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Extraction of Chitosan from *Bionectria sps,* Study of its Bioactivity and Dye Degradation ability

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Abstract

Recent advances in fermentation technology have led to new innovative techniques to obtain useful by-products from various soil microbes. Bionectria CBNR KRRR, 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 6.45g/L. The best yield of the chitosan so obtained is (31 mg/g or 3.1%). 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 93.5% inhibition in 72 hours.

Keywords: Chitosan, polysaccharides, pichavaram, antimicrobial, bioremediation.

Introduction

Chitosan is a natural polymer derived from chitin, the principal fiber component of the exoskeleton of shellfish. It is a polysaccharide formed primarily by repeated units of ß (1-4) 2amino-2-deoxy-D-glucose (or D-glucosamine). Chitosan in cell walls is produced through enzymatic deacetylation of chitin. Deacetylation is a common step in the modification of sugar chains, which may confer resistance to lysozyme action[1]. Chitosan has great potential in agriculture, medicine, biotechnology and pharmaceutical industries. The development of applications for chitosan in drug delivery has expanded rapidly in recent years[2-4]. The process of producing commercial chitosan involves several steps, such as, taking the shells of crustaceans, grinding them to a fine powder and deacetylating this powder using strong alkali[5]. This chemical approach appears to have a limited potential in production of chitosan for medical applications due to the inappropriate physicochemical properties. Therefore, the use of *Zygomycetes* mycelia has been proposed as a good alternative source of chitosan.

The antimicrobial activity of chitosan is described to be associated with molecular weight, degree of acetylation, concentration of chitosan and bacterial inoculum size was described[6,7] . It is reported that lower molecular weight chitosan is more effective against Gram-negative bacteria, whereas high molecular weight chitosan is effective against Gram-positive bacteria in atomic force microscopy of cell wall structure and nanoindentation study. The charge density on the cell surface is a determinant factor to establish the amount of adsorbed chitosan. More adsorbed chitosan would evidently result in greater changes in the structure and in the permeability of the cell membrane. This would suggest that the antibacterial mode of action is dependent upon the host microorganism[8].

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[9]. The discharging of these dyes into water resources evn 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[10]. 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[11]. 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.

Material and Methods

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. This area is rich in *Avicennia officinalis, Rhizophora mucaronata, Acanthus illicifoliu* and *Excoecaria agallocha* plants. 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. The alignment of the sequences was done by using CLUSTALW.

Extraction and characterization of Chitin and Chitosan: Culture medium: *Bionectria CBNR KRRR* was grown, for 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 *Bionectria CBNR KRRR* were harvested from cultures grown for seven days at 28ºC on Petri dishes containing PDA medium. A suspension was prepared and adjusted to 10^8 sporangioles / mL, using a hematocytometer for counting. For fungal submerse cultivation, 10mL sporangioles suspension (10^8 sporangioles / 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.

Chitin and 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 using the absorbance ratio A1655/A3450 and calculated according to equation[12]:

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 98% homology with *Bionectria* spp. The sequence was submitted to the Gene Bank under the accession number KF680540. To analyze the phylogenetic position of the 18S rRNA sequence. The phylogenetic tree was constructed using Mega5 by neighbor-joining tree using Kimura-2-parameter with 1000 bootstrap replication. The phylogentic relation was determined.

Extraction and characterization of Chitin and Chitosan: Biomass Production: The growth of the fungus *Bionectria CBNR BKRR* in three different media was observed for 14days at RT. The highest growth rate with Henderson and Anderson medium was found to be 6.45 g/L. This result is similar to the reported by Synowiecki et al., which obtained a yield biomass of *Mucor rouxii* grown in yeast extract and glucose 2% medium, for 48 hrs, to the 4 g, per litre of medium [13].

Chitin and Chitosan Extraction: The best yields of the polysaccharides (mg per gram of dry mycelia biomass) obtained with Henderson and Anderson medium was found to be 31 mg/g or 3.1% respectively. Thayza et al. reported that the best yields of the polysaccharides (mg per gram of dry mycelia biomass) are obtained with 48 hrs of culture for chitosan (66 mg/g or 6.6%) and with 72 hrs for chitin (440 mg/g or 44%) [14].

