without further purification. Silver Nitrate ($AgNO_3$-99.85%) was used as the silver precursor, which was obtained from Acros Organic (USA). The Pullulan powder (R&M Chemicals, UK) was applied as a solid support for Ag-NPs. All these aqueous solutions were used with double distilled water.

The synthesis was start by dissolving 5g of pullulan in 100ml of distilled water. The solution was then stirred for 60 minutes at 90°C. Silver nitrate ($AgNO_3$) solution with concentration of 0.1M was

prepared by adding 1.7g of $AgNO_3$ in 100ml of distilled water. It was then stirred for 60 minutes. The $AgNO_3$ solution was then added into pullulan solution and the mixture was further stirred for 1 hour.

Microwave irradiation was taking places by putting the solution into the microwave chamber. Equal volume of 30 mL AgNP/PL solution was irradiated in microwave (Panasonic NN-ST342M) at 250W for 1 minute. The microwave irradiation process was conducted at definite power of 250W and then repeated for different durations of time which are 3, 5, 7, 9, 12, 15 and 17 minutes.

The fundamental characterizations were conducted to further confirming the formation of Ag-NPs/PL. The analysis was start with UV-Vis spectroscopy to determine the silver peak. In this analysis, the Ag-NPs/PL were scanned were scanned from the 300-1000 nm with UV-Vis Spectrophotometer (UV-2600 Shimadzu, Japan) at medium rate scan.

The Ag-NPs/PL was also characterized to determine the crystallinity and purity of the sample. X-ray diffraction (XRD) morphological analyses was performed using XRD-Empyrean (PAN analytical, United Kingdom). In this analysis the XRD pattern was started to scan from 10^o to 80^o at 2θ angle.

Fourier transform infrared spectroscopy (FTIR; Shimadzu IRTracer-100) was used to obtain qualitative information about the functional groups and chemical characteristics of synthesized pullulan-silver nanoparticles. Potassium Bromide (KBR) method was used to study the characteristic vibration frequencies in infra-red range (500- 4000 cm^{-1}) wavelength range. Approximately 5 mg of the sample was mixed with 95 mg of KBR prior to compacting into a thin film using a hydraulic press for about 3 minutes. The SEM was performed using the JEOL JSM7600F (Japan) to study the morphology of Ag-NPs and pullulan.

The microbial activity assay of the Ag-NPs/PL biofilm against Gram-positive *Bacillus subtilis* was carried out by culture medium toxicity method. The biofilm sample with dimension of 1 x 1 cm was placed on the surface of an agar plate, seeded with 100 µL of test culture consisting microorganism. Petri dish covers were sealed by wax to avoid any kind of contamination. The petri dish was incubated for 24 hours and the microbial growth was followed by visual observation. The Natrium Agar (NA) was used as a medium with a temperature of 37 °C. The clear zone that formed around the film sample in the medium was recorded as an indication of inhibition against the microbial species.

3. Results and Discussion

The synthesis Ag-NPs/PL under microwave irradiation was controlled by adjusting the irradiation time. Figure 1(a) and (b) shows the solution samples before and after microwave irradiation. The synthesis of Ag-NPs/PL under microwave irradiation was controlled by adjusting the reaction time from 1 minutes to 17 minutes.

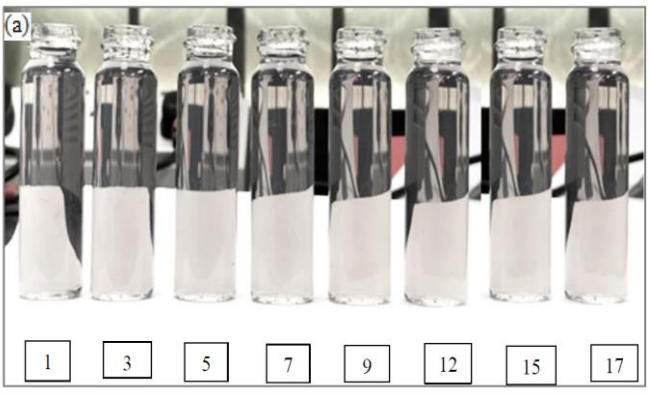

Figure 1(a). Ag-NPs/PL samples before microwave irradiation.

