Production and Evaluation of Chitosan Obtained From *Aspergillus spp.*

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ABSTRACT

Chitosan is a natural polysaccharide comprising copolymer of gluocosamine and N-acetyl glucosamine, and can be obtained by deacetylation of chitin. This study was focused on the production of fungal chitosan and its application. 8 different samples [F15, F24-3, F5, F5(3), F14, F1, F10, F11] of fungi were isolated and inoculated in respective medium of Potato Dextrose Broth, Incubated at 30°C for 7 days and the chitosan were extracted. Top 3 chitosan producer [F5, F15, F10] were selected and incubated for different time duration [72 h, 96 h, 120 h] and the chitosan were extracted. Out of them top 2 chitosan producer [F5, F15] were selected and further study was performed using them. The antimicrobial susceptibility test was performed to access effect of produced chitosan by testing against five bacterial test pathogens [*Staphylococcus aureus, Escherichia coli, Bacillus cereus, Salmonella typhi, Pseudomonas aeruginosa*] by Disc diffusion technique.Both Chitosan Samples F5 and F15 gave highest zone of inhibition against *Pseudomonas aeruginosa*and lowest zone against *Bacillus cereus*. The chitosan sample F5 was more effective as compared to that of chitosan sample F15. Sample F5 showed the effective inhibition. The antioxidant activity of selected samples [F5, F15] was also performed by using FRAP assay, which confirmed that chitosan obtained from isolate F5 has higher antioxidant activity. The comparison of the [SSF] Solid State Fermentation and [SMF] Submerged State Fermentation was done to check the production of chitosan in respective fermentation process. The experimental result concluded that chitosan production in SSF was more compared to SMF. The optimization of chitosan production was performed using different pH, temperature and nitrogen sources. At pH 6, temperature 37°C and Beef extract as Nitrogen Source showed highest production of Chitosan by both the isolates. Further IR spectra analysis also confirmed the presence of chitosan in compound that is extracted from both the isolates.

KEYWORDS: Fungal Chitosan, Antimicrobial activity, Antioxidant activity, SMF, SSF, Optimization.

1. INTRODUCTION

After the discovery of chitin came chitosan. It was first observed by Rouget during during his experiment on chitin. In 1878, Ledderhose showed chitin to be made of glucosamine and acetic acid. It was not actually until 1894 that Hoppe-Seylernamed the substance "Chitosan"."Chitosan is a cationic polymer derived by deacetylation of chitin obtained from Crustaceans"[1].

Chitosan is second most polymers used in industries after cellulose [2].The production of Chitosan using Fungi has received much attention due to the need for an alternative source of chitin to solve these problems [3]. It was investigated that in *Aspergillus spp.* cell wall constituents, chitin comprises of 42% and also researchers confirmed that the chitosan content of fungi depends on Fungal strains, mycelia age, cultivation medium and condition.

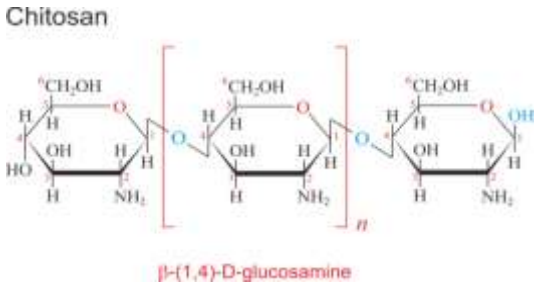

Figure 1: Structure of Chitosan [4]

Chitosan nanoparticles are also used in agricultural field; such as incorporation of NPK fertilizer into the nanoparticles to save fertilizer consumption [5]. Chitosan has been proven to be biodegradable, antimicrobial, antioxidant, non-toxic, non-antigenic and biocompatible [6].Solid State Fermentation **[SSF]** and Submerged State Fermentation **[SMF]** process used for the production of chitosan**.** It has been reported that the yield of chitosan using SSF is higher than that in SMF. Chitosan has been found to have an acceleratory effect on wound healing/ wound dressing process. It has also been used as a natural substance for the enhancement of seed germination and plant growth.Chitosan possesses repeating units of 1,4 linked 2-deoxy-2 aminoglucose. The amino group NH_2 can be protonated to NH_3^+ and readily form electrostatic interaction with anionic groups in an acidic environment. This property has been applied on edible film [7]. It has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 position respectively [8].

