Senthilkumar V et al. / International Journal of Phytopharmacology. **6(4), 2015, 196-202.**

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

EXTRACTION OF CHITOSAN FROM *Aspergillus terreus* sps, STUDY OF ITS ANTIMICROBIAL AND DYE DEGRADATION ABILITY

Senthilkumar V[1], Yesodha P[2], Krishnaveni B*[3]

[1]Head of Department, [2]Assistant Professor, *[3]Ph.D Research Scholar,
Department of Biotechnology, Maharaja Co-education, Arts and Science College, Perundurai, Erode, Tamilnadu, India.

ABSTRACT

Chitosan have wide-scale biomedical applications and are being studied extensively for their antimicrobial and bioremediation ability. *Aspergillus terreus* CBNRKR KF529976, isolated from the marine soils of Pichavaram, Tamil Nadu was used for the economic production of Chitosan using Hesseltine and Anderson medium. The polysaccharides were extracted by alkali-acid treatment, and characterized by infrared spectroscopy. The highest growth rate was with Henderson and Anderson medium with a mycelial dry weight of 12.50g/L. The best yield of the chitosan so obtained is (38.4 mg/g or 3.8%). The antimicrobial activity of Chitosan was tested against *E.coli* and *S.aureus* using Growth kinetics. It was found that the Extracted Chitosan have antimicrobial activity comparable to the Commercial Chitosan as well as the standard antibiotic used. Subsequently the extracted Chitosan was also tested for its photocatalytic ability to degrade dye-methylene blue and was found to exhibit 94% inhibition in 72 hours.

Key words: Chitosan, Polysaccharides, Pichavaram, Antimicrobial, Bioremediation.

INTRODUCTION

Chitosan is a cationic polymer derived by deacetylation of chitin obtained from crustaceans. Chitosan is the second most polymers used in industries after cellulose (Brugnerotto *et al*., 2001). Biodegradable and mucoadhesion properties of chitosan have recently led to increasing interest in the development of slow-release formulations for gastro-retentive drug delivery (Patel *et al*., 2005). Chitosan-based dosage forms of this kind could be useful in relation, *e.g.*, to the administration of antibiotics used for eradication of *Helicobacter pylori* in stomach. The present trend, in industrial applications, however, is toward producing high value products, such as cosmetics, drug carriers, feed additives, semi-permeable membranes, and pharmaceutics. The difference in value between the products and the low-cost polymer is one of the main driving forces pushing studies on new applications of chitosan (Brugnerotto *et al*., 2001).Biotechnology is currently attempting large-scale production of high value bio-products like monoclonal antibodies, Immobilization techniques, *etc*. Previously, it was investigated that in *Aspergillus niger* cell wall constituents, chitin comprises of 42% and also researchers confirmed that the chitosan content of fungi depends on fungal strains, mycelial age, cultivation medium and conditions (Pochanavanich *et al.*,2002). The chitosan quantity also depends on the extraction methods.

The antimicrobial activity of chitosan varies depending on their physical properties (degree of deacetylation (DD), and molecular weight), solvent, microorganism species and source. The antimicrobial activity is reported to vary depending on the methods involved in preparation of different DD and molecular weight of chitosan (Qin *et al.*, 2006; Chen *et al.*, 2002). The antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as the type of chitosan

Corresponding Author

Krishnaveni. B
Email: krishnavenibt88@gmail.com

(e.g., plain or derivative), degree of chitosan polymerization, host nutrient constituency, substrate chemical and/or nutrient composition, and environmental conditions such as substrate water activity (Cuero *et al.*, 1999). In an extensive research on the antimicrobial activity of chitosan prepared from shrimp against *E. coli*, it was found that higher temperature and acidic pH of foods increased the bactericidal effect of chitosan (Abu *et al.*, 2014). The mechanism of chitosan antibacterial action involving a cross-linkage between polycations of chitosan and the anions on the bacterial surface that changes membrane permeability and has been approved as a food additive in Japan and Korea since 1983 and 1995 respectively (Weiner *et al.*,1992).