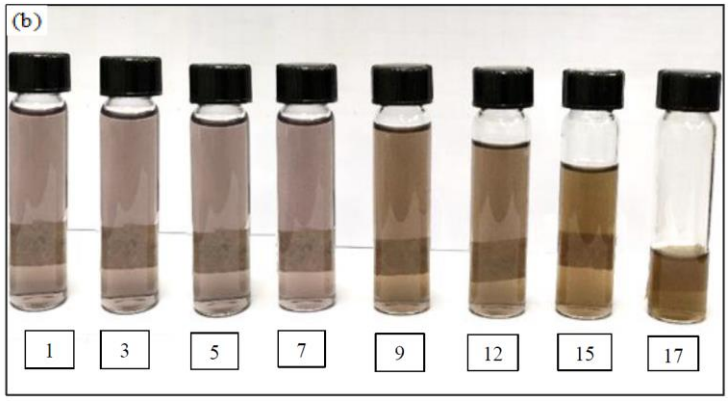

Figure 1(b). Ag-NPs/PL samples after microwave irradiation at 1, 3, 5, 7, 9, 12, 15 and 17 minutes (A1-A8).

The solution was found to be colourless before being treated with microwave irradiation. The reaction mixture colour was gradually changed from colourless to light brown, then to brown, and finally dark brown over different periods of time as shown in Figure 1(b). Dark coloured solution will confirm the formation of silver nanoparticles which can be prove by UV-Vis analysis. In short, it is obvious that pullulan is having the ability to reduce Ag^{+} ions to Ag^{0} (Ag-NPs) through microwave irradiation technques [5].

3.1. UV-visible spectroscopy analysis

The formation of silver nanoparticles was monitored by UV-vis spectroscopy. Figure 2 below shows the UV–vis spectra of silver nanoparticles at different reaction time of 1, 3, 5, 7, 9, 12, 15 and 17 minutes (A1-A8) and absorbance increases with respect to time. The absorbance peak start to observed after 7 minutes (A4) indicative of Ag-NPs formation.

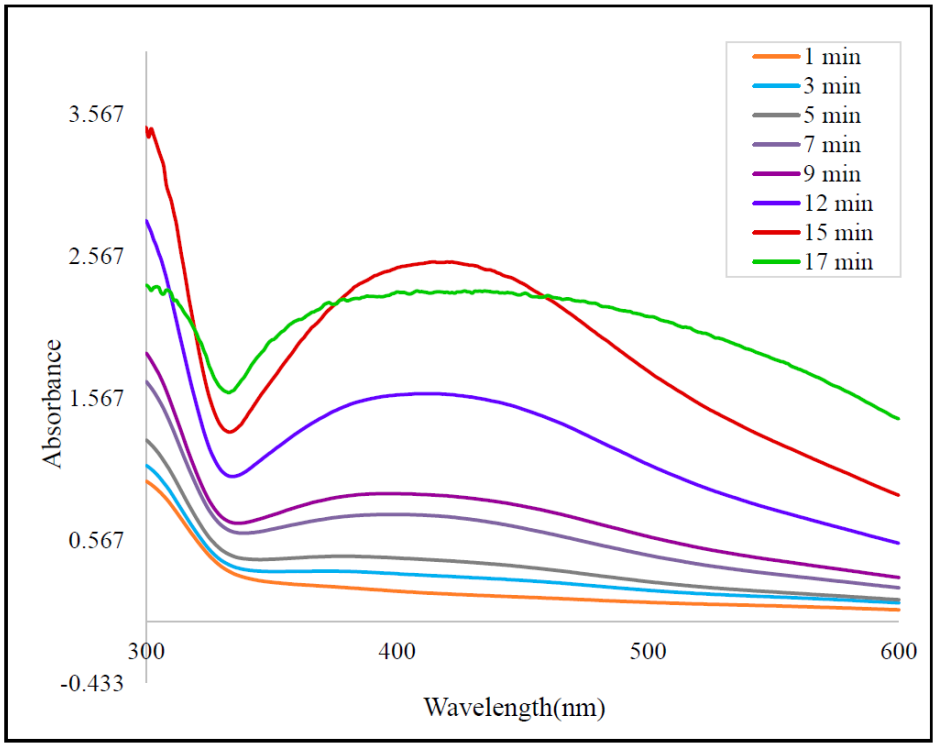

Figure 2. UV-Visible spectra of Ag-NPs/PL prepared at 1, 3, 5, 7, 9, 12, 15 and 17 minutes (A1-A8).

