IMPACT OF MUSHROOM *PLEUROTUS OSTREATUS* ON SYNTHESIS OF CHITOSAN & ITS EFFECTS IN ANTIOXIDANT & ANTIBACTERIAL ACTIVITY

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ABSTRACT

Chitosan extraction from fungi plays a major role in the world of industrial Mycology. Thus fungi are the promising alternative source of chitosan. Recent studies on exopolysaccharides and biopolymer from mushrooms have brought out many interesting biological activities. In the present study, research was made to isolate chitosan a biopolymer from edible mushroom like *Pleurotus ostreaus* which is commonly known as Oyster mushroom. The extraction of chitosan was done by three steps which includes deproteinization, demineralization and deacetylation. The extracted chitosan was analyzed according to its color, texture, nature and its solubility. The extracted chitosan was pale yellow in color and it is hygroscopic with flabby nature. Chitosan was soluble in 10% of acetic acid. The crude chitosan extract was measured for its maximum absortion by UV spectrometer and the maximum peak was observed at 252nm. FT-IR spectroscopy was also done to identify the structural groups present in the chitosan and was characterized in the middle region ranges from 1552-1639 Cm^{-1} in liquid form and 1566-1641Cm^{-1} in the form of powder. *In vitro* antibacterial screening of chitosan from *Pleurotus ostreatus* was performed against selected clinical isolates and the zone of inhibition was observed. This findings suggest that the *Pleurotus ostreaus* are capable of producing eco-friendly chitin and chitosan can be used in various fields such as nanomedicine, food industry, agriculture and in many research industries.

Keywords: Antibacterial activity, Chitosan, FT-IR spectroscopy, *Pleurotus ostreatus.*

INTRODUCTION

Mushrooms are macrofungi which has fruiting bodies. It can be either epigenous or hypogenous and visibly seen [5]. Many variety of mushrooms are edible with high protein, medicinal and nutritional values [2] .Edible mushrooms plays a major role in the source of food because it is easily digestible[20,12] and nowadays consumption of mushroom is increased greatly because it has balanced nutritional composition . Especially oyster mushroom plays a major role to overcome protein deficiency. It was first cultivated in Germany. The genus *Pleurotus* includes 40 different types of species. They are commonly known as "Oyster mushrooms". Among these species *Pleurotus ostreatus* is commonly used in this world. Because it has some special and peculiar qualities like good taste, high nutritional values, flavor and also because of its medicinal properties it is mainly used all over the world. And due to this unique property it is said to have antioxidant, anticancer, antidiabetic and antiviral activities. They help humans to protect and reduce their body from the oxidative damages [1]. The nutritive values of mushrooms varies from species to species and it actually depends on their growth requirement. When mushrooms are compared with beef and fresh vegetables it has high water content than the other. Water content in mushroom is around 93-95%, in beef it is about 70% and in fresh vegetables water content is 92%. It also contains high amount of potassium (K), calcium (Ca), iron (Fe) and phosphorous (P). Mushrooms are rich in vitamin B & D. They also possess high amount of proteins with low amount of caloric diet. Chitin and chitosan are the biopolymers that have received much research interest due to its numerous potential applications in agriculture, food industry, biomedicine, paper making and textile industry. Hence the production of chitin and chitosan from fungal mycelium has increased recently due to its significant advantages. For example, while crustacean waste supplies are limited by seasons and sites of fishing industry, fungal mycelium can be obtained by convenient fermentation process that does not have geographic or seasonal limitations [6]. Fungal mycelia have lower level of inorganic materials compared to crustacean wastes [16]; fungal chitin and chitosan are apparently more effective in including the plant immune response and are potentially more suitable for agricultural applications [10].Many fungal species, including *Absidia glauca, Absidia coerulea, Aspergillus niger, Mucor rouxii, Gongronlla butleri, Phycomyces blakesleeanus, Absidia*

blakesleeana, Rhizopus oryzae, Trichoderma reesei and Lentinus edodes have been investigated for the production of chitin and chitosan. The term "chitosan" refers to the group of polycationic polysaccharides with high molecular weight and different viscosities. Nowadays mushrooms are considered nutraceuticals or functional foods due to bioactive nutrient content. According to internal life sciences institute of Europe mushrooms are considered as functional foods since it has been proven that they have both beneficial and nutritional effects on one or more functions of the body, thus improving health, well-being and lowering risk of illness.