Dyes are widely used in many industries such as food, textiles, rubber, paper, plastics and so on. About over 7.105 to 10.000 different commercial dyes and pigments are produced annually all around the world. It has been estimated that about 10-15% of these dyes is lost during the dyeing process and released with the effluent (Luo *et al.*, 2011). The discharging of these dyes into water resources even in small amounts can affect aquatic life and the food chain. Dyes can also cause allergic dermatitis and skin irritation. Some of them have been reported to be carcinogenic and mutagenic for aquatic organisms (Dogan *et al.*, 2009). Chitosan is being used as an attractive source of adsorbents. Besides being natural and plentiful, chitosan possesses interesting characteristics that also make it an effective adsorbent for the removal of dye as it has outstanding adsorption capacities. It can be manufactured in the form of films, membranes, fibers, sponges, gels, beads and nano-particles, or supported on inert materials (Crini *et al.*, 2008). This study, aimed the utilization of newer fungal strains to get maximum biomass and excess production of chitosan along with testing its anti-microbial and dye degradation ability.

METHODS AND MATERIALS

Collection of Samples and Isolation of fungi

Pichavaram (Lat.11°428'E; Long.79°798`E), Cuddalore (dt) of Tamil nadu is home to the second largest Mangrove forest in the world, and it is one of the unique eco-tourism spots in South India. Marine Mangrove sediments were collected from rhizosphere as well as non rhizosphere region of various parts of Pichavaram. The surface layer of the sediment was removed and the central portions of sediments were transferred into sterile plastic bags. The samples were taken separately for serial dilution. Ten grams of sample was suspended in 90 ml of sterile distilled water. The suspension was considered as 10^{-1} dilution. About 0.1ml of the serially diluted sample was spread over the Potato Dextrose Agar (Potato Infusion 200, Dextrose 20, Agar 15 g/L) pH was adjusted to 5.6 ± 0.2.The medium was supplemented with 20 µg ml^{-1} Ciproflaxin to minimize the fungal and yeast contaminations respectively . After inoculation, the plates were incubated in an inverted position for 5-7 days at 25 ± 2^0C. The fungal isolates were observed and the colony morphology was recorded with respect to color, shape, size and nature of colony. Fungal isolates were microscopically characterized by Lactophenol Cotton Blue mounting. The cell morphology was recorded with respect to spore chain morphology, hyphae and mycelium structure.

Isolation and Identification of Test Fungus

Individual fungal colonies were picked and further purified by subculturing on potato dextrose agar medium. Further identity of fungus was confirmed by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing using ABI-Big Dye Termintor v3.1 Cycle Sequencing Kit in the ABI 3100 automated sequencer by National Fungal Culture Collection of India (NFCCI), Pune, India. ITS region was amplified by using universal fungal primer set, (Forward Primer) 5'-GACTCAACACGGGGAAACT-3' and (Reverse primer) 5'-AGAAA GGAGG TGATC CAGCC-3'. Polymerase chain reaction amplified regions were sequenced. The analysis of nucleotide sequence was done in Blast-n site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) .The alignment of the sequences was done by using CLUSTALW (www.ebi.ac.uk/clustalw).

Extraction and characterization of Chitosan

Culture medium

A. terreus CBNRKR KF529976 was grown, for chitin and chitosan production, in the following culture media: Hesseltine and Anderson (HA medium)- (glucose (40 g); asparagine (2 g); chloridrate of thiamine (0.05 mg); potassium phosphate (0.50 g) and magnesium sulphate (0.25 g) per litre of distilled water, pH 5.2)

Microbiological methods

Growth profile. The sporangioles of *A. terreus* CBNRKR KF529976 were harvested from cultures grown for seven days at 28ºC on Petri dishes containing PDA medium. A suspension was prepared and adjusted to10^8 sporangioles/mL, using a hematocytometer for counting. For fungal submerse cultivation, 10 mL sporangioles suspension (10^8sporangioles/ mL) were inoculated in Erlenmeyer flask of 1000 mL containing 290 mL of culture media, and the flasks were incubated at 28ºC in an orbital shaker at 150 rpm, during 96 hrs. The mycelia were harvested, washed twice in distilled and deionised water by filtration, utilizing a silkscreen nylon membrane (120 F), and were submitted to lyophilization process. After lyophilization the biomass was maintained in a vacuum dissecator until constant weight.

