PREVENTION OF MICROBIAL INFECTION IN BROILER CHICKEN USING GREEN SYNTHESIS OF TITANIUM DIOXIDE NANOPARTICLES

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Abstract: Green synthesized metal oxide nanoparticles have diverse applications. When coupled with structures like Poly Ethylene Glycol increases the unique properties which paved way in the food industry for safe food packaging. The present study focusses on antibacterial activity of titanium dioxide nanoparticles synthesized using *O. basilicum* leaves. The nanoparticles were characterized as 338nm absorption in UV spectroscopy, spherical shape in SEM characterization, 99 nm size based on particle size analyser. The second phase of the study involved the study of antimicrobial property of the synthesized nanoparticles against the microbes isolated from the broiler chicken. It gave very clear zones of inhibition indicating antibacterial activity. Three different types of colonies revealed different species which were subjected to morphological and biochemical analysis. The species were identified as *Bacillus species* and *Salmonella species*. The last phase of the study was to coat the titanium nanoparticles on Poly ethylene glycol (PEG) by the chemical process Aminopropyl triethoxy silane (facilitates dipole-cation binding). The coated nanoparticle produced antimicrobial film with the help of Polyvinyl alcohol (PVA). Both PEG and PVA is crosslinked using citric acid (crosslinking agent) and concentrated hydrochloric acid. The film was obtained after 96 hours of drying which is proved to be an antimicrobial film to extend the shelf life of broiler chicken.

IndexTerms **–** Green synthesis, titanium dioxide, Coating, Poly ethylene glycol, Polyvinyl alcohol, dipole-cation biding.

1. INTRODUCTION

Nanotechnology is an emerging and rapidly growing field because of its greatest application in the biological field (Santhoshkumar *et al.,* 2014). Likewise, metal oxide nanoparticles have various application in different fields. When couple with other structures like poly ethylene glycol it paved way in the food industry especially in packaging (Galstyan *et al.,* 2018). One such metal, titanium dioxide belongs to the family of transition metal oxide and its chemical formula is TiO_2 (Pelaez *et al.,* 2012). It is much more effective in photocatalyst in the form of nanoparticles than the bulk powder and is achieved by a large surface area and high crystallinity. The high exposure of heat for long duration affects the photocatalytic activity of titanium dioxide films. It is non-toxic in nature (Gupta & Tripathi 2011) and biologically more stable and inert within the human body (Bharathi and Palaniswamy 2020).

Food safety is one of the increasing public health through worldwide. Foodborne microbial infection caused 20 million people annually throughout the world which could be solved by food safety measures only (Pal, 2017). Nano structed metal-oxide thin films are mainly involved in realization of gas sensors like CO, CH_4 and aromatic hydrocarbons and also involved in polymer-based composites has various applications like packaging in food industries etc. (Alagarasi 2011).

With the constant increase in poultry meat consumption, ensuring the microbial safety of poultry carcasses and cuts is essential (Mohamed *et al.,* 2014). Common bacterial communities found are *Salmonella* and *Campylobacter* (Rouger *et al.,* 2017). Freezing canning, food irradiation, smoked food, has various disadvantages like loss of some nutrients, eating freezer-burned foods is safe, but it is unappetizing because of these disadvantages it paved for a new way to decrease the bacterial contamination in chicken using nanoparticles.

Green synthesis of nanoparticles is being widely used due to advantages like easily available, safe to handle and possess a broad viability of metabolites (Sundrarajan & Gowri 2011). In this study *Ocimum basilicum* leaf extract were used for the synthesis of titanium dioxide nanoparticles. It can be prepared in the cost-effective manner and it is a novel antibacterial material. Coating of nanoparticles on the surface of food materials or packaging papers has many protective features. Recently, titanium dioxide nanoparticles are being used in food industry because of its photocatalytic property (Bharathi *et al.*, 2019).

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

The fresh leaves of *Ocimum basilicum* (Basil) was collected and washed thoroughly with tap water and again washed with distilled water.