Chitin is the second most abundant biopolymer in nature found in shells of crustaceans in the cuticle of insects and fungi cell walls. In the fungi cell walls mushrooms have linear chain polymer composed by 1,4-linked 2-acetamido-2deoxy-beta –D-glucopyranose units and classified as Gamma-chitin [4,3]. Chitin and its derivative chitosan are natural amino polysaccharides, which has unique structures, multi-dimensional properties, highly sophisticated functions and a wide range of applications in biomedical area and other industrial area. The most important characteristics of this biomaterial are excellent biocompatibility, haemostatic activity and biodegradability with ecological safely and low toxicity. In addition chitin and chitosan have versatile biological activities and low immunogenicity [17].

Recently increasing attention has been paid to develop and test coating with antimicrobial properties in order to improve food safety and shelf life. Active biomolecule such as chitosan and its derivatives have a significant role in a food product. Chitosan has a great potential for a wide range of application due to its biodegradability, biocompatibility, antimicrobial activity, non-toxicity and versatile chemical and physical properties.

The present study focused on an ecofriendly method of chitosan extraction from edible fungi, *Pleurotus ostreatus* then the extracted chitosan were characterized and its antimicrobial & antioxidant studies were evaluated.

MATERIALS AND METHODS

Cultivation of Oyster Mushroom

Substrate Preparation and Treatment

The wheat or paddy straw is chopped in 3-5cm long by hand or mechanically as a base. The chopped straw is filled into gunny bags and soaked for 12-24 hours, then the straw is treated in boiled water for 15-25 minutes. This is done to find the capacity of straw to absorb and retain the moisture [19].

Spawn Preparation

10kg of wheat grains are boiled for 15 minutes in 15L of water and then allowed to soak for another 15 minutes without heating. The excess water is drained off and the grains are cooled in sieves. The grains should be turned several times with a spoon for quick cooling. The cooled grains are mixed with the gypsum and 30g of calcium carbonate. The gypsum prevents the grains from sticking together and the calcium Carbonate is necessary to correct the pH. The prepared grains are filled into half liter milk bottles or polypropylene bags (150-200g per bottle or bag) and autoclaved for 2 hours at 121^0 C. After sterilization, the material should have the pH value of 7. The bottles are inoculated with grains and bits of agar medium colonized with mycelium, and then incubated at $22\text{-}24^0$ C in a dark place. [14]. The mycelium completely spreads through the grains about 2 weeks.

Polythene Bag

Transparent polythene bag of 125-150 gauge with a dimension of 80 cm is suitable for Oyster cultivation. Bags of 60 x 40 cm may also be used for this purpose. These bags can be also reused for the second crop after proper cleaning.

Raising of Bags

One end of the bag was tied with the rubber band tightly. And the moistened substrate is put inside the bag with the height of 15 cm. substrate is gently pressed and one part of each spawn (50g) spread at the periphery closed to polythene. For each bag out of 2kg of dry straw, 200g of spawn was used. Likewise 5 to 6 layers were prepared and finally the ends were tied tightly. 15-20 small holes (0.5cm diameter) was made on all sides for the

purpose of gas exchange. This type of spawning is called layer spawning. Then the bags are incubated in a well ventilated room at 25^0 C. And importantly during the mycelial growth bags should not be opened [24]

Harvesting

Premordia (small eggs) appears within 4-5 days of opening the bag that indicates the harvestable stage 3-4 days later. The mushroom should be harvested when the caps begin to fold inwards. Picking is done by twisting the mushroom gently without disturbing the surrounding fruit bodies. Crops should not be watered before harvesting. The second crop appears after 7-10 days. Hence within 45 days crop period, 3-4 crops are expected. This crops are then used for chitosan preparation [7].

Sample Preparation

The mushroom was completely washed in running water and were wrapped in newspaper and stored in moisture free open places. They were air-dried in shade and cut in small pieces of around 2 to 3 cm across using a machete. Then they were ground using metal mortar and pestle. The smashed pieces were further dried at 35ºC for an hour and immediately powdered in a grinder. This powdered mushroom was used for our further studies.