Chitosan is well established as an excellent natural absorbent because its amine ($-NH_2$) and hydroxy (-OH) groups may serve as coordination sites to form complexes with various heavy metal ions [9]. In order to prepare the high quality chitosan, it is desirable that the microbial biomass be produced in substantially control manner, having relatively uniform temperature and nutrient levels [10].

This study was focused on extraction of chitosan from fungi. Isolated chitosan was further subjected to antimicrobial and antioxidant activity study. Comparative analysis of effect of use of SSF and SMF on chitosan production and optimization was also performed.

2.MATERIALS AND METHODS

3-5 days old Culture of *Aspergillus spp*., Potato Dextrose Broth, Tween 80 solution (0.1%)**,** NaOH, Deionized water**,** Glacial acetic acid, Mueller- Hinton agar plate**,** 24 hours old culture of*Salmonella typhi, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus,* Sterile paper disc, Extracted Chitosan sample, Phosphate buffer**,** Potassium ferricyanide, Tri-Chloro Acetic acid**,** Ferric chloride**,** ButylatedHydroxy Toluene, Soya bean residue, Bromocresolpurple, HCL, Peptone, Beef Extract, Yeast extract.

2.1. PREPARATION OF STOCK CULTURE

8 different samples of *Aspergillus spp.*[F15, F24-3, F5, F5(3), F14, F1, F10, F11] were collected from the previous batch of laboratory.The stocked culture wasprepared by streaking the potato Dextrose Agar slants with the suspension of 8 different sample of *Aspergillus spp.* respectively. All the slants were incubated at room temperature for 5-7 days. 8 flasks containing Potato Dextrose broth were inoculated with 8 different fungal suspensions, prepared with 0.1% Tween 80 solution. All the flasks were incubated on the rotatory shaker at 30^{o}C with 180 rpm for 7 days.

2.2. EXTRACTION OF CHITOSAN

After the fungal biomass was collected, the extraction of chitosan from each of the fungal biomass was carried out. Equal amount of (4%w/v) NaOH solution was mixed with equal amount of biomass then the flask containing biomass in NaOH solution placed in the pre-heated oven at about 120^{o}C for 30 minutes. After heating, warm solution filtered and the residual was completely washed with deionized water. This pretreated biomass containing chitosan-glucan complex then rinse with water much time, until the pH of filtered solution reached to less than pH 9. Then glacial acetic acid was added till pH 3.5 obtained. Crude chitosan from chitosan - glucan complex was extracted by centrifugation. Then the crude chitosan was washed with distilled water. After that air dried at 20^{o}C to a constant weight. Similarlythis process was performed for the remaining samples. All the extracted chitosan were weighed and top 3 chitosan producer were noted [11].

2.3. EXTRACTION OF CHITOSAN AT DIFFERENT TIME INTERVAL

Extraction method was performed for different incubation period(i.e 72 hours, 96 hours, 120 hours) for each of that top 3 chitosan producer. Then checked from which sample the extraction of total chitosan was more. Top 2 chitosan producer were selected and various activities were performed.

2.4. ANTIMICROBIAL SUSCEPTIBILITY TEST BY DISC DIFFUSION TECHNIQUE

Bacterial suspension, swabbed onto entire surface of Mueller- Hinton Agar plate (pH-5.9) with sterile swab. Dilutions of chitosan sampleswere prepared with the help of acetic acid to achieve 1% and 3%). Sterile paper disc was impregnated with 1% and 3% concentration and placed on the surface of the Mueller- Hinton Agar using sterile pair of forceps. All the plates were incubated at 37°C for 24 hours and inhibition zone was measured with the help of zone-meter [12].