Chitosan extraction. The process of extraction involved deproteination with 2% w/v sodium hydroxide solution (30:1 v/w, 90ºC, 2 hrs), separation of alkali insoluble fraction (AIF) by centrifugation (4000 rpm,15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid 40:1 v/w, 60ºC, 6 hrs), separation of crude chitin by centrifugation (4000 xg, 15 min) and precipitation of chitosan from the extract at pH 9.0, adjusted with a 4 M NaOH solution. Crude chitin and chitosan were washed on a coarse sintered-glass funnel with distilled water, ethanol and acetone and air-dried at 20ºC.

Chitin and chitosan characterization

Infrared spectroscopy (Deacetylation degree – DD %).

The degree of deacetylation for microbial Chitosan was determined using the infrared spectroscopy according to Roberts et al., 1992 using the absorbance ratio A1655/A3450 and calculated:

A (%) = (A1655/A3450) x 100 / 1.33

Two milligrams sample of fungal chitin and chitosan, which had been dried overnight at 60ºC under reduced pressure were thoroughly blended with 100 mg of KBr, to produce 0.5 mm thick disks. The disks were dried for 24 hrs at 110ºC under reduced pressure. Infrared spectrometer was recorded with a Bruker 66 Spectrometer, using a 100 mg KBr disks for reference at STIC, Cochin University of Science and Technology. The intensity of maximum absorption bands were determined by the baseline method.

Comparative study of antimicrobial activity of Chitosan

Addition of Chitosan

3% (v/v) 100 ml acetic acid preparation: 3ml concentrated (99%) acetic acid was taken into a conical flask and made up to 100ml volume mark by distilled water.

1.5gm chitosan was taken into two test tube (sterile) and 10ml 3% acetic acid was poured into it gradually. To increase the solubility the solution stirred and heat was also applied in water bath at 40º C. The solution of chitosan was then added into the test tubes. The upper soluble portion of each sample was added with medium, we did not take the supernatant from the test tube. 0.5 ml of commercial chitosan solution and 0.5 ml of extracted chitin and chitosan was taken by micropipette and added to the respective test tubes. Both for chitosan, from these test tubes one was used as standard (media), one for negative control (media+commercial chitosan CCS), one for gram negative bacteria inoculation (media+commercial chitosan+ gram negative), and one for gram positive bacteria inoculation (media+commmecial chitosan+ gram positive). Of the remaining test tubes one for (media+ extracted chitosan ECS+ gram negative) and other for (media+ extracted chitosan+ gram positive).More two tubes were used for (media+antibiotic disk+gram negative) and (media+ antibiotic disk+ gram positive).

Incubation

After successful inoculation we incubated the test tubes in an incubator, and the temperature was set at 37 ^{o} C. After each 4 hours later we took the turbidimetric measurement by spectrophotometer.

Photo catalytic Degradation of Dye

Typically 10mg of Methylene Blue dye was added to 1000 mL of double distilled water used as stock solution. About 10 mg of extracted Chitosan was added to 100 mL of dye solutions. A control was also maintained without addition of silver nanoparticles. Before exposing to irradiation, the reaction suspension was well mixed by being magnetically stirred for 30min to clearly make the equilibrium of the working solution. Afterwards, the dispersion was put under the sunlight and monitored from morning to evening sunset. At specific time intervals, aliquots of 2-3 mL suspension were filtered and used to evaluate the photocatalytic degradation of dye. The absorbance spectrum of the supernatant was subsequently measured using UV-Vis spectrophotometer at the different wavelength. Concentration of dye during degradation was calculated by the absorbance value at 660 nm.