EXTRACTION OF CHITOSAN

Deproteinization

The 10 grams of dried and powdered Oyster mushroom obtained was soaked in boiling 4% sodium hydroxide for 1 hr. The Sample was removed and then allowed to cool at room temperature for 30 minutes.

Demineralization

The sample obtained was demineralized using 1% hydrochloric acid with 4 times its quantity. They were then soaked for 24 hrs to remove minerals. The above samples were treated with 50 ml of 2% sodium hydroxide for 1 hr. The remaining sample were washed with deionized water and then drained off.

Deacetylation

The process was then carried out by adding 50% sodium hydroxide to the obtained sample on a hot plate and boil it for 2 hrs at 100 degree Celsius. The sample was then allowed to cool at room temperature for 30 minutes. Then they were washed continuously with 50% sodium hydroxide. The sample obtained is filtered (chitosan is obtained). The sample was left uncovered, and oven dried for 6 hrs at 110^0 C.

CHARACTERIZATION OF CHITOSAN USING SPECTRAL ANALYSIS TECHNIQUES

UV-Visible Spectroscopy

The sample was subjected to UV-Visible spectroscopy using Perkin Elmer Lambda 35, double beam UV-Visible Spectrophotometer. Distilled water was used as the blank solution and 10% glacial acetic acid was used as the reference solution. The absorbance of the sample was measured in the range of 200-800 nm. The peaks obtained for the sample was compared with Spectral properties of the Standard chitosan and the results were interpreted.

FT-IR Spectroscopy

The prepared biopolymer chitosan was analyzed by shimadzu FTIR 8300 spectrophotometer in the wavelength between 400cm^{-1} and 4000cm^{-1} and in the solid state using potassium bromide pellets.

DPPH radical scavenging activity method

The scavenging activity of mushrooms was estimated by taking an aliquot of 1.5 ml of sample extracts at different concentrations(100, 150, 250mg/ml) were added to test tubes with 3.5 ml of 0.1mM DPPH radical in methanol. The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. The reaction mixture was determined at 515 nm with UV-visible spectrophotometer. Extraction solvent was used as blank while mixture without extract served as control. Ascorbic acid was used as a standard. The scavenging effect was calculated based on the following equation:

Scavenging effect (%) = 1- [(Absorbance sample/ Absorbance control) x 100]

INVITRO ANTIMICROBIAL ACTIVITY

Test Microorganisms

Six bacterial strains used in the present study were *Staphylococcus aureus, Klebsiella pneumoniae, Methicillin Resistant Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and *Acenitobacter baumanii* were maintained in nutrient agar.

Antibacterial Assay

The effect of chitosan extracts from *Pleurotus ostreatus* tested for their antibacterial activity on the several bacterial strains by Agar well diffusion method.

Agar Well Diffusion Method

In this method, the antimicrobials present in the chitosan extracts are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Petri plates containing 20ml Muller Hinton medium were prepared aseptically and seeded with 24hr culture of bacterial strains. Wells were cut with 6mm cork borer and 20 µl of the chitosan crude extracts (100,200 and 300mg/ml in 10% glacial acetic acid) were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well [13].

Result and Discussion

The mushrooms are harvested after 25 days (Plate-1(a-c)) and further processed for extraction of chitosan. *Pleurotus ostreatus* cultivated in more than seventy countries, and it is one of the most commonly and widely consumed mushrooms in the world. The production of biopolymer from fungi depends on its species and culture conditions. Filamentous fungi have been considered an attractive source of chitin and chitosan for industrial application. The synthesis of chitosan involves various chemical steps such as preparation of the chitin from the mycelial mat of *Pleurotus ostreatus* which will be the initiation of the chitosan synthesis, the removal of the proteins in the mycelium followed by demineralization for the removal of the carbon and other salts present in the crude form which will be preceded by the deacetylation of the chitin that would result in chitosan. The regular

chitosan is obtained by following the above steps. The extracted chitosan was characterized based on color, nature, texture and its solubility. The color of the extracted chitosan is pale yellow, hygroscopic in nature and has a flabby texture (Plate 2). They are insoluble or sparingly soluble in water. Chitosan was found to dissolve in 10% acetic acid. The solubility of chitosan in various acid agreed with the observation of Guibal, 2004. The total yield of chitosan from dried powdered mushroom is 0.2 g.