The absorbance peak at 400-420 nm is the indication of the silver formation in the mixture. As shown in Figure 2, the surface plasmon resonance (SPR) peak start to appear in formulation A4 (7 minutes microwave irradiation). The Ag-NPs absorbed radiation in the visible regions of 400–420 nm due to the strong SPR transition. The appearance of the SPR transition in this region confirmed the production of Ag-NPs [6]. The intensity of the SPR peaks increased as the reaction time increased. It is believed that the prominent and sharp peak are translated to a higher concentration Ag-NPs produced. In addition, increasing trend of the intensity peaks and inducing their shift toward lower wavelengths will formed a smaller particle size, narrow size distribution, and monodisperse of silver nanoparticles [7]. It is also believed, the fast transfer of energy during microwave irradiation obviously induces more localized growth of the nanoparticles in the presence of pullulan molecules which can be seen in formulation A7 [5]

3.2. Fourier Transform Infrared Spectroscopyanalysis

FTIR spectrum measurement was also carried out to examine the possible functional groups of pullulans responsible for reduction and stabilization of the Ag-NPs. Figure 3 shows the FTIR spectrum of the Ag-NPs/PL for 9, 12 and 15 minutes (A5-A7).

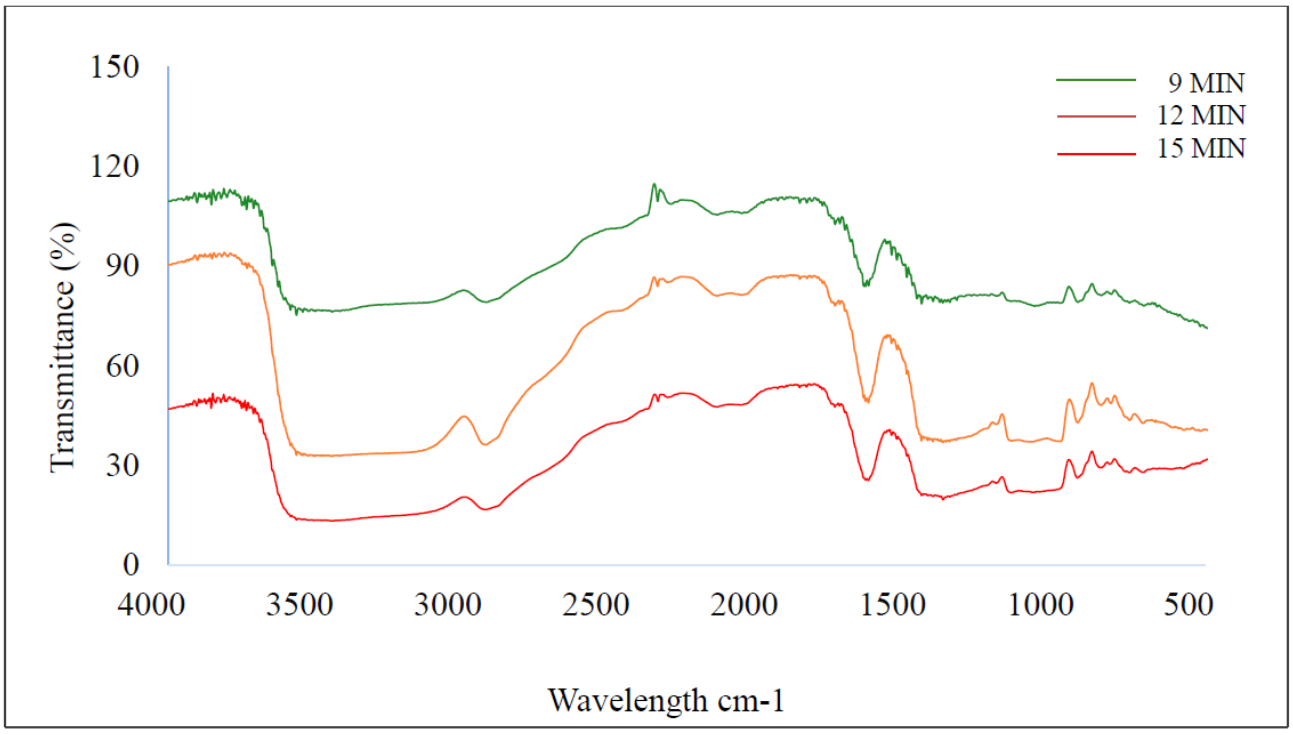

Figure 3. FTIR spectrum of Ag-NPs/PL prepared at 9, 12, and 15 minutes microwave irradiation

The Ag-NPs/PL have shown characteristic peak from 3680-2975 cm^{-1} as shown in Figure 3. Broad spectra were observed between 3600-2959 cm^{-1}, indicating hydroxyl group (O-H). In contrast, Jung, Jeong, and Kim (2008) found that the hydroxyl (O-H) and C-H stretching frequency of pure pullulan exhibits distinct peaks at 3356 cm^{-1} and 2943 cm^{-1} [8, 9]. Another instant peak observed between 1699-1543 cm^{-1} prove the presence of carbonyl (C=O) and C=C stretching frequencies. These observations suggest a strong interaction of Ag with pullulan functional group.