Percentage of dye degradation was estimated by the following formula:

$$\% \text{ Decolourization} = 100 \times [(C_0 - C)/ C_0]$$

Where C_0 is the initial concentration of dye solution and C is the concentration of dye solution after photocatalytic degradation.

RESULTS AND DISCUSSION

Morphological identification of the fungal isolates obtained from the soil sample

The isolated fungi were purified by repeated sub-culturing on the Potato Dextrose Agar medium at regular intervals and incubating at 29°C. The isolates were identified based on the colony morphology, microscopic observation and molecular identification. The identification was done based on 18S rRNA gene sequencing. The 18S rRNA sequences of the isolates were compared with the data present in NCBI. The BLASTn of the isolates was showing 100% homology with *Aspergillus* spp. The sequence was submitted to the Gene Bank under the accession number KF529976.

Extraction of Chitosan

Biomass Production

The growth of the fungus *A. terreus CBNRKR* KF529976 in Henderson and Anderson medium was

observed for 14days at RT. The growth rate was with Henderson and Anderson medium with a mycelial dry weight of 12.50g/L. The result is also in agreement with the growth curve of *C. elegans* (IFM 46109) established by Andrade *et al*. 2000 and Franco *et al.* 2005 using the same culture medium, which referred biomass yield of 11.0 and 11.6 g/L, respectively.

Chitosan Extraction

The best yields of the polysaccharides (mg per gram of dry mycelia biomass) are obtained with Henderson and Anderson medium for chitosan (38.4 mg/g or 3.8%).The best yield of chitosan per 1 g of biomass from *C. elegans* (UCP 542) are obtained Sabouraud sucrose medium for chitosan 58.9 mg/g respectively.

Chitosan characterization

Infrared spectroscopy

In this study, the IR spectra of the three isolated samples of chitosan were analyzed and compared with the IR spectrum of commercial chitosan (Figure. 1).

Chitosan samples from HA medium show bands at 3438 cm^{-1} indicating strong dimeric OH stretch. The next band at 1640 cm^{-1} indicates the presence of Amide Region I. The peaks around 1559 cm^{-1} are due to stretching vibrations of C-O group (Amide II).The bands at 1410 cm-1 indicate strong presence of aromatic C-C stretch. Andrade et al. 2000; Amorim *et al.* 2001; Franco *et al.* 2005 indicated that the most significant parts of chitin and chitosan spectra are those showing the amide bands at approximately 1665 cm^{-1}, 1555 cm^{-1}, 1313 cm^{-1}, which could be assigned to the C = O stretching, the N-H deformation in the CONH plane and the CN bond stretching plus CH wagging.

Deacetylation degree – DD %

In the present study Chitosan grown in Hesseltine and Anderson medium were found to have 20% DD. Amorim *et al.* 2001, Pochanavanich and Suntornsuk 2002 and Franco *et al.* 2004, reported deacetylation degree of chitosan from fungi between 80 to 90% DD.

Comparative study of antimicrobial activity of Chitosan

The recorded absorbance for *S. aureus* and *E. coli* are given in the following tables (Table 1). Commercial Chitosan (CCS) and Antibiotic (Erythromycin) showed higher absorbance than the Extracted Chitosan (ECS) when tested against *E.coli*. (Table 2, Figure 3).Similar results were observed in the case of *S.aureus* where Commercial Chitosan (CCS) and Antibiotic (Erythromycin) showed higher absorbance than the Extracted Chitosan (ECS) (Table 3, Figure 4). Similar results were obtained by Abu Tareq et al. 2013 indicating that *S. aureus* with chitosan recorded 0.28 and chitin with *S.aureus* found 0.64. So chitosan is about 2.2 times more active against *S.aureus* than chitin. While chitosan is about 3.0 times more active against *E.coli* than chitin. Masihul *et al.*, 2012 reported that chitosan and vancomycin together showed slightly raised antibacterial effect against gram negative *E. coli*, the difference between antibacterial activity against both the gram positive *S. aureus* and gram negative *E. coli* very little as negligible.