Characterizations of Chitosan

The UV Visible spectrum of the chitosan exhibited maximum absorption at 252nm which indicates the conversion of glucosamine (GlcN) into glucosamine acetate unit (Figure.1). The λ max of chitosan (252 nm) shifts to longer wavelength which indicates the chemical interaction of chitosan and acetic acid even at room temperature. A similar effect was reported by Sharma *et al.*, (2009) who had studied the interaction of chitosan with ammonium sulfate and t-butanol.

Infrared (IR) spectroscopy is one of the most important and widely used analytical techniques to determine their functional group & its structure which is their atomic molecules. The infrared spectrum is commonly obtained by passing infrared electromagnetic radiation through a sample that possesses a permanent or induced dipole moment and determining what fraction of the incident radiation is absorbed at a particular energy. The infrared spectra of the chitosan extracted from *Pleurotus ostreatus* were characterized by three significant amide bands at 1652,and 1568 cm−1, which corresponded to the C O secondary amide stretch (Amide I), C O secondary amide stretch (AmideI), and NH2 deformation, C–N–stretching in secondary, respectively (Figure 2). The absorbance bands of 3430, 2926 and 1416 cm-1 indicated the N-H stretching, OH and CH deformation ring , C-H stretching- alkane groups-CH3,CH2, and N-H Stretch- Primary amine group, respectively. From this data quantitative analyses and structure of the compound can be employed. The fact of certain groups of atoms presenting bands at or near the same frequency and the unique IR fingerprint of molecules with the aid of other techniques, elucidate the structure of a compound and it was similar to the finding of Paulino *et al.*,[15]

Antioxidant Activity

The DPPH method was used to determine the free radical scavenging activity of *Pleurotus ostreatus*. The radical scavenging activity (RSA) of mushroom extracts was tested and the % RSA for *Pleurotus ostreatus* are

presented in (Figure 3). The Antioxidant compounds exists in the sample will transfer electron or hydrogen atom to react with DPPH. The odd electron in DPPH free radical gives a strong absorption maximum at 517nm. The dark purple color is reduced to pale violet color. RSA of mushroom sample for 100mg is 59.09%, 150mg is 65.9%, 250 mg is 79.54%. As the concentration of extract was increased the scavenging activity towards DPPH was also elevated.

The phenolic compounds could be an important indicator of antioxidant capacity [22] And some of them are gentisic acid, gallic acid, caffeic acid, rutin & cinnamic acid [11]. Higher antioxidant activity could be exhibited with the presence of the greater numbers of hydroxyl groups in the phenolic [18]. Consumption of *pleurotus ostreatus* can be beneficial for protecting body from harmful free-radicals compounds which can create oxidative damage to the body.

ANTIMCROBIAL ACTIVITY

In vitro antibacterial screening of chitosan from *Pleurotus ostreatus* against selected clinical isolates were performed and zone of inhibition were given in graph (Figure 4:a-f). The highest zone of inhibition was observed in *Bacillus subtilis, P. aeuroginosa* followed by *K. pneumonia, and Aceintobacter baumanii.* The antimicrobial activity of chitosan and their derivatives against gram positive and gram negative bacteria has received considerable attention in recent years. Several mechanisms are the responsible for the inhibition of microbial cells by chitosan. The interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. It has been demonstrated by electron microscopy that the site of action is the outer membrane of gram-negative bacteria. Recently, the bactericidal effect is also partially mediated by ompA, an outer protein of bacteria that is responsible for cell surface integrity [9]. It is also due to decrease in pH level and change in osmotic pressure in peptidoglycan layer of cell wall, which does cellular destruction and cell lysis. This results also suggest that higher molecular weight of the polymer chitosan shows greater antibacterial activity because positive charge present in amino group of chitosan may interact with the sites on the cell surface causing disturbances in cellular permeability[23].In our present study, the extracted chitosan showed encouraging results against bacterial with maximum inhibitory activity.