2.5. ANTIOXIDANT ACTIVITY

Chitosan solution were prepared with the help of acetic acid to achieve 100% and 50% concentration, In that 1 ml of 0.2M phosphate buffer (pH = 6.6) and 1 ml of 1% potassium ferricyanide were added and reaction mixture incubated at 50°C for 20 minutes, followed by addition of 1 ml of 10% trichloro acetic acid and centrifuged at 2000 rpm for 10 minutes.Aliquot of supernatant (1 ml) was mixed with 1 ml of deionized water, followed by addition of 0.25 ml of 0.1 % (v/v) ferric chloride and absorbance was measured at wavelength of 700 nm [13].

2.6. SOLID STATE FERMENTATION

Soya bean residue was used as the solid substrate for solid culture medium of fermentation. The initial pH of the soya bean residue was 6.5. A 30g of dry substrate weighed in 500ml flasks and the substrate moisture was adjusted by adding distilled water to 35% to 50%. The flasks were shaken to homogenize the solid medium and autoclaved at 121°C for 20min. 1ml of spore suspension, in sterile condition was inoculated into flasks and shaken to distribute the spores. The flasks were cotton plugged and remained static during incubation up to 7 days at 30°C. The extraction of Chitosan was carried out and assay was performed.

2.7. SUBMERGED FERMENTATION

1ml of spore suspension under sterile condition was inoculated into sterile flasks containing Sabouraud Dextrose broth medium and shaken to distribute the spores. The flasks incubated at 30°C upto 7 days and the extraction of chitosan was carried out and assay was performed.

2.8. ASSAY OF CHITOSAN

After extraction, assay of Chitosan was performed. In that, the powdered sample of SSF and SMF was wetted with 1 ml of deionized water and allowed to soak for 15min to allow possible swelling of the matrix. 0.5ml of the dye solution was slowly passed through the sintered funnels and excess dye solution was drained out with deionized water. Then 1 ml of 1N HCL and 1ml of 1N NaOH were added. The absorbance of blue color was measured at 530nm.

2.9. OPTIMIZATION OF PARAMETERS FOR CHITOSAN PRODUCTION

A) EFFECT OF pHON CHITOSAN PRODUCTION

The effect of pH Chitosan production was studied by incubation at various pH values of the medium pH such as 4, 5, 6. The fermentation media after inoculation with spore of *Aspergillus spp.* was kept and incubated at room temperature for 7 days. The Chitosan was extracted and analyzed spectrophotometrically.

B)EFFECT OF TEMPERATURE ON CHITOSAN PRODUCTION

The effect of temperature on Chitosan production was studied by incubating the flasks at various temperatures such as 37°C and Room Temperature for 7 days. The Chitosan was extracted and analyzed spectrophotometrically.

C) EFFECT OF DIFFERENT NITROGEN SOURCES ON CHITOSAN PRODUCTION

The effect of Nitrogen Sources on Chitosan production was studied by using Nitrogen Sources such as Peptone, Beef extract, Yeast extract [14].

2.10. CHARACTERIZATION OF CHITOSAN BY FTIR [FOURIER-TRANSFORM INFRARED SPECTROSCOPY]

To understand the overall chemical nature of the extracted mycotoxin, Fourier Transform Infrared Spectroscopy (FTIR) was used. It was done at centre of Excellence, Vapi. Infrared absorption spectra was recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a resolution and wave number accuracy of 4 and 0.01cm^{-1} respectively.

3. RESULTS AND DISCUSSION

3.1. EXTRACTION OF CHITOSAN

For the process of extraction, 8 different samples of ***Aspergillusspp*[F15, F24-3, F5, F5(3), F14, F1, F10, F11]**were collected from Laboratory isolated by the previous batch student and inoculated in respective medium of Potato Dextrose Broth, Incubated at 30°C for 7 days and the chitosan was extracted from each. Top 3 chitosan producer [F5, F15, F10] were selected and incubated for different time duration [72 h, 96 h, 120 h] and the chitosan were extracted. Out of them top 2 chitosan producer [F5, F15] were selected and various activities were performed using them [2]