2.2 PREPARATION OF LEAF EXTRACT

50g of fresh leaves were boiled in 125ml of distilled water for 2 hours at 70℃. The extract was filtered using What man No.1 filter paper.

2.3 SYNTHESIS OF TITANIUM DIOXIDE NANOPARTICLES

10ml of titanium tetra isopropoxide was added to 250ml of distilled water, the distilled water should be continuously stirred for half an hour. After this the leaf extract should be added to the solution till the solution achieves a pH 7. The whole mixture was continuously stirred for 4 hours at 980 RPM. After this process the whole mixture should be kept aside for 12 hours. Then next day the settled wet cake is obtained at dried at 600℃ in muffle furnace. After which powder will be changed from orange to white powder.

2.4 CHARACTERIZATION OF NANOPARTICLES

The synthesized nanoparticles were characterized using **UV visible spectroscopy** - It is a valuable tool for structural characterization of titanium dioxide and is a fundamental technique to ascertain the formation of stable metal nanoparticles in aqueous medium based on Surface Plasmon Resonances. **Particle size analyser** - to know about the size of the nanoparticles. particle size of the nanoparticles was determined by DLS technique using a sub-micrometre particle analyser and **SEM** - to identify the size of the nanoparticles at higher magnification. The particle size and distribution are most commonly measures using electron microscopy.

2.5 ISOLATION OF BACTERIA FROM CHICKEN

5g of chicken was purchased from the nearby market and it is stored in fridge for 3 hours. After the incubation time the raw chicken was grinded with phosphate buffer saline. Serial dilution technique was performed to isolate the bacteria from the broiler chicken. For serial dilution, 1ml of chicken grinded phosphate saline was added to the 99ml of sterile distilled water this is serve as 10^{-1} the tubes were maintained from 10^{-2} to 10^{-6} by adding 1ml of water from 10^{-1} to next dilution tube 10^{-2} this process was continued up to 10^{-6}. Nutrient medium was prepared and sterilized and poured in a petriplate and allowed for solidify. 0.1ml of the serially diluted sample were taken and spread in a respective plate. The plates were incubated at 37℃ for 14-28 hours. After the incubation time the plates were observed and different colonies were isolated and pure cultured were obtained using streaking method. The different single was taken in loop and streaked in a new nutrient agar plate, quadrant streaking was used to get pure culture.

2.6 BIOCHEMICAL ANALYSIS OF ISOLATED BACTERIA

The basic biochemical procedure was carried out to identify the unknown bacteria isolated from raw chicken.

2.6.1 Carbohydrate fermentation

Lactose broth were prepared and 5 drops of phenol red was added as an indicator and Durham's tubes were added without air bubble and inoculated the culture and tubes were kept for incubation at 37℃ for 18-24hours

2.6.2 Triple sugar iron

Triple sugar iron slants were prepared and autoclaved and the bacterial colonies were streaked and kept for incubation at 37℃ for 18-24 hours.

2.6.3 IMVIC test

1) Indole production:

Tryptone broth were prepared and autoclaved and our bacterial colonies were inoculated in the broth and kept for incubation at 37℃ for 18-24 hours. After the incubation Kovacs reagent is added to conform the indole production.

2)Methyl Red:

MR-VP broth was prepared and autoclaved and the inoculated. The inoculated tubes were kept for incubation at 37℃ for 18-24 hours. After the incubation methyl red indictor is added.

3)Voges-Proskauer:

MR-VP broth was prepared and autoclaved and the inoculated. The inoculated tubes were kept for incubation at 37℃ for 18-24 hours. After the incubation Barrett's reagent is added.

4)Citrate Utilization:

Simon citrate agar slants were prepared and inoculated. The inoculated tubes were kept for incubation at 37℃ for 18-24 hours.

2.6.4 Hydrogen Sulphide test

SIM agar is prepared and autoclaved. The tubes were inoculated with the bacterial culture and kept for incubation at 37℃ for 18-24 hours.