Chitosan characterization: **Infrared spectroscopy:** In this study, the IR spectra of the isolated sample of chitosan were analyzed and compared with the IR spectrum of commercial chitosan.

Chitosan samples from HA medium show bands at 3416 cm^{-1} indicating strong dimeric OH stretch. The next band at 1631 cm^{-1} indicates the presence of Amide Region I. The peaks around 1572 cm^{-1} are due to stretching vibrations of C-O group (Amide II). The bands at 1412 cm-1 indicate strong presence of aromatic C-C stretch. Andrade et al.; Amorim et al.; Franco et al. 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[15-17].

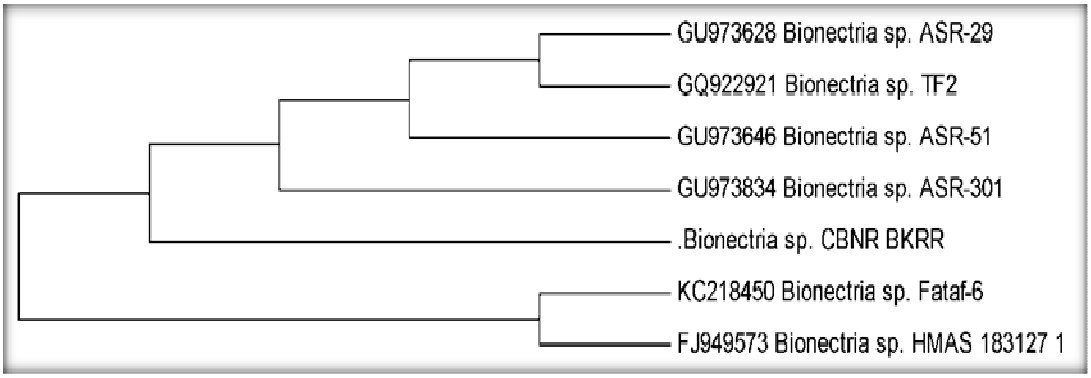

Figure-1
Phylogenetic tree of Bionectria CBNR BKRR showing homology with *Bionectria* spp