Plate 1 (a-c): Cultivation and growth stages of *Pleurotus ostreatus*

(Oyster Mushroom)

(a) Initial formation of fruit bodies

(b) Formation of Mycelium

(c) Mature Mushroom for harvesting

Plate 2: Extracted Chitosan from *Pleurotus ostreatus*

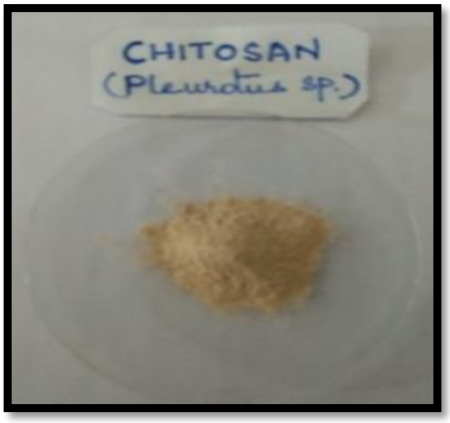


Fig 1: UV Visible Spectral Data of Chitosan from *Pleurotus ostreatus*

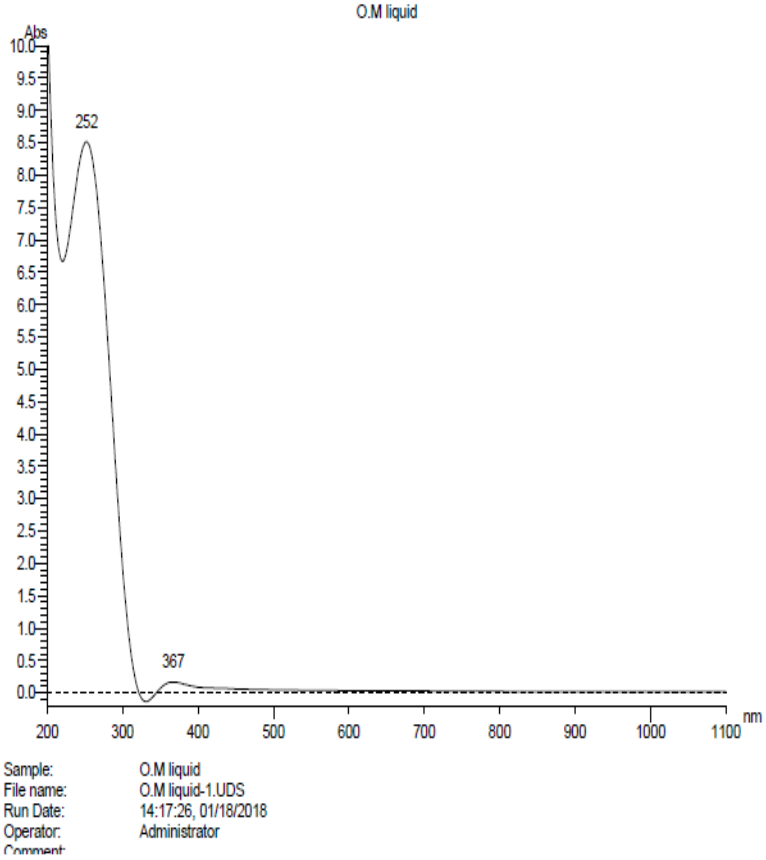


Fig 2: FTIR Spectral Data of Chitosan from *Pleurotus ostreatus*

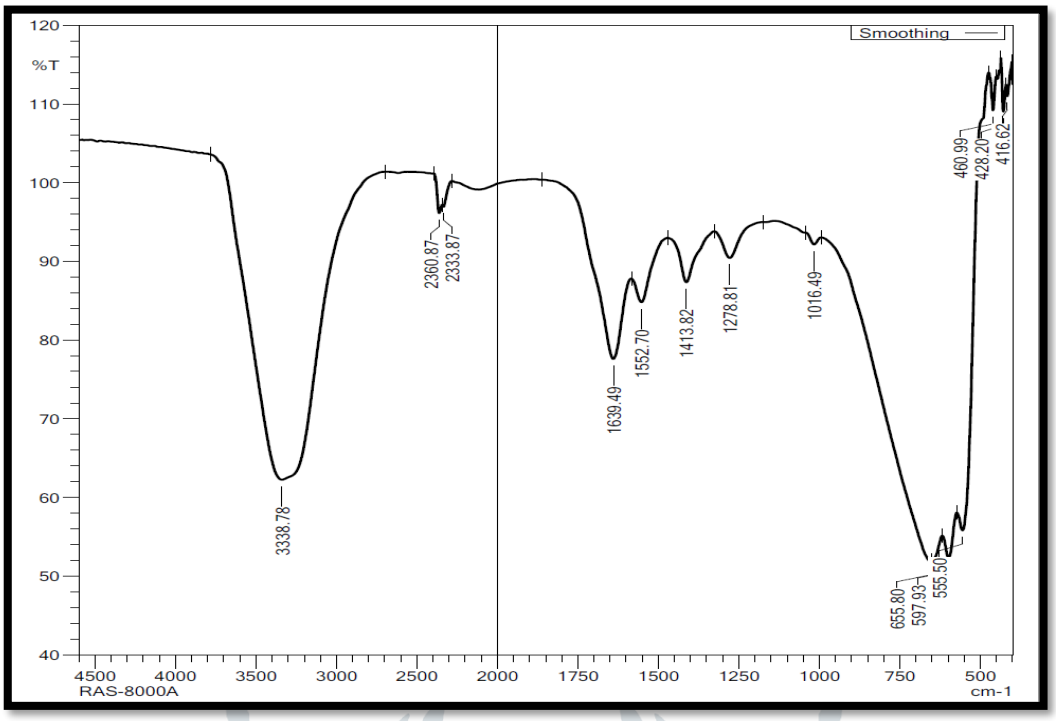


Fig :3 Showing in vitro antioxidant activity by DPPH assay

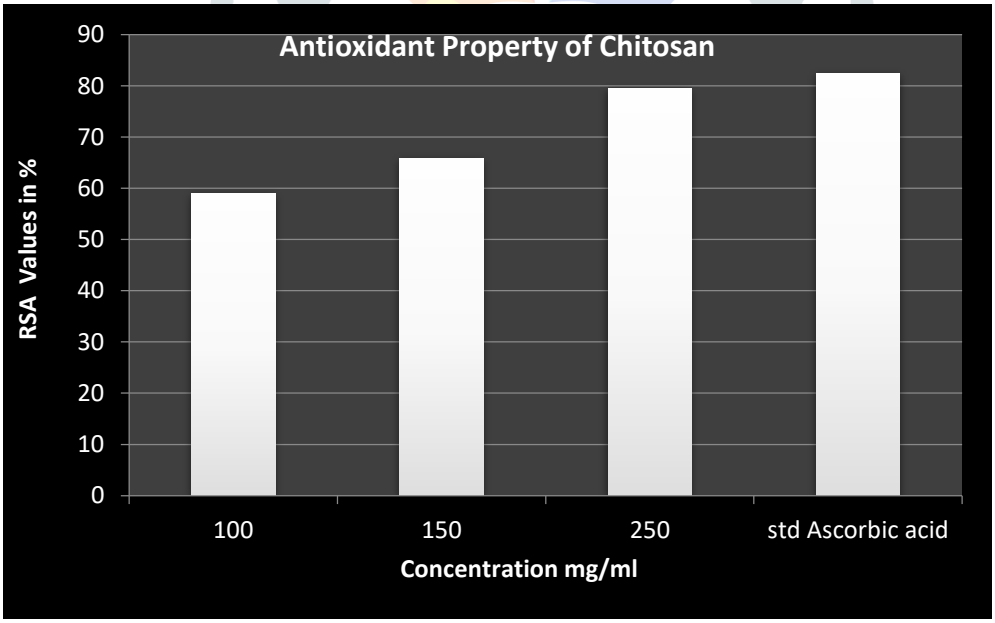
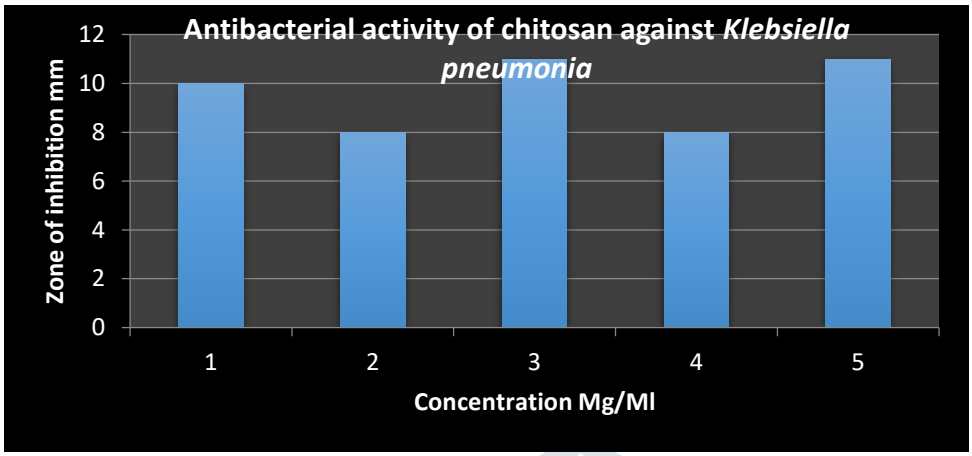
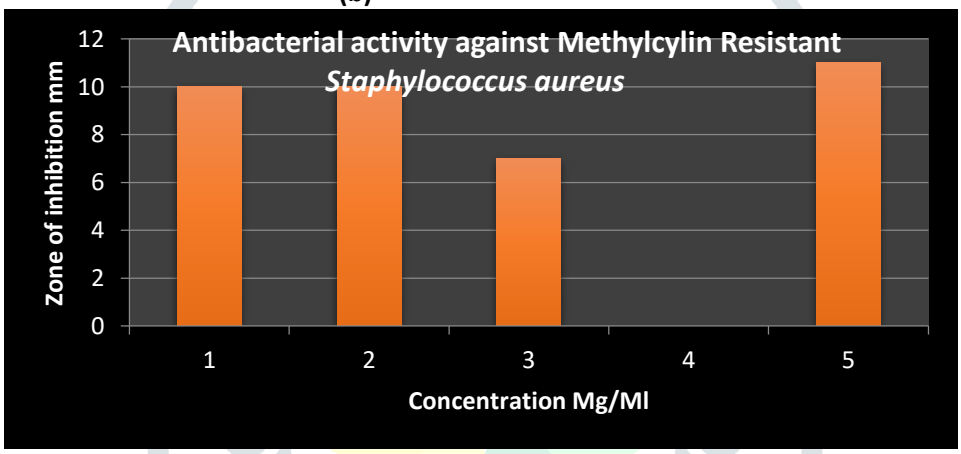