2.6.5 Urease test

Urease broth were prepared and inoculated with cultures. The inoculated tubes were kept for incubation at 37℃ for 18-24 hours.

2.7 MORPHOLOGICAL ANALYSIS OF ISOLATED BACTERIA

2.7.1 Staining analysis

Gram staining was performed. Take a loop full of culture and smeared a culture in a clean grease free slide and it is heat fixed. Added few drops of crystal violet stain, after 1 minute washed with distilled water and few drops of Grams iodine was added and again washed with distilled water and next few drops of decolourizer (100% ethanol) was added to it and then washed, at last counter dye safranin was added and wait for a minute and washed with distilled water and viewed with light microscope. This picture was compared with Cappuccino manual to identify the group of the bacteria. Gram staining was also used to identify the morphology and cell wall nature of the bacteria, whether it is gram positive or gram negative.

2.7.2 Pure culture analysis

Nutrient agar plates were prepared and a single colony was taken in loop from mother culture and quadrant streaking was performed and the plates were inoculated at 37℃ for 12- 24 hours. The grown colonies were compared with Cappuccino manual to identify the specific species of microbe.

2.8 ANTIMICROBIAL ACTIVITY OF NANOPARTICLES

With the help of well diffusion method the antimicrobial activity of titanium dioxide nanoparticles was estimated. The different concentration like 100mg/ml, 200mg/ml, 300mg/ml were used. First nutrient agar plates were prepared and the bacterial culture from broth is swabbed equally and with the help of gel puncher the wells were prepared. Positive control was Ciprofloxacin and negative control was sterile distilled water. Into the wells the different concentration of nanoparticles was added and kept for incubation to observe and measure the zone of inhibition.

2.9 COATING PROCESS

2.2g of Polyethylene glycol and 0.45ml of Aminopropyl triethoxysilane were dissolved in anhydrous dimethyl formamide and solution was kept at room temperature for 3 days (Solution 1). Then, 0.5g of nanoparticles were dispersed in 25ml of dimethyl formamide and added to solution 1. This mixture was kept at

continuous stirring for 48 hours at room temperature, then centrifuged at 10,000 rpm for 10 mins. The obtained pellet was air dried to get the product (polyethylene glycol powder coated with titanium dioxide nanoparticles).

2.10 PREPARATION OF FILM

For preparation of film, 4g of poly vinyl alcohol was added to 30ml of deionized water and mix the substance inside the glass beaker, placed into an autoclave at 120℃ for 30 mins. After this process 1.5 g of polyethylene glycol coated with nanoparticles were taken and added to the autoclaved polyvinyl alcohol. After solubilization polyvinyl alcohol and polyethylene glycol were crosslinked by adding 0.5g citric acid and add immediately 1 ml of concentrated hydrochloric acid and the mixture was transferred to petriplate and air dried for 96 hours.

3. RESULTS AND DISCUSSION

3.1 SAMPLE PREPARATION

50g of *Ocimum basilicum* leaves were weighed and washed with tap water. The leaves were dipped with 125ml of distilled water and was boiled for 2 hours at 70℃. The extract was filtered using what man no 1 filter paper. The extract was used as a reducing agent for the synthesis of TiO_2 nanoparticles.

3.2 SYNTHESIS OF TITANIUM DIOXIDE NANOPARTICLES

The *Ocimum basilicum* leaf extract changes its colour from brown to orange when added to titanium tetra isopropoxide, and it indicates the synthesis of titanium dioxide nanoparticles. Then the synthesised titanium dioxide was purified by calcination and the powder changes from orange to white.

Kantheti and Alapati (2018) have worked with the synthesis of TiO_2 NPs using *Ocimum basilicum* leaf extract with water as a solvent is used to reduce the titanium tetraisoproxide as a TiO_2 NPs where similar results as above were noted. In another study by Sundrarajan and Gowri in 2011 who used *Nyctanthes arbor-tristis* leaf extract is prepared with ethanol as a solvent it is used as a reducing agent to breakdown the titanium tetraisopropoxide results in the formation of TiO_2 nanoparticles have seen potential results.