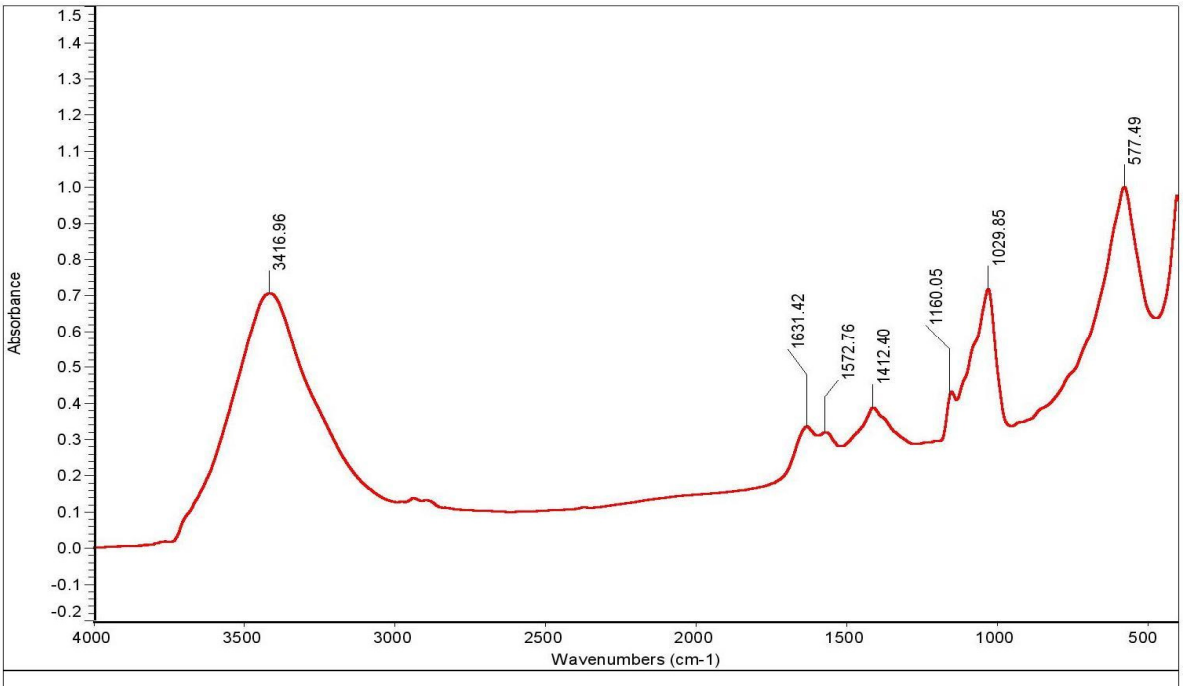

Figure-2
FTIR Spectrum of Chitosan extracted from *Bionectria* CBNR BKRR

In the present study Chitosan grown in Hesseltine and Anderson medium were found to have 27% DD. Amorim et al.; Pochanavanich and Suntornsuk, and Franco et al. reported deacetylation degree of chitosan from fungi between 80 to 90% DD[18,17].

Comparative study of antimicrobial activity of Chitosan: The recorded absorbance for *S. aureus* and *E. coli* are given in the tables-1, 2, 3.

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	0.702	0.739	0.891
8	0.791	0.808	1.355
12	0.807	1.048	1.454
16	0.881	1.062	1.512
20	0.937	1.119	1.528
24	0.995	1.224	1.616

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	0.663	0.324	1.434
8	0.791	0.952	1.443
12	0.823	1.102	1.478
16	0.885	1.161	1.484
20	0.905	1.214	1.495
24	1.160	1.225	1.574

The O.D values measured for Commercial Chitosan (CCS) and Extracted Chitosan (ECS) showed lower absorbance than the Antibiotic (Erythromycin) when tested against *E.coli.*.The O.D values measured for Commercial Chitosan (CCS) and Antibiotic (Erythromycin) showed higher absorbance than the Extracted Chitosan (ECS) when tested against *S.aureus.* Masihul et al., 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[19]. Similar results were obtained by Abu Tareq et al., 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[20].