Fig 6 (a-f) : Antibacterial Assay of Chitosan

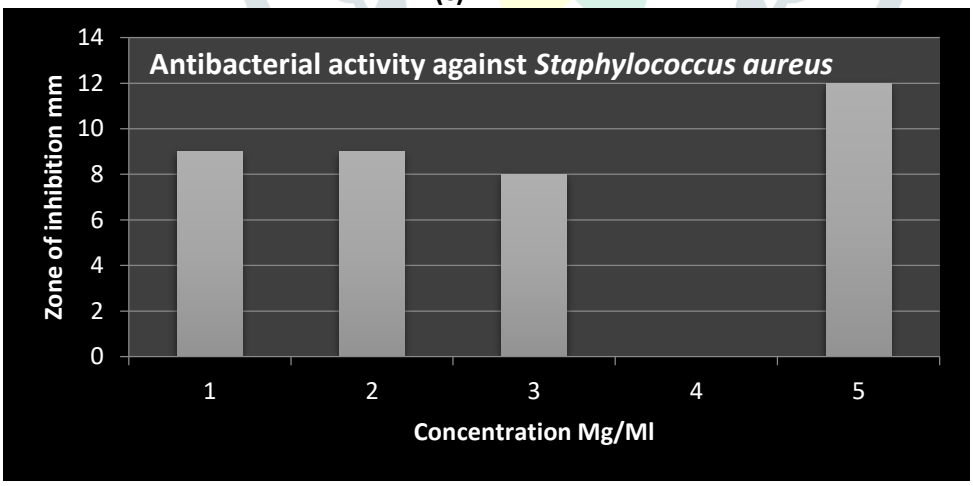
(a)



