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Biosynthesis of Silver Nanoparticles Using Lactobacillus Acidophilus and White Rot Fungus- A Comparative Study

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Abstract: ***the Future perspective of nanotechnology is to enhance the biological approach. In the present investigation biosynthesis of silver nanoparticles was evolved by bioreduction of silver by mixture (AgNO3+ filtrate). The conformation of silver nanoparticle synthesis was characterized using SPR band shown in UV-Visible spectroscopy and FTIR analysis. SEM micrographs depict puff shaped structure ranging from 20-50nm in size. Antimicrobial activity of biosynthesized Ag nanoparticle showed inhibitory activity against strains. While comparing strains, effective ant cancerous activity was found in white rot fungus. This, in turn, gives the insight to develop new formulations for the conventional method in pharmaceutical companies.***

Keywords: Biosynthesis, Silver Nanoparticles, UV-VIS, FTIR, SEM, Antimicrobial Activity.

I. INTRODUCTION

Nanotechnology is an emerging field growing day by day in various aspects which are considered as attractive building blocks for materialistic architectures. It is the application of science at the molecular level and promising field in emerging new applications in medicine. Nano biotechnology combines with a biological approach to generate nanosized particles having specific functionality. Due to a major outbreak of infectious disease caused by several pathogens in and around the environment many pharma companies are searching for novel antibacterial agents which can be done with the help of nanoscience. Compared to biological molecules such as enzymes, nanoparticles are smaller than hundred nanometres in size. For drug delivery applications silver and gold nanoparticles are a powerful and promising tool. Multidrug-resistant bacteria has raised a demand for the need to identify antimicrobial agents[10].Nanoparticles have unique properties such as large surface to volume ratio, absorption in the visible range, controlled drug delivery make nanoparticles very useful in human life. The synthesis of nanoparticles is characterized by top-down and bottom-up approaches. In top-down methods, bulk materials are reduced in size in the range of nanoscale and in bottom-up approach, the starting materials are grown to larger structures by joining atoms and molecules [1]. Silver nanoparticles are more superior disinfectants which significantly reduces many bacterial infections for longer extent compared to common penicillin and tetracycline [2]. Silver is a good antimicrobial agent, non-toxic natural inorganic metal and possesses low toxicity [9]. As a reducing and capping agent prokaryotes and eukaryotes explore as a potential bio-factory for nanoparticle synthesis [7]. Moreover, there is a hypothesis that nanoparticles are toxic due to its combination of specific properties free ions gets released by then they enter into a human body so they can be used in toxicity testing [8].

In this present research work, the novelty lies in the fact that we have used culture filtrate to develop a simple, inexpensive and eco-friendly approach for the synthesis of AgNP's using *Lactobacillus acidophilus* and white rot fungus. The study exemplifies systemic analysis of antibacterial, antifungal and anticancerous activities of biologically prepared

AgNP's. We also found the efficacy test by comparing coated drug and non-coated drug. This paper focuses mainly on the emerging performance of white rot fungus and now it's clear that it improves the antibiotic efficacy by increasing the drug concentration with the addition to the attachment of nanoparticles.

II. MATERIALS AND METHODS

A. Culture Collection

A *Lactobacillus acidophilus* and white rot fungus (*Pleurotus platypus*) were obtained from Centre for Biological and Nanoscience Research (CBNR), Coimbatore.

B. Preparation of Silver Nanoparticles

Briefly, 0.50 g of silver-containing glass powder was dispersed in 50mL of an aqueous solution of 0.25, 1, or 4.0, the weight percentage of glucose in a 100mL glass vial. The mixture was at 121^{o}C for 20 min. The mixture was then gradually cooled to room temperature and centrifuged at 3000 rpm for 10 min. The supernatant containing the AgNP suspension was removed and stored in the dark at 4^{0}C.