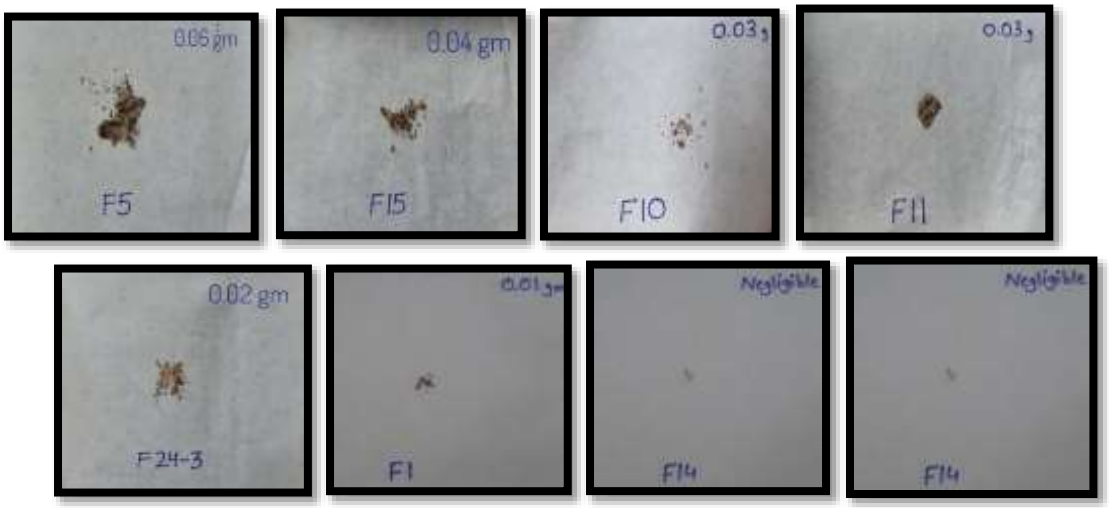

Figure 2: Result of Production of Chitosan after 7 days from 50 ml Potato Dextrose Broth

Table 1: Result of Production of Chitosan after 7 days from 50 ml Potato Dextrose Broth

SAMPLES	QUANTITY
F5	0.06 g
F15	0.04 g
F10	0.03 g
F11	0.03 g
F24-3	0.02 g
F1	0.01 g
F14	-
F5(3)	-

3.1.1. EXTRACTION OF CHITOSAN AT DIFFERENT TIME INTERVAL

As the production of fungal biomass was more at 120 h as compared to that of 72 h and 96 h, thus in the present study the maximum production of chitosan was observed at 120 h. F5 gave more production of Chitosan as compared to that of F15. According to the study report [2], best production of Chitosan was observed at 120 h.

Table 2: Result of Total production of Chitosan from Top 3 samples

ISOLATES	PRODUCTION OF CHITOSAN AT DIFFERENT TIME DURATION			TOTAL PRODUCTION
	72 h	96 h	120 h	
F5	0.8 g	0.10 g	0.12 g	0.3 g
F15	0.01 g	0.03 g	0.1 g	0.14 g
F10	-	Approx 0.01 g	0.01 g	~ 0.02 g

Out of Top 3 Chitosan producers [F5, F15, F10], Top 2 Chitosan producers [F5, F15] were selected on the basis of total production of Chitosan. Various Parameters were performed, using Top 2 Chitosan producers.

3.2. ANTIMICROBIAL SUSCEPTIBILITY TEST

In the present study, the antimicrobial susceptibility test with extracted chitosan samples [F5 and F15] were tested against five bacterial test organisms [*Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Pseudomonas aeruginosa*] by Disc diffusion technique. Both Chitosan Sample [F5 and F15] showed the highest zone against *Pseudomonas aeruginosa* and lowest zone against *Bacillus cereus*. The result showed that the *Pseudomonas aeruginosa* was more susceptible than that of *Bacillus cereus.* The Chitosan sample F5 showed the effective zone of inhibition as compared to that of Chitosan sample F15, while according to the previous research [12], Researchers observed that the diameter of the inhibition zone of produced Chitosan loaded with acetic acid was highest against *Pseudomonas aeruginosa* and lowest against *Escherichia coli.*