3.3 CHARACTERIZATION OF TITANIUM DIOXIDE NANOPARTICLES

Several techniques have been used to characterize the size, crystal structure, elemental composition and a variety of other physical properties of nanoparticles.

A) UV- Visible Spectroscopy: The synthesized TiO_2 NPs were analysed with the help of UV-Visible spectroscopy. In UV-Visible spec nanoparticles along with solvent (distilled water) is placed at room temperature after 10 minutes the absorption spectrum of titanium dioxide formed showed peak at 338 nm as shown in Fig 1. The stable titanium dioxide was synthesised and confirmed using UV-Vis spectrophotometer.

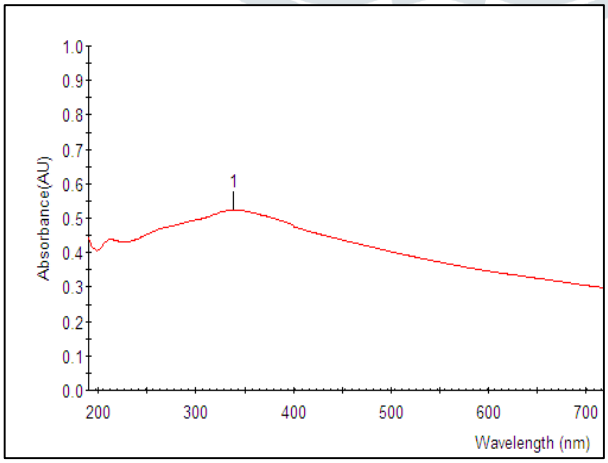

Fig 1: Absorbance of TiO2 Np

Similar studies confirmed that titanium dioxide nanoparticles synthesized using *Moringa Oleifera* leaf extract which is measured at 318 nm (Patidar and Jain 2017). TiO_2 nanoparticles synthesized using *Cassia fistula* leaf extract contained peak at 350nm when exposed to UV visible spectroscopy (Swathi *et al.*, 2019). From the above few references that are stated, it can be confirmed that the presence of TiO_2 nanoparticles ranges from 300 to 400nm range where sharp peaks are observed from the few studies which were carried out previously. Hence, it strongly supports the current study where the values of peaks obtained were 338 nm.

B) Particle size analyser: The particle size analyser works on the principle of dynamic light scattering and it is used to interpret average particle size of the nanoparticles. With the help of particle size analyser at 24.9℃ with water as a diluent with the help of DLS method average particle size of the synthesized nanoparticles is ranged of 99 nm as shown in Fig 2.

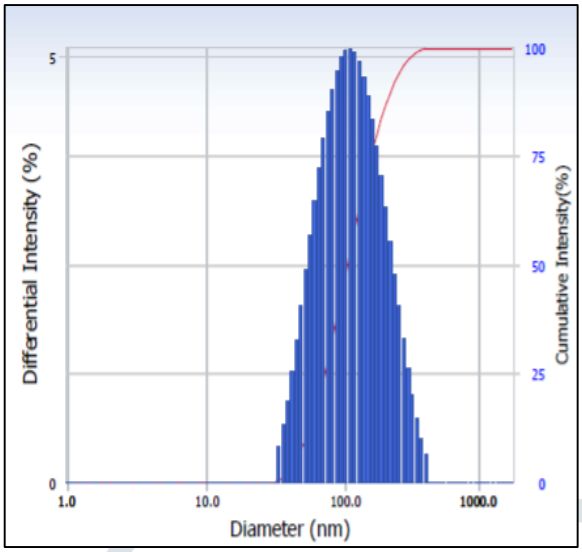

Fig 2: Particle size analyser of TiO2 NPs

The particle size of synthesized TiO_2 powder using *Nyctanthes arbor-tristis* leaves extract measured by Particle size analyser using DLS method was approximately 150nm (Sundrarajan and Gowri 2011). Another study showed that the average particle size of the synthesized nanoparticles after calcination was 52nm where they had used *Ocimum basilicum.* The method used for the synthesis was principle of dynamic light scattering (Kantheti and Alapati 2018). It is confirmed that the average particle size of the TiO_2 nanoparticles was in the range from 50 to 150nm as it is in line with the current study that we have performed with *O.basilicum* leaves.