The other peaks in the range between 1160 cm^{-1} to 1000 cm^{-1} due to vibration of the O-H stretching. Wave numbers in all peaks observed in the FTIR spectra of pullulan stabilized Ag-NPs differed from the FTIR spectrum results for pure pullulan. All peaks observed in the FT-IR

spectrum of pullulan-stabilized Ag-NPs differed from the FT-IR spectrum results for pure pullulan [8, 10]. These observations suggest a strong interaction of Ag with the pullulan functional groups. In addition, no new peak was observed in the FT-IR spectrum, suggesting that no new bond formation occurred between the pullulan and Ag-NPs. Pandey et al. (2012) reported that the O-H group of polymers had efficient coordination ability with silver ions [11-14]. These indications confirm the good interaction of silver with the pullulan functional groups.

3.3. X-ray diffraction analysis

The crystalline nature of the Ag-NPs/PL confirmed by the X-ray diffraction (XRD) analysis. The quality of the produced nanoparticles lies in the purity and crystallinity of the colloidal Ag-NPs/PL. Formulation A7 is selected to be analyzed in XRD due to prominent peak observed in UV-Vis analysis [15]. Figure 4 shows XRD pattern of Ag-NPs/PL showing peak indices and 2θ values. Five peaks were observed in XRD scanning at 2θ values of 38.89, 46.34, 54.86, 64.50 and 78.02° corresponding to 111, 200, 210, 220 and 311 planes of silver indicates that resultant particles are face centered cubic (FCC) structure [16,17]. No spurious diffractions due to crystallographic impurities are found [18]. All the reflections correspond to pure silver metal with face centered cubic symmetry [14].

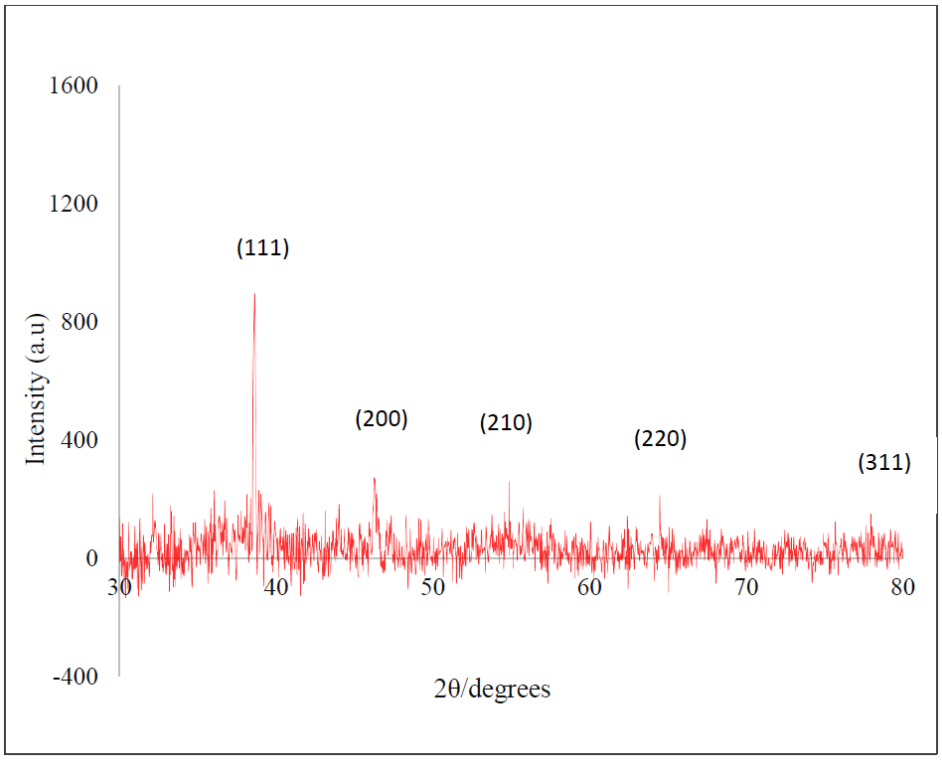

Figure 4. XRD pattern of Ag-NPs/PL at 15 minutes microwave irradiation

3.4. Antimicrobial Activity Analysis

To evaluate the antimicrobial activity study, the sample is exposed to the microorganism by using an agar diffusion method. The result in Figure 5 (a) shows the reaction of the samples towards *Bacillus Subtillis* pathogen before incubation and Figure 5 (b) after incubations of samples with pathogen. From the analysis, a clear zone was formed around the film sample after 2 days incubation at a constant temperature of 37°C. Table 1 showed the diameter of the clear zone that formed after 24 hours exposure to *Bacillus Subtillis*. The antimicrobial activity against *Bacillus*