C. Biosynthesis of Silver Nanoparticles

The obtained fungal species was inoculated in MGYP media (Makeup 20 ml volume using distilled water by adding 0.4gm Meat extract,0.3gm glucose,0.06gm yeast extract and 0.1gm peptone) and incubated at room temperature for a period of 5days in order to get enormous fungal growth. Using Whatman filter paper the fungal mat is discarded thereby the filtrate was used for the study. Silver nitrate solution was added to the filtrate in different concentrations namely 1mm and 2mm and incubated at dark room until the color change was observed. Then they were centrifuged at 5000rpm for 10 minutes and the pellets were air dried thoroughly. Subsequently, *Lactobacillus acidophilus* was inoculated in 10ml of nutrient broth, then they were centrifuged for 5000rpm for 10 minutes. To the supernatant, silver nitrate solution was added namely in 1mm and 2mm concentration. Finally, the resulting colloidal suspension was characterized using various techniques.

D. Characterization of Silver Nanoparticles

I. UV-Visible Spectrometer (UV-VIS)

Bioreduction of pure silver ions was examined by visual observation of color change and further confirmed by sharp peaks. The spectra between (250-500nm) ranges were scanned to find absorbance peak. A small aliquot of the sample was taken in a quartz cuvette and wavelength was observed with distilled water as a reference. All samples were loaded into a 1cm path length quartz cuvette for UV-VISIBLE spectrophotometers reading and scanned from 300-500nm at a scanning speed of 0.5 nm interval. The decolorized water was used as a blank.

II. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR was used to identify possible interactions between Ag salts and protein molecules. FTIR spectroscopy analysis of biosynthesized silver nanoparticles was performed for the identification of functional groups capping in the silver nanoparticles The sample were determined using the infrared spectroscopy using the absorbance ratio A1655/A3450 and calculated according to the equation:

A (%) = (A1655/A3450)x100/1.33

Two milligrams of the sample were dried overnight at 60^{0}C under reduced pressure 0.5 mm thick discs. The discs were dried for 24hrs at 110^{0}C under reduced pressure. The infrared spectrometer was recorded with a Bruker 66 Spectrophotometer, using a 100 mg KBr disc for reference. The intensity of maximum absorption bands was determined by baseline method.

III. Scanning Electron Microscopy (SEM)

Scanning electron microscope (JEOL/EO, JSM-6390, Japan, magnification range 1500, acceleration voltage 20kv) was used to evaluate the surface and shape characteristics of the particles after prior coating with silver. The elemental film composition was analyzed using energy dispersive spectrometer (JEOL, JED-2300) at SAIF, Cochin, India. UV-VISIBLE absorption spectra of the samples were recorded in the wavelength range of 300-500 nm using UV spectrophotometer (UV-VISIBLE Perkin Elmer Lambda) at the Centre for Biosciences and Nanoscience Research, Coimbatore, India.

E. Antibacterial activity

The antibacterial activity of silver nanoparticles synthesized using white rot fungi was investigated against various pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus subtitles.* The volume of 50ml of nutrient agar(1.4gms of nutrient agar make up to 50ml using distilled water) was prepared and plated thereby the bacterial cultures were spread using a sterile cotton swab. Then the disk was placed at the center and subsequently, four wells were made, in that 20 µl of 1mm, 2mm silver nanoparticles and control was added respectively. The plates were incubated at 37^{0}C for a period of 24hours in order to observe the zone of inhibition.

F. Antifungal activity

The antifungal activity of AgNP's was performed using *Aspergillus niger* and *Aspergillus flavus.* A volume of 50ml of malt agar (2.25gms of malt agar make up to 50ml using distilled water) was prepared and plated thereby creating four wells. To the wells control and silver nanoparticles was added to 20 µl in each well. The plates were incubated at room temperature for 5 days in order to observe the zone of inhibition.

G. Drug Preparation using Ofloxacin

Ofloxacin is a drug used as eye drops which reduce eye irritation even to treat certain infections like bronchitis, pneumonia, and infections of skin, bladder, urinary tract and prostate. This drug is an antibiotic and it is added in few drops to the

biosynthesized AgNP's in the concentration of 1mg/ml**.** A semisolid mixture of silver nanoparticles of both *Lactobacillus acidophilus* and white rot fungus were used for drug coating.