Photocatalytic Degradation of Dye

Visual Observation

Photocatalytic degradation of methylene blue was carried out by using extracted Chitosan under solar light. Dye degradation was initially identified by color change. Initially, the color of dye shows deep blue color changed into light blue after the 1 h of incubation while exposed to solar light. Thereafter light blue was changed into light sheen. Finally, the degradation process was completed at 72 h and was identified by the change of reaction mixture color to colorless.

UV-Vis Spectrophotometer

Photocatalytic activity of extracted Chitosan on degradation of dye was demonstrated by using the dye methylene blue, at different time in the visible region. The absorption spectrum showed the decreased peaks for methylene blue at different time intervals. The percentage of degradation efficiency of Chitosan was calculated as 94% at 72 hrs. The degradation percentage was increased as increasing the exposure time of Dye-Chitosan complex in sunlight (Table 4). Absorption peak for methylene blue dye was centered at 660 nm in visible region which diminished and finally it disappeared while increasing the reaction time, which indicates that the dye had been degraded. Similar results were reported by Vanaja *et al.*, 2014 wherein the degradation efficiency for silver nanoparticles synthesized from *Morinda tintoria* was found to be 95.3% at the end of 72 hours incubation.

Table 1. Determination of O. D Value Standards (4h-24h)

Time in hours	Media	Media+Chitosan
4	Nil	Nil
8	Nil	Nil

12	Nil	Nil
16	Nil	Nil
20	Nil	Nil
24	Nil	Nil

Table 2. Determination of O. D Value for *E.Coli* (4h-24h)

Time in hours	Media+CCS+*E.coli*	Media+ECS+*E.coli*	Media+AB+*E.coli*
4	0.735	0.609	0.834
8	0.831	0.832	0.913
12	0.974	0.841	0.929
16	1.046	0.862	1.031
20	1.095	0.943	1.106
24	1.110	1.151	1.214

Table 3. Determination of O. D Value for *S.aureus* (4h-24h)

Time in hours	Media+CCS+ *S.aureus*	Media+ECS+ *S.aureus*	Media+AB+ *S.aureus*
4	0.832	0.818	0.845
8	1.176	0.858	0.920
12	1.189	0.874	0.955
16	1.295	1.012	1.021
20	1.344	1.041	1.034
24	1.366	1.225	1.045

Table 4. Methylene blue degradation (%) by extracted Chitosan (10 mg)

Exposure Time	Amount of dye degradation (%)
1h	1.45
2h	2.09
3h	4.6
4h	9.5
21h	18.2
22h	19
23h	20.1
24h	36.3
41h	41.4
42h	47.5
44h	49.2
45h	57
46h	59
48h	60.1
65h	67
66h	73.5
70h	85
71h	90.2
72h	94