C) Scanning Electron Microscope: SEM is used to record the photomicrograph images of synthesized TiO_2 nanoparticles. Sample is prepared to withstand the high vacuum condition and high energy beam of electrons. Then the specimen is placed in stage and viewed under higher magnification showed the presence of TiO_2 NP which was spherical in shape as shown in Fig 3.

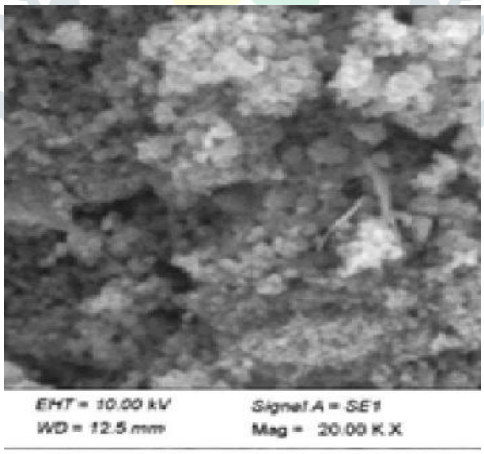

Fig 3: SEM image of TiO2 NPs.

The formation of TiO_2 NP using *Punica granatum* and their morphological dimensions in the SEM study demonstrated that the shapes were uniform and spherical (Begum *et al.*, 2009). Another study shows that the synthesized TiO_2 NPs using *Psidium guajava* in the SEM images resulted in smooth and spherical particles (Santhoshkumar *et al.*, 2014). SEM study of synthesized TiO_2 NPs using *Cassia fistula* clearly showed agglomerated and spherical particles (Swathi *et al.*, 2019). All these studies showed that TiO_2 nanoparticles mostly resulted in spherical shapes irrespective of the type of samples used for the study.

3.4 ISOLATION OF BACTERIA FROM POULTRY MEAT

Sample preparation:

1g of poultry meat (weighed, washed) transferred to a sterile motor and pestle. Added 5ml of phosphate buffer saline and homogenised. From that, 1 ml was pipetted out and made up to 99ml with sterile distilled water and used for serial dilution.

Organism	Gram Staining	Carbo-hydrate fermentation	Triple sugar iron	Indole production	MR	VP	Citrate Utilization	H_2S	Urease Test
Colony 1	Rod+	_	_	_	_	_	_	_	_
Colony 2	Rod -	_	_	_	+	_	+	+	_
Colony 3	Rod -	_	_	_	+	_	+	+	_

Serial dilution:

With the help of serial dilution and spread plate method, different types of bacterial colonies were isolated and grew in Nutrient agar medium plates. Three different colonies which were different in their colours were isolated. Pure culture was obtained to get single colonies using quadrant streaking. Later it was compared with cappuccino manual to identify the species.

Frozen chicken samples exposed to a 10-fold serial dilution further observed total number of five microorganisms that were isolated - *Escherichia coli* (27.82%), *salmonella sp* (13.64%), *Staphylococcus aureus* (18.52%), *Bacillus subtilis* (17.83%), *Klebsiella sp* (1.84%) (Arueyingho 2019). In another study conducted by Nagarajan *et al*. (2018) aimed to isolate bacterial colonies from poultry meat and fish and total bacterial count was performed by Lazy Susan plating method. A 10-fold serial dilution followed by colony forming units were counted after 24hours and expressed in CFU/mg which showed the presence of *E.* coli, *Staphylococcus aureus* (Nagarajan et al., 2018).