H. Efficacy study

The efficacy test was performed to test the better efficient coated nanoparticle from two species using Muller Hinton agar in the concentration of 38gm in 1000ml distilled water and separated in two plates. One plate was spread using *E.coli* and another plate was spread using *Staphylococcus aureus*. Four wells were made on each plate, two wells were filled with drug-coated nanoparticle sample and other two wells were filled with nanoparticle sample. The zone of inhibition was measured after 24 hours.

I. Anticancerous activity

To study the anticancerous activity DMEM (Dulbecco's modified Eagle's medium) media was prepared and used.

The HeLa cell line was added to DMEM and then transfer to T-flask and kept in a CO2 incubator for 4 to 5 days. The anti-cancerous activity of white rot fungus can be detected by using MTT assay. Add 500µl of the supernatant of drug-coated sample and silver nanoparticles. To that tube 500µl of DMEM medium was added and 100µl of MTT dye is added. Then it was allowed to incubate at 37^0 C for 24 hours. The absorbance of the cell line medium was measured at 570nm. Then the sample was observed by using a microscope.

$$\%of\ viability\ calculated = \frac{Optical\ density\ of\ treated\ cell}{Optical\ \ density\ of\ control} \times 100$$

III. RESULTS AND DISCUSSION

A. Biosynthesis of AgNP's

For the synthesis of silver nanoparticles, *Pleurotus platypus* was inoculated in MGYP media and incubated at room temperature for a period of 5days. Using Whatman filter paper the fungal mat was discarded thereby the biomass was filtered. Silver nitrate solution was added to the filtrate in different concentrations namely 1mm and 2mm and incubated at dark room until the color change was observed. Then they were centrifuged at 5000rpm for 10 minutes and the pellets were air dried thoroughly. Subsequently, *Lactobacillus acidophilus* was inoculated in 10ml fresh nutrient broth and then they were centrifuged at 5000 rpm for 10 minutes. To the supernatant, silver nitrate solution was added namely in 1mm and 2mm concentration. Silver nanoparticle production was monitored and control (without silver) was also run along with the experimental flask. The current method is superior to other available methods based on the higher yield and pure quality**.**

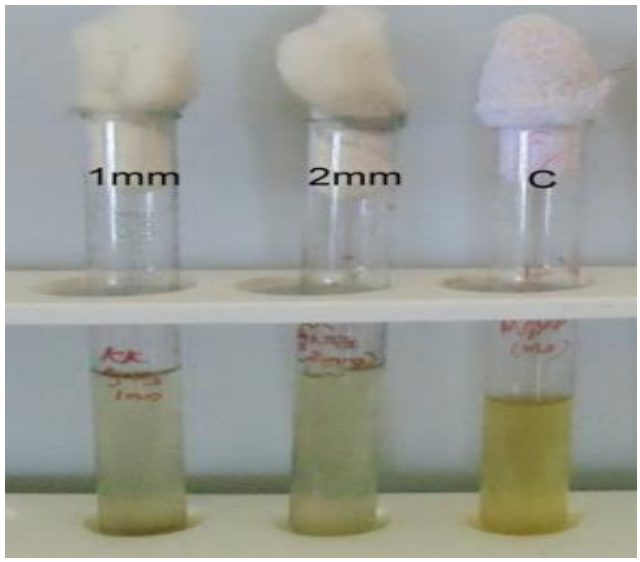

Fig.1 Synthesis of silver nanoparticles from white rot fungus (1mm silver nanoparticle, 2mm silver nanoparticle, and control)

B. Characterization of AgNP's

I. UV-Visible Spectrometer (UV-VIS)

The process of reaction between the metal ions and biosynthesized AgNP's was monitored using UV spectra through the aqueous solution. After 24hours of incubation color change was observed for 1mm white rot fungus and 1mm *Lactobacillus acidophilus*. The preliminary characterization of 1mm white rot fungus and 1mm *Lactobacillus acidophilus* were taken using UV-VIS spectra. The absorbance was taken at 250-500nm. In a study [11] said that the surface plasmon of AgNP's occurred at 435nm confirming the presence of silver. In our present study, the peak value of 1mm white rot fungus was attained at 465 and 1mm *Lactobacillus acidophilus* at 412nm.For further analysis 1mm, white rot fungus was used since it is more effective than the other.