Figure 3: Result of Antimicrobial Susceptibility test

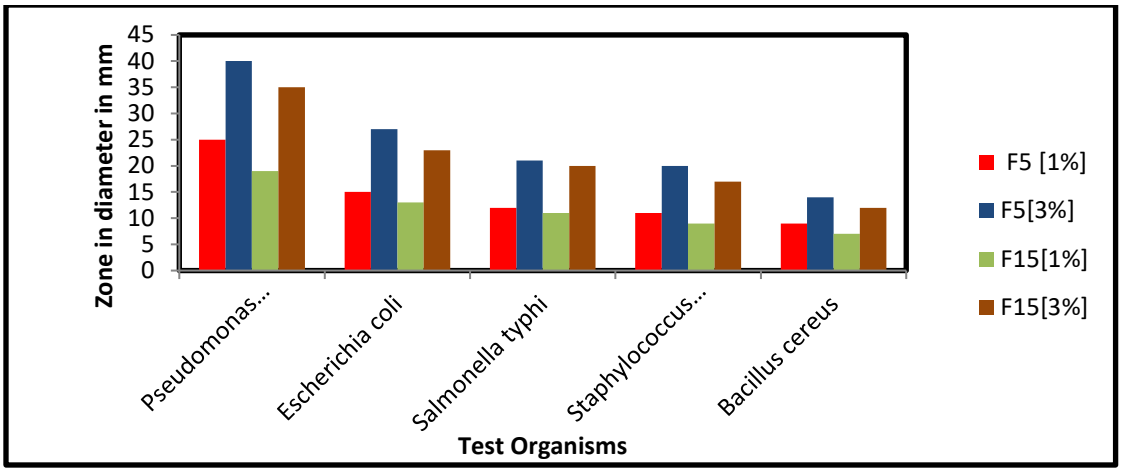

Figure 4: Result of Antimicrobial Susceptibility test

3.3. ANTIOXIDANT ACTIVITY

The Antioxidant activities of produced Chitosan samples from isolates [F5 and F15] were performed by using Reducing Power Assay. It was observed that the Antioxidant activity was increase as concentration of Chitosan sample increased. The Chitosan Sample F5 showed higher Antioxidant activity than sample F15. According to the study report [13], Researchers observed that the Antioxidant activity was increase as the concentration of Chitosan sample increase. In other study report [3], researchers observed that the absorbance increase as the concentration of Chitosan sample increase, while in other study report [15] also researchers observed that as the concentration of chitosan increase, antioxidant activity increase.

Figure 5: Result of Antioxidant activity for samples F5 and F15 and **Standard Antioxidant [ButylatedHydroxy Toluene]**

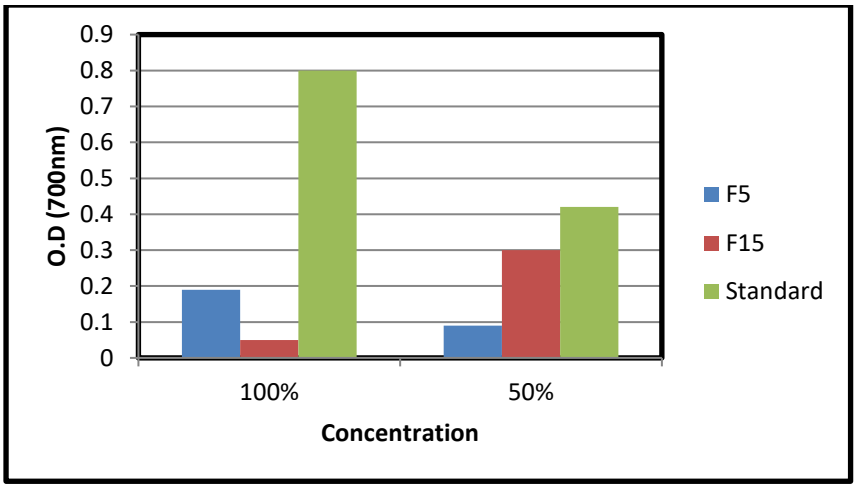

Figure 6: Result of Antioxidant activity

3.4. SOLID STATE FERMENTATION AND SUBMERGED FERMENTATION

Submerged Fermentation and Solid State Fermentation were performed for samples F5 and F15. The amount of Chitosan produced in both fermentation methods were evaluated and compared. In the present study the yield of Chitosan was found more in Solid State Fermentation than Submerged Fermentation because the more amount of mycelia produced in SSF compared to that of SMF. The Isolate F5 showed the effective result in both the fermentation methods. According to the study report [3], the yield of Chitosan was more using Solid State Fermentation as compared to that of Submerged Fermentation. So in comparison with the study report, the results were similar.