3.6 BIOCHEMICAL ANALYSIS OF ISOLATED BACTERIA

The isolated bacteria were biochemically characterized and identified based on Cappuccino manual. Carbohydrate fermentation, triple sugar iron, IMViC (Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test), Hydrogen sulphide, Urease test were done and the results are compiled in Table 1.

Table 1: Biochemical analysis of bacteria isolated from broiler chicken

Note: + indicates Positive for the test, - indicates Negative for the test

Similar studies show the same results negative for urease and Voges-Proskauer, methyl red, indole, H_2S production for *Bacillus sp.,* isolated from bacterial contaminated silkworm (*Bombyx mori*) (Guan-Nan Li *et al.,* 2015). Same results showed for *Bacillus sp.* isolated from coalmine soil negative for citrate, methyl red, Voges-Proskauer, nitrate, urea hydrolysis and oxidation/fermentation tests (Dhanjal and Cameotra, 2010).

Biochemical characterization for colony 2 and 3, Gram negative rod-shaped bacteria it is negative for Carbohydrate fermentation, Triple sugar iron, Indole production, VP and Urease test and positive results for MR, citrate and H_2S test it shows the colony 2 & 3 indicates the *Salmonella sp*.

From previous literature it is evident that *Salmonella sp.* isolated from food shows similar results - positive for hydrogen sulphide and citrate and negative for lactose fermentation, Indole and VP test (Zadernowska and Chajęcka 2012). One more study conducted by Kar *et al.*, (2017) isolated microbe from

turkey flock shows similar results positive for MR and citrate as well as negative for Triple sugar iron, Indole production, VP, Urease test and lactose fermentation test.

3.7 MORPHOLOGICAL ANALYSIS OF ISOLATED BACTERIA

3.7.1 Staining analysis

Gram staining was used to identify whether the bacteria is Gram positive or Gram negative. From the above isolated 3 different colonies, first colony is Gram positive and rod shaped, second and third were Gram negative, rod shaped. This was confirmed using the Cappuccino manual as the first colony is *Bacillus sp.*, second and third colonies belongs to *Salmonella sp.*,

3.7.2 Pure culture analysis:

In nutrient agar plate, pure culture colonies were grown and then the colonies were compared with Cappuccino manual. It was confirmed that first colony was *Bacillus sp.*, second and third colonies belong to *Salmonella sp.*

3.8 ANTIBACTERIAL ACTIVITY OF NANOPARTICLES

The synthesized nanoparticles were subjected to a series of antibacterial activity against the isolated *Bacillus sp.*, *Salmonella species* and the results were noted. The synthesized TiO_2 NP produced zone of clearance against both Gram positive and Gram-negative organisms isolated from poultry meat where the concentration of TiO_2 nanoparticles increases zone of inhibition (Fig 4). The diameter of zone of inhibition was measured and it is mentioned in Table 2 which shows the antimicrobial activity of TiO_2 nanoparticles against the bacteria isolated from poultry meat (colony 1, colony 2, colony 3) about 3.5cm, 3.7cm, 3.2cm in the higher concentration of 300mg/ml respectively.