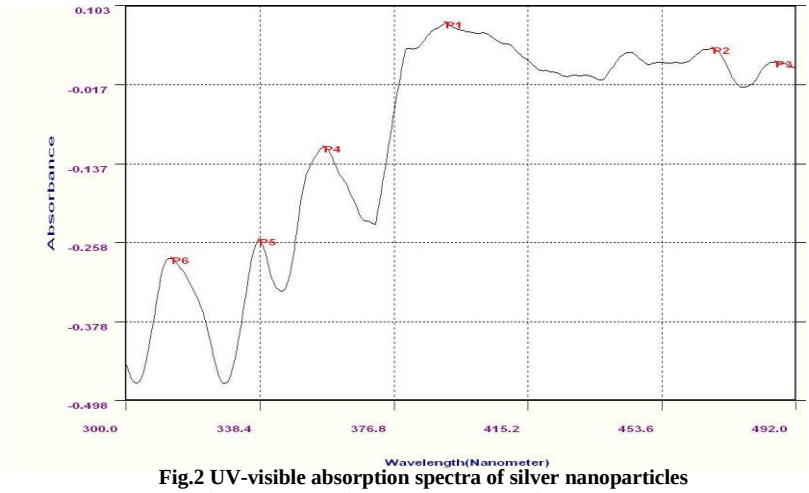

Fig.2 UV-visible absorption spectra of silver nanoparticles from white rot fungus after 24 hrs of incubation

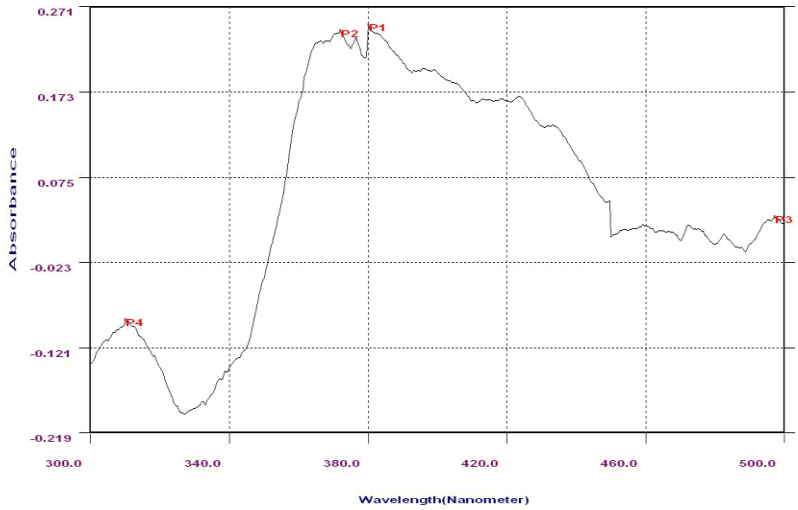

Fig.3 UV-visible absorption spectra of silver nanoparticles from Lactobacillus acidophilus after 24 hrs of incubation

II. FTIR Analysis

Usually, proteins present in silver nanoparticle binds to nanoparticle either by means of free amine groups or cysteine residues. FTIR analysis was carried out basically to identify biomolecules responsible for effective stabilization and capping of synthesized AgNP's [4]. The silver nanoparticles absorb strongly at 1679, 1539, 1452, 1430 and 1040cm^{-1}.It is evident that based on absorption, the capping series could be different. In the present study, FTIR(Thermo Nicolet Model -6700) results revealed that the absorption bands at 3417, 2924, 1627, 1501, 1015.41, 1015 and 570.04 cm^{-1} which are associated with stretching vibrations of primary and secondary amines, C-N, C-H, N-H stretching or bending vibrations.

