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Research article

CHITOSAN EXTRACTION FROM *F.solani sps,* STUDY OF ITS ANTIBACTERIAL 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. *F.solani 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 14 g/L. The best yield of the chitosan so obtained is (33.57 mg/g or 3.3%). 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 94.5% 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 (Muzzarelli 1977). 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.

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 (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).

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 at 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. 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 Chitin and Chitosan

Culture medium

F.solani 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 *F.solani 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, 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.

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 (Roberts, 1992).

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:

% 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 99% homology with *Fusarium* spp. The sequence was submitted to the Gene Bank under the name *Fusarium solani CBNR BKRR* and is awaiting accession number.

Extraction and characterization of Chitin and Chitosan

Biomass Production

The growth of the fungus *F.solani 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 14 g/L. This result is similar to the reported by Synowiecki et al., 1997 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.

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 33.57 mg/g or 3.3% respectively. Thayza et al., 2007 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%).

Chitosan characterization

Infrared spectroscopy

In this study, the IR spectra of the isolated sample of chitosan were analyzed (Figure.1) .Chitosan samples from HA medium show bands at 3442.59 cm^{-1} indicating strong dimeric OH stretch. The next band at 1657 cm^{-1} indicates the presence of Amide Region I. The peaks around 1558 cm^{-1} are due to stretching vibrations of C-O group (Amide II).The bands at 1378 cm^{-1} indicate strong presence of aromatic C-H 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
40 % DD. Amorim et al., 2001; Pochanavanich and Suntornsuk 2002, and Franco et al., 2005 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, 2, 3).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 Extracted Chitosan (ECS) showed lower absorbance than Antibiotic (Erythromycin) when tested against *S.aureus.* 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. 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 (Figure.2, 3).