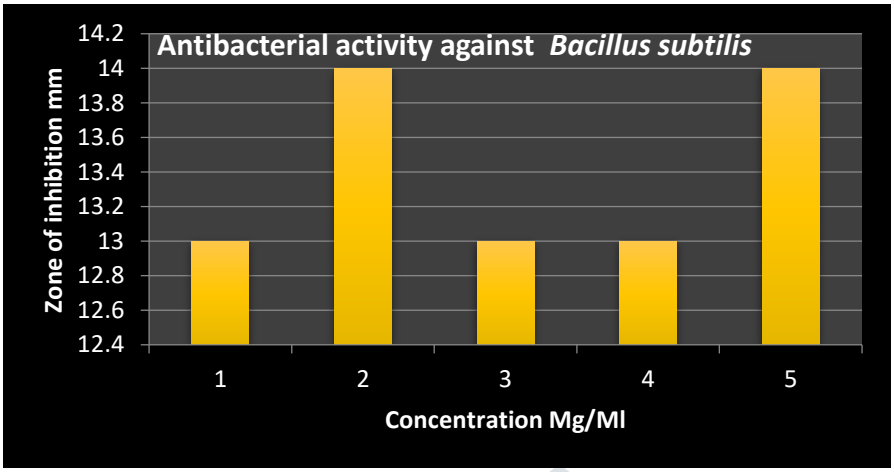
(b)



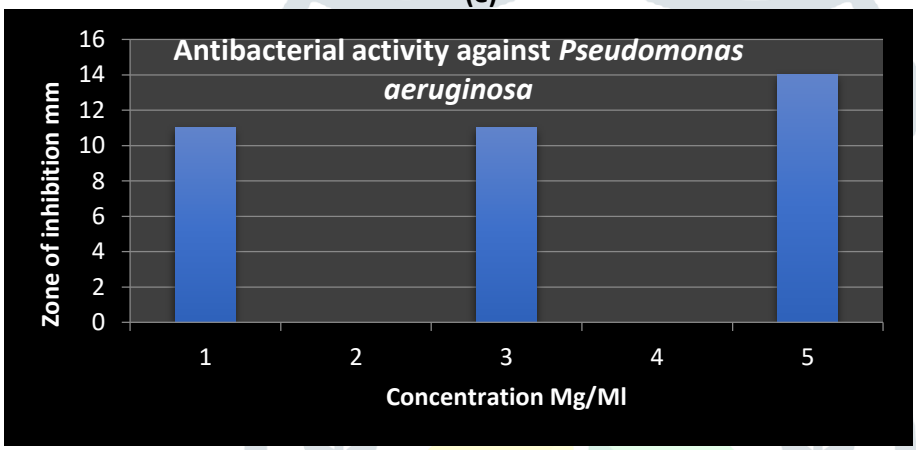
(c)



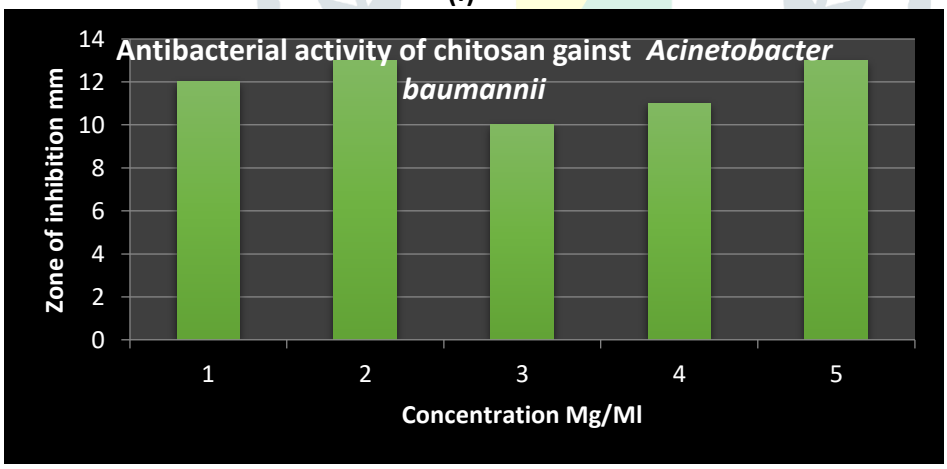
(d)



(e)



(f)



1.100mg/ml 2. 200mg/ml 3.300mg/ml 4.Ab (Amoxycillin 100mg/ml)

5. Control(10%acetic acid)

CONCLUSION

From our study we have concluded that chitosan which was produced from an edible mushroom (*Pleurotus ostreatus*) highly beneficial product. And it also posses some properties such as antioxidants, antibacterial and so on. Due to its peculiar properties it is used mainly in agricultural and medicinal field. Still

further research like nano conversion and chitosan as a synthetic polymers and likewise many researches are also yet to be done.

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