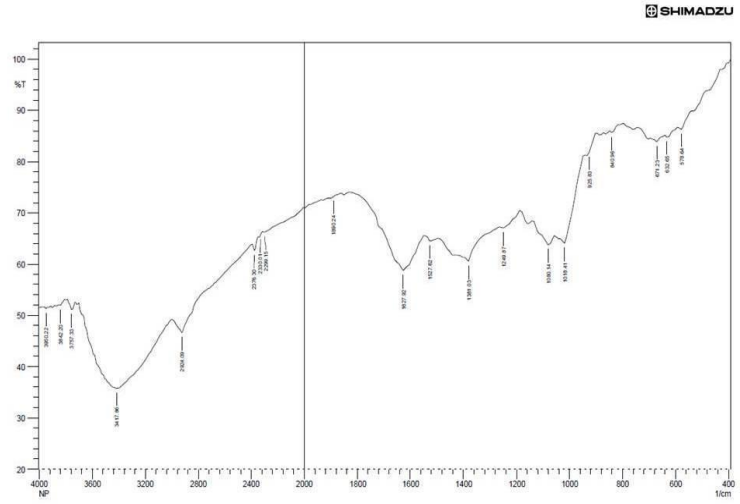

Fig.4 FTIR spectrum of silver nanoparticles synthesized from white rot fungus

Table.1 Detection of various functional group by FTIR from synthesized silver nanoparticles of white rot fungus

S.No	Assignment of functional group	Group frequency cm^{-1}
1	O-H stretch, Primary two bands	3417
2	C-H stretch, alkane	2924
3	N-H bend, amine	1627
4	C-H bend, alkane	1000.14
5	C-N bend, amine	1015.41
6	Alkene	570.04

III. SEM Analysis

Silver nanoparticles synthesized using white rot fungus was allowed to dry completely and used for further study. At high vacuum, the specimen is usually fixed in the solution of a buffered chemical fixative such as glyceraldehyde. The dry specimen was mounted on the adhesive resin. In the existing investigation, the SEM micrographs of nanoparticle obtained in the filtrate showed that silver nanoparticles are spherically shaped without aggregation with a size of 5-50nm [3]. In the present study, the SEM micrographs showed that silver nanoparticles were well distributed without any aggregation with an average size of about 20-50nm.This study concluded that silver nanoparticles play a significant role in the field of biology. SEM has given an insight into the morphology and size detail of biosynthesized silver nanoparticle.

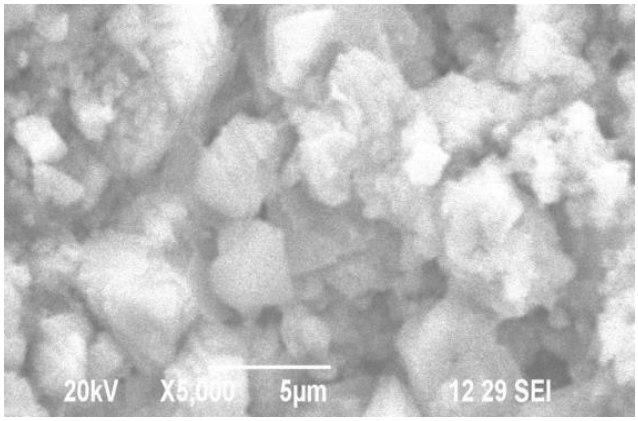

Figure.5 SEM image of AgNP's biosynthesized from white rot fungus

C. Antibacterial Activity

Antibacterial activity was evaluated using AgNP's synthesized by white rot fungus against various pathogenic bacteria such as *Bacillus subtitles, E.coli* and *Staphylococcus aureus* by the good diffusion method. Here AgNP's of two different concentrations, control and silver was loaded into the wells in a volume of 40µl.In this analysis, AgNP's displayed antibacterial activity and the maximum zone of inhibition of 0.8cm in 2mm AgNP's and 0.6cm in 1mm AgNP's against *E.coli* was found. Satisfactory zone of inhibition was seen in *B.subtilus* and *S.*aureus. The antimicrobial activity was reported due to the penetration of AgNP's into bacteria which damages the cell membrane and cell contents will be released [5].