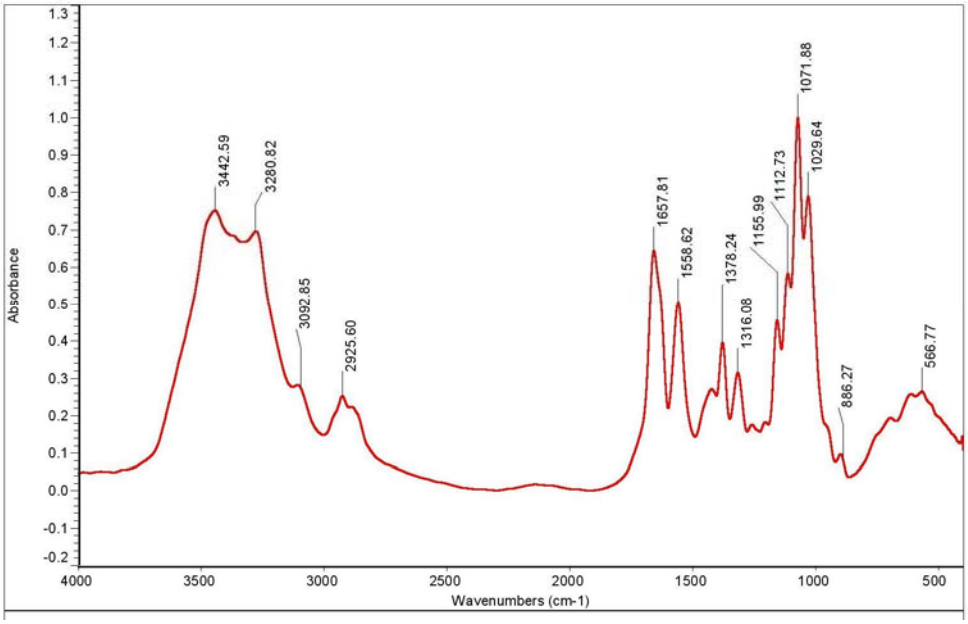

Figure-1: FTIR Spectrum of Chitosan extracted from *F.solani* CBNR BKRR

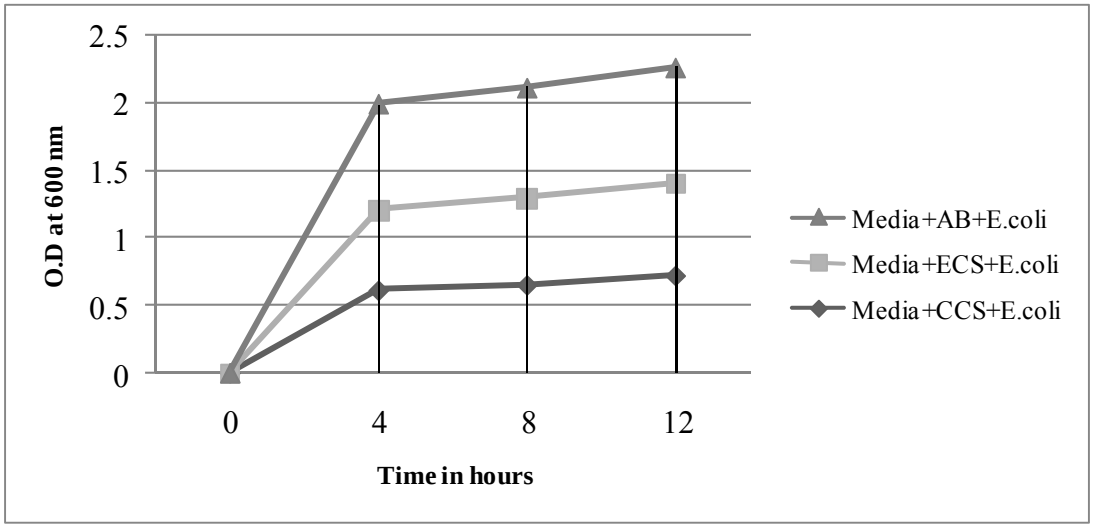

Figure-2: Comparative study of antimicrobial activity of Chitosan against *E.coli*

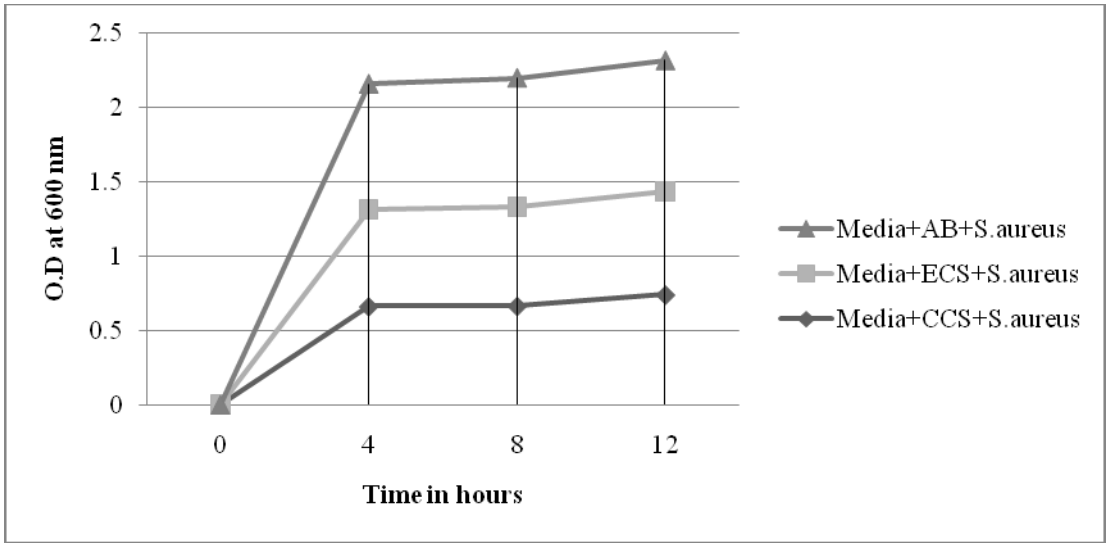

Figure-3: Comparative study of antimicrobial activity Chitosan against *S.aureus*

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	0.616	0.592	0.787
8	0.656	0.639	0.819
12	0.726	0.680	0.857
16	0.790	0.744	0.860
20	0.813	0.890	0.973
24	0.853	0.895	1.010

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	0.661	0.654	0.846
8	0.664	0.665	0.872
12	0.740	0.696	0.882
16	0.750	0.731	0.912
20	0.753	0.750	0.975
24	0.919	0.759	1.056

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

Exposure Time	Amount of dye degradation (%)
1h	1.56
2h	1.99
3h	3.6
4h	8.5
21h	11.2
22h	18
23h	21.1
24h	35.4
41h	42.4
42h	49.5
44h	50.2
45h	52
46h	57
48h	61.2
65h	65
66h	82.5
70h	86
71h	91.2
72h	94.5

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 (Table 4**).**

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 94.5% 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 93.4 % at the end of 72 hours incubation.

CONCLUSION

Fungi are abundantly available plethora for the production of industrially important secondary metabolites. These results present a cost effective methodology for production of the polysaccharides-Chitosan from marine fungi and have potential application as antibiotics and in bioremediation.

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