Fig 1. FTIR Spectrum of Chitosan extracted from *A.terrues* CBNRKR KF529976

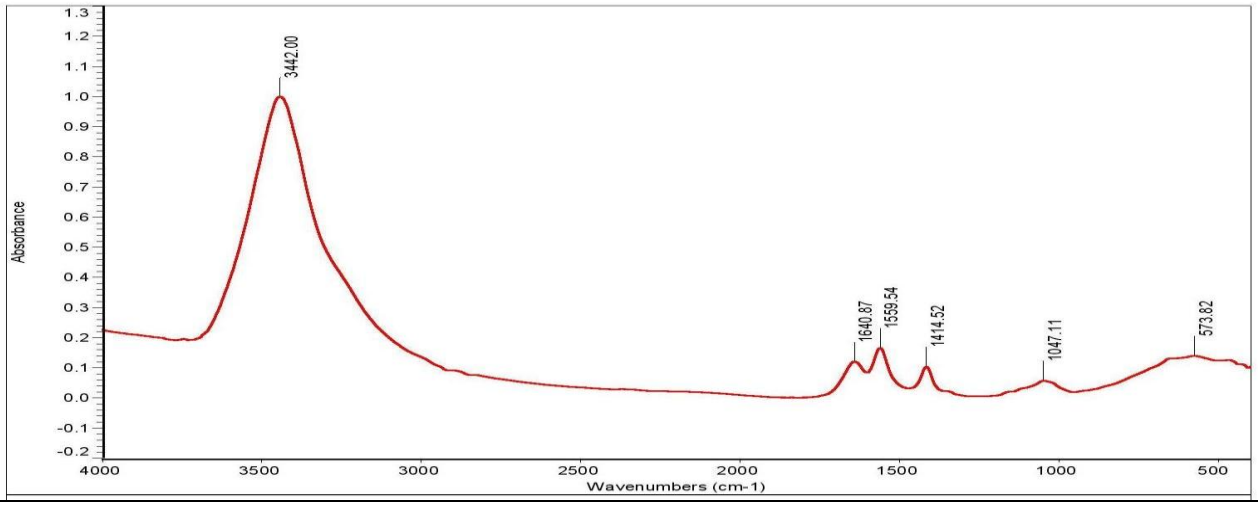


Fig 2. Comparative study of antimicrobial activity of Chitosan against *E.coli*

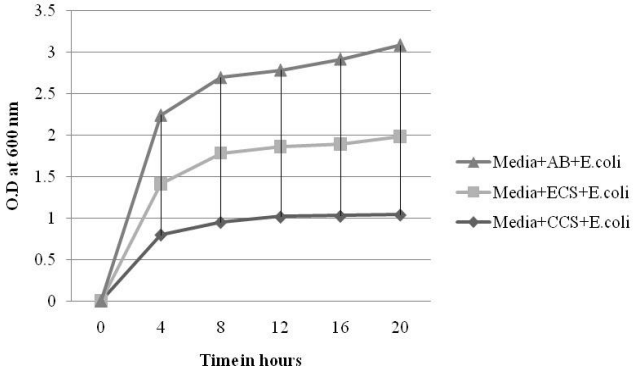
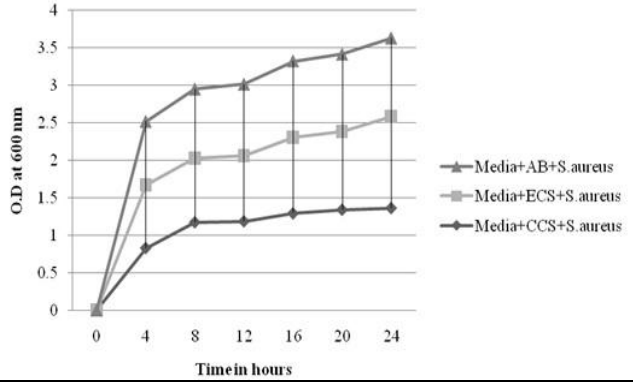


Fig 3. Comparative study of antimicrobial activity of Chitosan against *S.aureus*



CONCLUSION

Fungi are abundantly available plethora of microbial sources which can be used for producing industrially important secondary metabolites. The present study aimed to work on developing viable methodology for extraction of Chitosan and thereby exploit its potential applicability as antibiotic and in bioremediation.

ACKNOWLEDGEMENT: None.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Abu Tareq M, Masihul Alam M, Salim Raza M, Tanvir Sarwar Z. Comparative study of antibacterial activity of chitin and chemically treated chitosan prepared from shrimp (*Macrobrachium rosenbergii*) shell waste. *International Journal of Virology and Microbiology*, 2013, 1-9.

Amorim R, *et al*. Faster chitosan production by Mucoralean strains in submerged culture. *Brazilian Journal of Microbiology*, 32, 2001, 20-23.