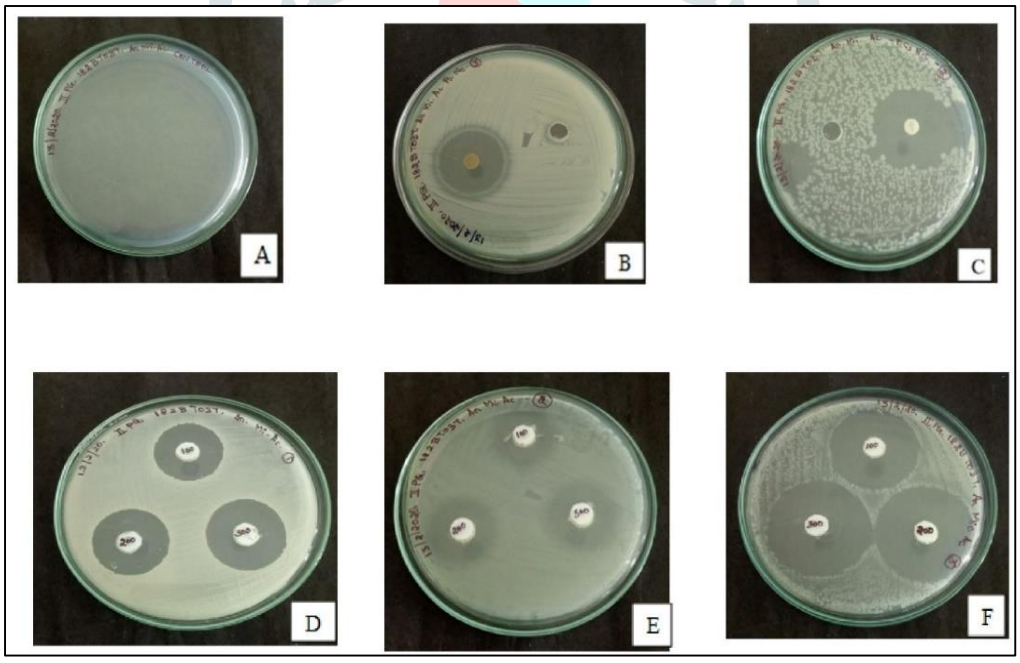

Fig 4: A) Control B) Positive & Negative control for colony 1 C) Positive & Negative Control 2&3 D) TiO_2 NPs against Colony 1 E) TiO_2 NPs against Colony 2 F) TiO_2 against Colony 3

Table 2: Antibacterial activity of TiO_2 NPs.

Bacterial colony	Zone of Inhibition		
	100mg/ml	200mg/ml	300mg/ml
Colony 1	2.5cm	2.4cm	3.5cm
Colony 2	2.7cm	2.8cm	3.7cm

Colony 3	2.6cm	2.7cm	3.2cm

In earlier studies TiO_2 Nps were synthesized using *Vigna radiata* showed the antibacterial activity against *P. aeruginosa* (15 mm) at 19.0 µg /ml, *C. albicans* (25 mm) at 9.7 µg /ml. *B. subtilis* (15mm) at 19.5 µg/ml. (Piskin *et al.*, 2013). Another study also shows synthesized TiO_2 NPs using *Planomicrobium sp.* has antibacterial activity against *Bacillus substilis* (14mm) and *Klebseilla planticola* (17mm) at 200 µl concentration of TiO_2 nanoparticles (Annadurai *et al.*, 2013). All these above-mentioned references confirmed that antibacterial activity of TiO_2 NPs in the current study are in line with the literature survey conducted.

3.9 PEG COATED NANOPARTICLE

Nanoparticles coated with polyethylene glycol (PEG) was prepared by dipole-cation binding. PEG-modified magnetic nanoparticle was synthesized by 48 h agitation with Aminopropyl triethoxysilane (APTS) and Dimethylformamide. During this time, acrylated PEG was first reacted with APTS and then condensation reaction was carried out between the triethoxysilyl-terminated PEG and hydroxyl groups on the surface of magnetite nanoparticles. Surface modifying of magnetic nanoparticle with methoxy polyethylene glycol provides stability and enhanced biocompatibility for magnetic nanoparticles.

As per the observations of Khoee and Kavand (2014) iron nanoparticles coated with polyethylene glycol were connected to methoxy poly (ethylene glycol) (mPEG) via a new method mPEG was acrylated; at first and Michael reaction was carried out between acrylated mPEG and 3-aminopropyl triethoxysilane as a coupling agent and found that the coupled nanoparticles have the potential to be used in different biomedical applications.