Subtillis contributed to a reduction of the growth rates of aerobic coliform [19]. The clear zone surrounding the film indicates antimicrobial diffusion from the film and subsequent growth [20].

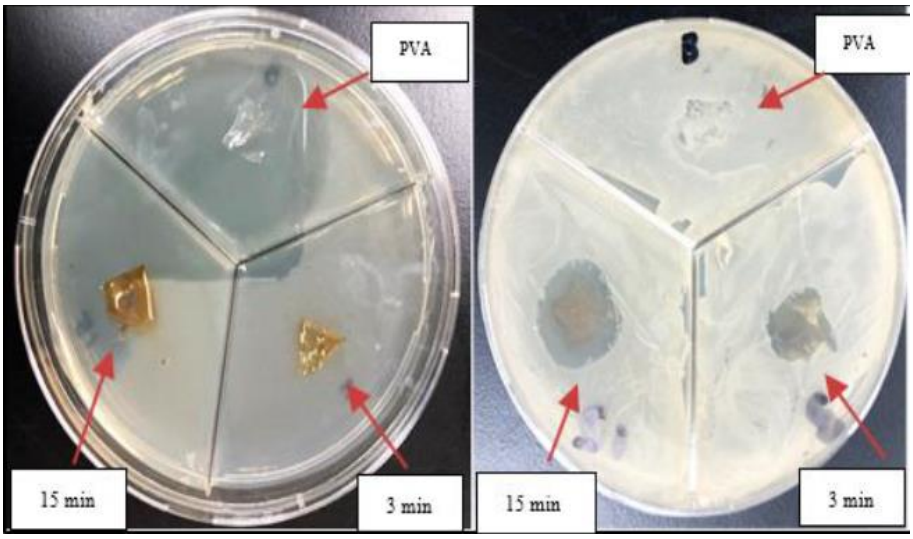

Figure 5. Photograph of antimicrobial test results against *Staphylococcus aureus* – before (a) and after exposed to microbe (b)

The clear zone was prominently seen as shown in Figure 5. The transparent zone was translated to average diameter zone which were tabulated in Table 1. At higher microwave irradiation times (15 minutes) the clear zone diameter was higher (16 mm) compared to 25 kGy (12 mm) γ-doses. The outcomes of this analysis indicate that the Ag-NPs/PL embedded in biofilm have good potential for antimicrobial applications. Antibacterial activity in Ag-NPs/PL was primarily affected by the role of silver ion (Ag+) which was released by Ag-NPs (2021). The diameter of inhibition zone may vary to one another for the same sample due to the response of the biofilm to the wet condition of the agar [21]. The biofilm reacts to water and crumple inwards, causing the contact area of the biofilm with the bacteria-spread agar to be varies thus the bacteria growth and the inhibition zone are expected and proven to be varies to one another [22].

Table 1. Average diameter of inhibition zone

Sample	Average diameter of inhibition zone (mm)
Film	0 ±0
A2 (3 minutes)	11 ±1.259
A7 (15 minutes)	16 ±1.324

4. Conclusions

Synthesis of Ag-NPs on pullulan was developed using a very safe, simple, eco-friendly, and energy saving method. The stable Ag-NPs on pullulan were prepared without using any chemical reducing agent and solely using the microwave irradiation to reduce the sample into nanoparticles. Presence of Ag-NPs was validated by using characterization analysis of UV-Vis, FTIR and XRD. The antimicrobial properties of the biofilm were established by the clear inhibition zone of *Bacillus Subtillis* during the antibacterial test, indicating the biofilm ability to inhibit and retard the growth of microorganisms. These experimental results suggest that pullulan reduced Ag-NPs can be used as potent antimicrobial agent for food packaging applications.

Funding
This research was funded by Nippon Sheet Glass Foundation, grant number R.K130000.7343.4B635

Acknowledgement
The authors are grateful for the financial and technical support from the Nippon Sheet Glass Foundation (grant number R.K130000.7343.4B635) and Malaysia-Japan International Institute of Technology (MJIIT).

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