Amorim RVS, *et al*. Screening of chitin deacetylase from Mucoralean strains (Zygomycetes) and its relationship to cell growth rate. *Journal of Industrial Microbiology and Biotechnology*, 32, 2005, 19-23.

Andrade V, *et al*. Effect of medium components and time of cultivation on chitin production by *Mucor circinelloides* (*Mucor javanicus* IFO 4570) - A factorial study. *Revista Iberoamericana de Micología*, 20, 2003, 149-153.

Andrade VS, *et al*. A factorial designs analysis of chitin production by *Cunninghamella elegans*. *Canadian Journal of Microbiology*, 46, 2000, 1042-1045.

Brugnerotto J, Lizardi J, Goycoolea F M, Arguelles Monal W, Desbrieres J and Rinaudo M. An infrared investigation in relation with chitin and chitosan characterization. *Polymer*, 42, 2001, 3569-3580.

Chen Y M, Chung YC, Wang LW, Chen KT, Li SY. Antibacterial activity of chitosan- based matrixes on oral pathogens. *Health A Tox Hazard Subst Environ Eng. J. Environ Sci*, 37, 2002, 1379-1390.

Collier, L., Balows, A and M. Sussman. *Topley & Wilson's Microbiology and Microbial Infections*, Arnold, London, Sydney, Auckland, New York, 1998.

Crini G. Application of chitosan, a natural aminopolysaccharide process using batch studies: A review of recent literature. *Prog. Polym. Sci*., 33, 2008, 399.

Cuero RG. Antimicrobial action of exogenous chitosan. *EXS*, 87, 1999, 315-333.

Del Blanco LF, *et al*. Influence of the deacetylation degree on chitosan emulsification properties. *Colloid Polymer Science*, 277, 1999, 1087-1092.

Dogan M. Adsorption of methylene blue onto hazelnut shell: Kinetics, mechanisms and activation parameters. *J. of Hazard. Mater*, 164, 2009, 172.

Franco L, *et al*. Heavy metal biosorption by chitin and chitosan isolated from *Cunninghamella* elegans (IFM 46109). *Brazilian Journal of Microbiology*, 35, 2004, 243-247.

Franco LD, *et al. Cunningamella elegans* (IFM 46109) como fonte de quitina e quitosana. *Revista Analytica*, 4, 2005, 40-44.

Luo X, *et al*. Removal of water-soluble acid dyes from water environment using a novel magnetic molecularly imprinted polymer. *J. of Hazard. Mater,* 187, 2011, 274.

Masihul Alam M, *et al*. Comparative study of antibacterial activity of narrow-spectrum antibiotic and chemically treated chitosan prepared from giant freshwater prawn waste. *As. J. Food Ag-Ind.*, 5, 2012, 540-546.

Patel JK, Patel RP, Amin AF and Patel MM. Formulation and evaluation of mucoadhesion glipizide microsphere. *AAPS Pharm Sci Tech*, 6, 2005, E49-E55.

Pochanavanich P. Fungal chitosan production and its characterization. *Letters in Applied Microbiology*, 35, 2002, 17-21

Qin CQ, Li HR, Xiao Q, Liu Y, Zhu JC, Du YM. Water solubility of chitosan and its antimicrobial activity. *Carbohydrate Polymers*, 63, 2006, 367-374.

Rashna Gulshan MD, *et al*. Comparative study of antibacterial activity of vancomycin and chemically treated chitosan prepared from shrimp (*Macrobrachium rosenbergii)* waste, International Journal of Nutrition and Food Sciences, 2 (6), 2013, 307-311.

Roberts, George AF. Chitin Chemistry. London, MacMillan Press, 1992, 352.

St-Germain G and Summerbell R. Identifying Filamentous Fungi - A Clinical Laboratory Handbook, 1st ed. Star Publishing Company, Belmont, California, 1996.

Vanaja M, *et al*. Degradation of Methylene Blue Using Biologically Synthesized Silver Nanoparticles, Bioinorganic Chemistry and Applications, 742346.

Weiner ML. An overview of the regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredients, 1992.