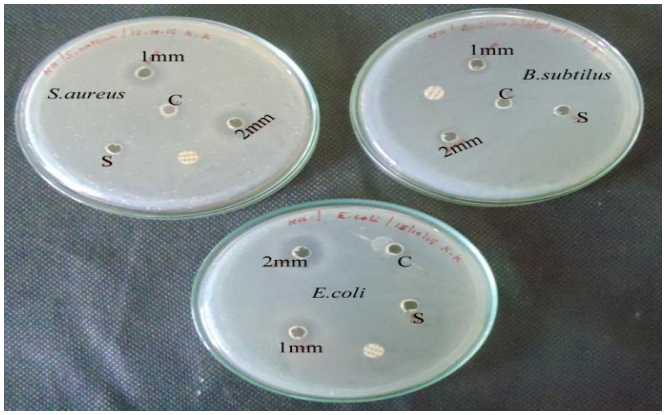

Fig.6 Antibacterial activity of silver nanoparticles on bacterial strains of white rot fungus

Table.2 Antibacterial activity of silver nanoparticles on bacterial strains of white rot fungus

Bacterial strain	Measurement of zone of inhibition(cm)			
	1mm	2mm	Silver	Control
E.coli	0.6	0.8	-	-
S.aureus	0.4	0.6	-	-
B.subtilus	0.2	0.3	-	-

D. Antifungal Activity

In a study antimicrobial test was the evaluation of activity of silver nanoparticles against clinically isolated organism like *Aspergillus niger* and *Aspergillus flavus*. They are the human pathogens responsible for many nosocomial infections and biofilm forming species. In the present study, the synthesized silver nanoparticles against *A.niger* and *A.flavus* shows effective antifungal activity. Malt agar was prepared and plated thereby four wells created, 40µl of following concentrations were loaded into the well such as 1mm AgNP's, 2mm AgNP's, silver and control. After 24hours of incubation maximum zone formation of 0.5cm was found against *A.niger* of 2mm AgNP's and in *A.flavus*(2mm AgNP's). It shows both the pathogens are effective against the biosynthesized silver nanoparticles. There is no zone formation in control.

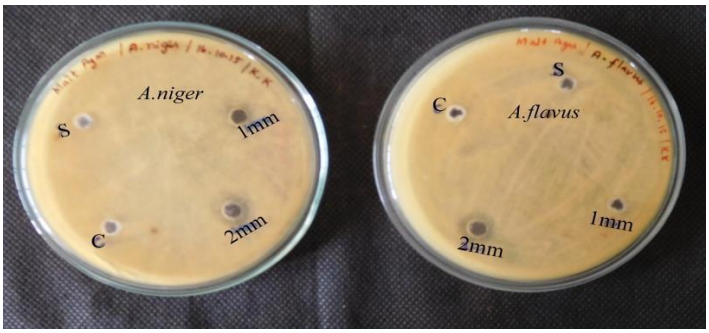

Fig.7 Antifungal activity of silver nanoparticles on fungal strains of white rot fungus

Table.3 Antifungal activity of silver nanoparticles on fungal strains of white rot fungus

Fungal strain	Measurement of zone of inhibition(cm)			
	1mm	2mm	Silver	Control
A.niger	0.3	0.5	-	-
A.flavus	0.1	0.5	-	-

E. Efficacy study of nanoparticles coated with drug

The efficacy test was performed using Muller Hinton agar to test the better efficient coated nanoparticle. Thereby ofloxacin drug was added to biosynthesized silver nanoparticles of white rot fungi and *Lactobacillus acidophilus.* One plate was spread using *E.coli* and another plate was spread using *Staphylococcus aureus*. Four wells were made on each plate, two wells were filled with drug-coated nanoparticle sample and other two wells were filled with nanoparticle sample. Maximum zone of inhibition was found in white rot fungi coated with a drug against *S.aureus*. It is found that the silver nanoparticles enhances the reaction rates of antibiotics in a synergistic mode as well as in its own way on the clinically isolated pathogens. The anticancer drug coated with the nanocomposites the anticancerous activity enhanced. Anti-cancerous drugs have lot of side effects, but very small amount drug coated with these nanocomposites, side effects are reduced. So now it used in cancer treatment, chemotherapy in foreign countries. This can be potentially applied for the development of new therapeutic agents.