Another study showed the PEG coated silica nanoparticles were used to load the drug ibuprofen. The silica nanoparticles synthesized through Stober's method were in the range of 10 – 150 nm (Thangaraja *et al.*, 2010).

3.10 PREPARATION OF POLY VINYL ALCOHOL (PVA) FILM

Poly vinyl alcohol is crosslinked with PEG coated with nanoparticles with the help of citric acid and concentrated HCl. Citric acid has demonstrated to be an apt crosslinking agent for the production of polyvinyl alcohol film. Fig 5 (A) showed the formed film crosslinked with PEG and Fig 5 (B) showed the presence of PVA film.

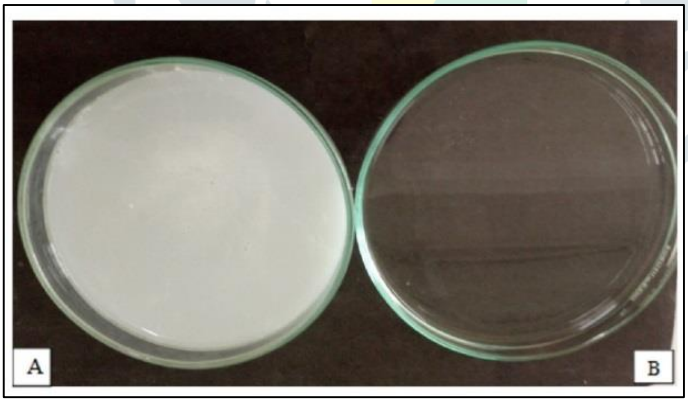

Fig 5: Poly vinyl alcohol Film A) PVA crosslinked with PEG B) PVA film without PEG.

Earlier study showed similar film formation aimed at develop a thin and water-resistant food-grade poly (vinyl alcohol) (PVOH)-based matrix. Film was prepared by blending PVOH and 7.20% (wt/wt of PVOH) of poly (ethylene glycol) (PEG) with citric acid as crosslinking agent. Film-forming solution was then casted onto a flat surface and the obtained film was 60 µm in thickness and showed a good transparency (Musetti *et al.*, 2014).

An observation by Saha *et al.*, (2016) to develop nontoxic nanocomposite films with good antimicrobial property was done. They used cellulose acetate (CA), polyethylene glycol (PEG) and cetyltrimethylammonium bromide (CTAB) modified montmorillonite (OMMT) to prepare the nanocomposite films. The films composed of 20 wt% PEG in CA matrix gave optimum results in terms of mechanical properties. Nanocomposites possess good antimicrobial activity as well as no toxicity upon human blood, so can be used as active packaging material.

4. CONCLUSION

TiO_2 nanoparticles have diverse applications; with its photocatalyst activity it can destroy the membrane of cells, degrade the virus's protein capsid and kill bacteria up to 99.97%. These metal oxide nanoparticles and its antimicrobial activity is due to these mechanisms like interfering in trans-membrane electron transfer, disrupting in cell penetration, dissolving heavy metals leads to cell damage.

In this current study, TiO_2 nanoparticles were synthesized using *Ocimum basilicum* leaves. Microbes were isolated from broiler chicken and identified to be *Bacillus sp.*, and *Salmonella sp.*, with the help of biochemical characterization and morphological analysis. Antimicrobial activity of TiO_2 nanoparticles was analysed which showed excellent zone of inhibition in the presence of *O. basilicum* TiO_2 nanoparticles for the for the packing. The synthesized nanoparticles were coated with Poly ethylene glycol (PEG) with the help of chemical process where citric acid is used as coupling agent to couple the polyvinyl alcohol and coated PEG. The prepared film possessed antimicrobial activity which was used as a packing material for broiler meat to increase the shelf life of the broiler chicken. Hence, the conclusion of the current study on *Ocimum basilicum* leaf extract is that synthesis of nanoparticles is a boon to the society in the field of food industry for the preparation of food packaging material apart from other applications.

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