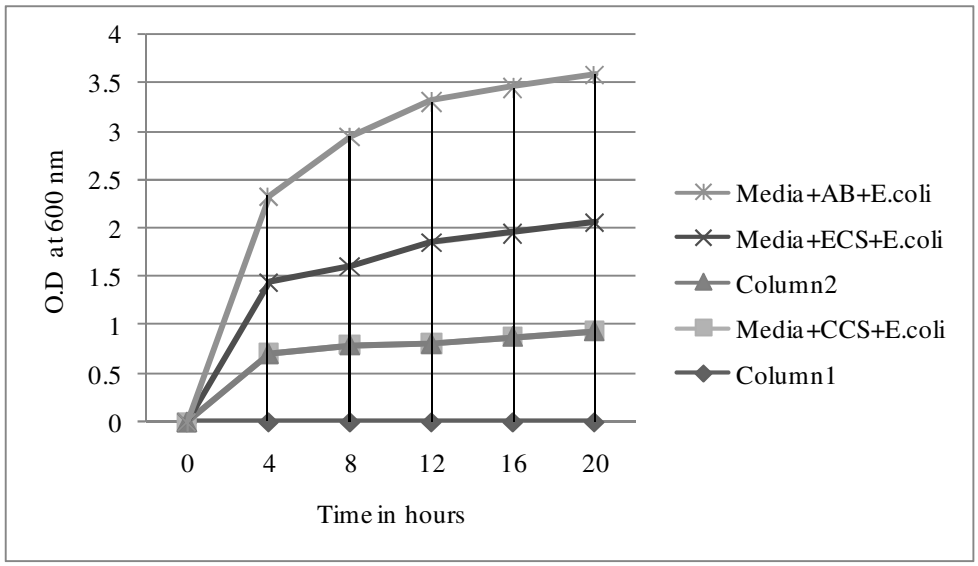

Figure-3
Comparative study of antimicrobial activity of Chitosan against E.coli

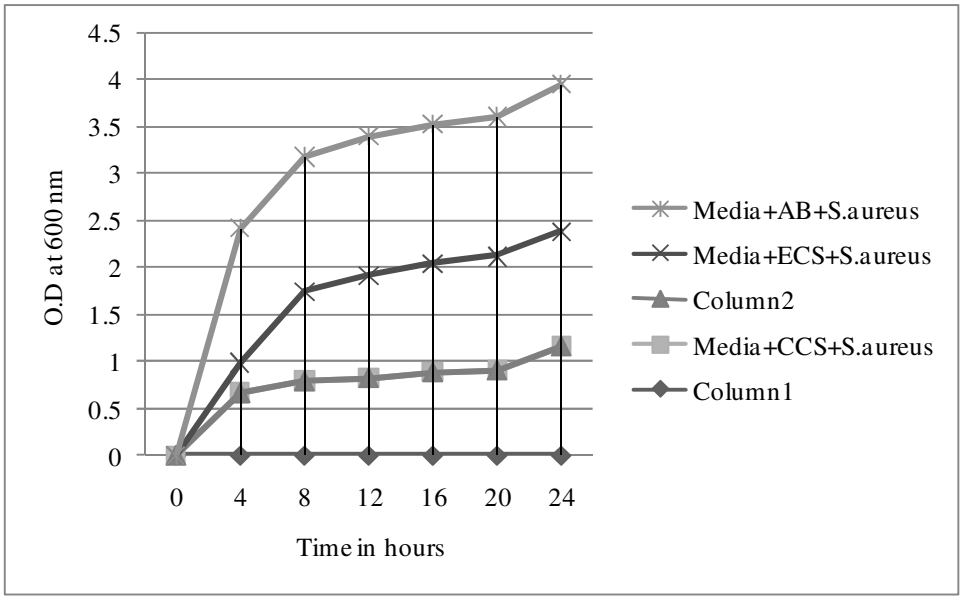

Figure-4
Comparative study of antimicrobial activity Chitosan against S.aureus

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 of blue. 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 93.5% at 72 hrs. Absorption peak for methylene blue dye was centered at 660 nm in visible region which diminished and finally it disappeared 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 [21].

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

Exposure Time	Amount of dye degradation (%)
1h	1.56
2h	2.76
3h	4.5
4h	10.5
21h	17.2
22h	18.5
23h	21.4
24h	35.3
41h	46.4
42h	49.5
44h	51.3
45h	56.4
46h	58
48h	61.4
65h	70
66h	75
70h	89
71h	91.2
72h	93.5

Conclusion

Fungi are abundantly available sources for the production of industrially important secondary metabolites. These results present an economically viable methodology for production of the polysaccharides-Chitin and Chitosan from marine fungi and have potential application as antibiotics and in bioremediation.

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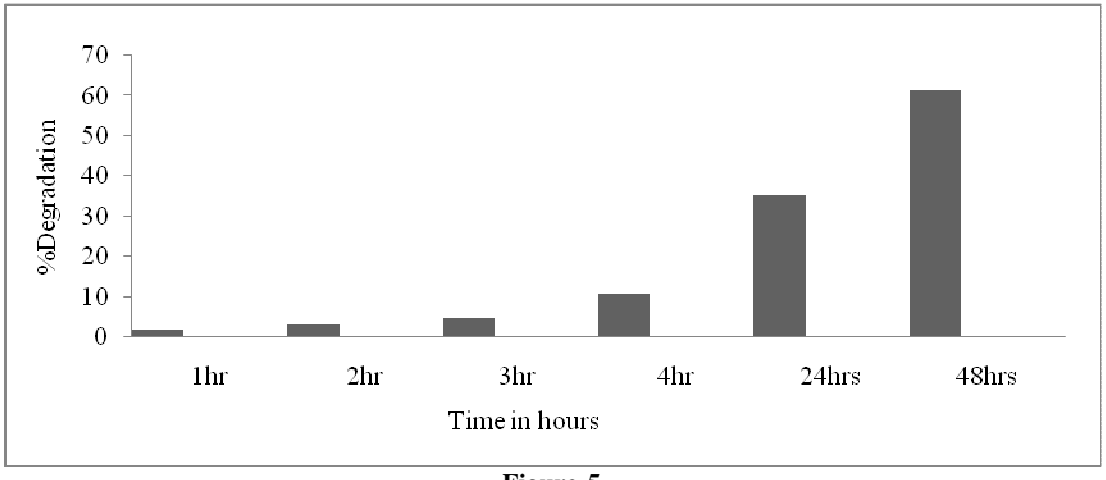

Figure-5
Methylene blue degradation (%) by extracted Chitosan (10 mg)

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