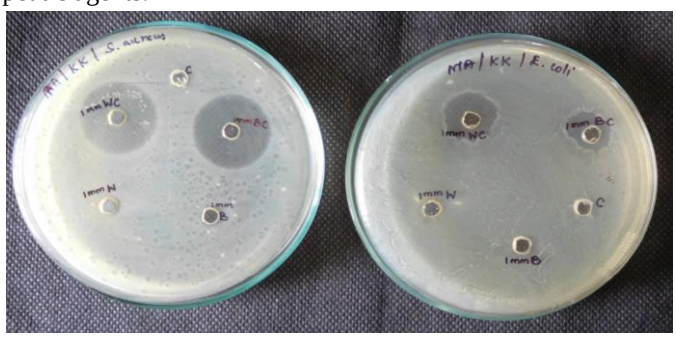

Fig.8 Efficacy study of white rot fungus and Lactobacillus acidophilus against pathogenic bacteria(WC-White rot drug coated, BC-*Lactobacillus acidophilus* drug coated, W-White rot non-drug-coated and B-*Lactobacillus acidophilus* non-drug-coated)

Table.4 Efficacy study of white rot fungus and Lactobacillus acidophilus against pathogenic bacteria

Bacterial strain	Measurement of zone of inhibition(cm)				
	WC	W	BC	B	C
E.coli	0.6	0.3	0.6	0.2	-
S.aureus	1	-	0.9	-	-

F. MTT assay results

In this test, the anti-cancerous activity of white rot fungus and drug coated white rot fungus can be measured. Three Eppendorf tubes are taken and 500µl cell line +500µl dye was added in control tube. Then in other two tubes, 100 µl dye and 500 µl coated and the non-coated drug was taken separately. After overnight incubation, all the three tubes are taken for UV reading at 570nm.

$$\% \text{ of viability measured} = \frac{\text{Optical density of treated cell x100}}{\text{Optical density of control}}$$

The standard control value=0.386

Drug coated white rot fungus

In % of viability $= \frac{0.006 \times 100}{0.386} = 1.5\%$ (live cells)

Non-drug coated white rot fungus

In % of viability $= \frac{0.018 \times 100}{0.386} = 4.6\%$

Fig.9 Microscopic view of dead cells in MTT assay

Drug-coated white rot fungus has high activity against HeLa cell lines (breast cancerous cell). Only 1.5% viable cells were remaining. Nondrug coated contains 4.6% viable cells. It indicates that white rot fungus also has a high amount of cancerous activities. Using UV Spectrophotometer, absorbance values for all concentrations were obtained. The readings were then calculated to yield the viability percentage, using the standard formula. These works indicate that white rot fungus has an antiproliferative effect on HeLa cell lines. DNA fragmentation assay could establish the reason for cytotoxicity is due to the induction of apoptosis (programmed cell death) or necrosis.

CONCLUSION

White rot fungus (*Pleurotus platypus*) and *Lactobacillus acidophilus* were biosynthesized using silver nitrate and synthesis were confirmed using UV-VIS, FTIR and SEM studies. Antibacterial activity of white rot fungus against bacterial strains was confirmed. The results showed it have high antibacterial activity. The anti-cancerous activity of drug-coated samples was measured and concluded that drug-coated white rot fungus has higher anti-cancerous activity against HeLa cell lines, only 1.5% of viable cells were remaining. Anti-cancerous drugs have lot of side effects, but very small amount drug coated with these nanocomposites, side effects are reduced. So now it used in cancer treatment, chemotherapy in foreign countries. This can be potentially applied for the development of new therapeutic agents. Now a day's several studies on *Pleurotus platypus* are taking place due to their wider application.

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