Figure 7: Production of Chitosan from Submerged Fermentation and Solid State Fermentation

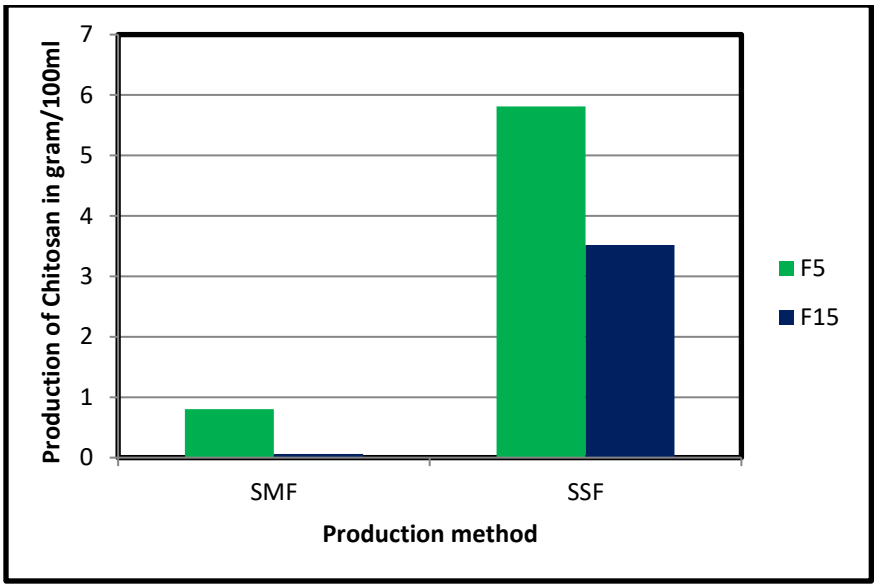

Figure 8: Result of Extraction of Chitosan from SSF and SMF

Table 3: Result of production of Chitosan from SSF and SMF

SAMPLES / TYPES OF FERMENTATION	PRODUCTION IN µg/ml	
	SSF	**SMF**
F5	**1050**	**F5**
F15	**700**	**F15**

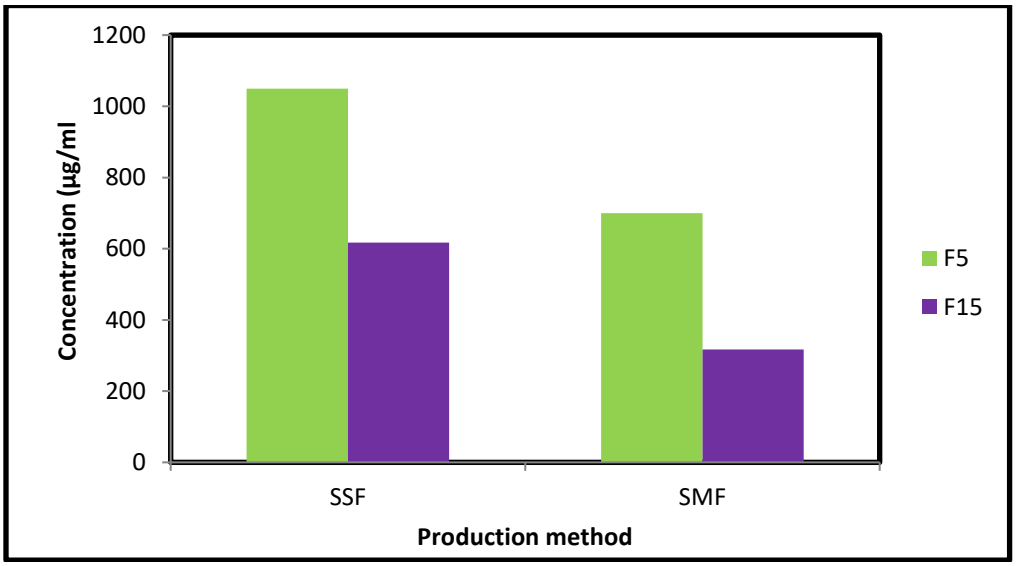

Figure 9: Result of production of Chitosan from SSF and SMF

3.5.OPTIMIZATION OF VARIOUS PARAMETERS FOR THE PRODUCTION OF CHITOSAN

3.5.1.EFFECT OF pHON CHITOSAN PRODUCTION

In this study the effect of pH on Chitosan production was observed. It was noted that the production of Chitosan was more at pH 6, given by both isolates whereas according to the study [3], the production of Chitosan was more at pH 6.5. In other study report [16], Researchers observed that the production of Chitosan was more at pH range in between 5.5-6.5, while in the study report [17] researchers observed the yield of chitosan was best at pH 5.5.

Figure 10: Result of Production of Chitosan samples [F5 andF15] at Different pH such as pH 4, 5, 6.

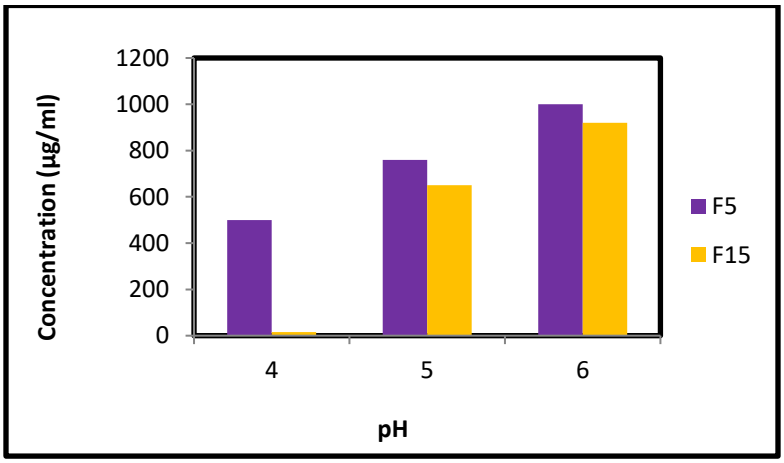

Figure 11: Result of Effect of pH on Chitosan production

3.5.2. EFFECT OF TEMPERATURE ON CHITOSAN PRODUCTION

In this study the effect of pH on Chitosan production was observed. It was noted both the isolates gave the best production of Chitosan at 37°C. Chitosan sample F5 showed the effective result as compared to that of the sample F15, whereas according to the study report [2], best production of Chitosan was observed at 30°C, while in the study report [18], researchers observed that yield of chitosan increased from 20°C till 30°C and further decreased at 35°C.

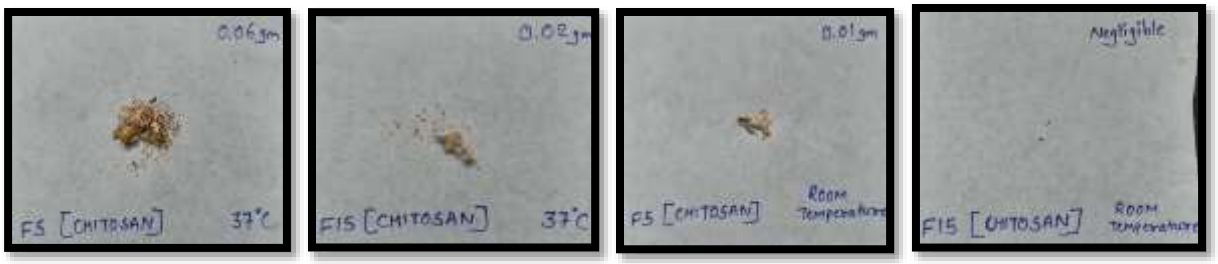

Figure 12: Result of Production of Chitosan samples [F5 and F15] at 37°C and Room Temperature

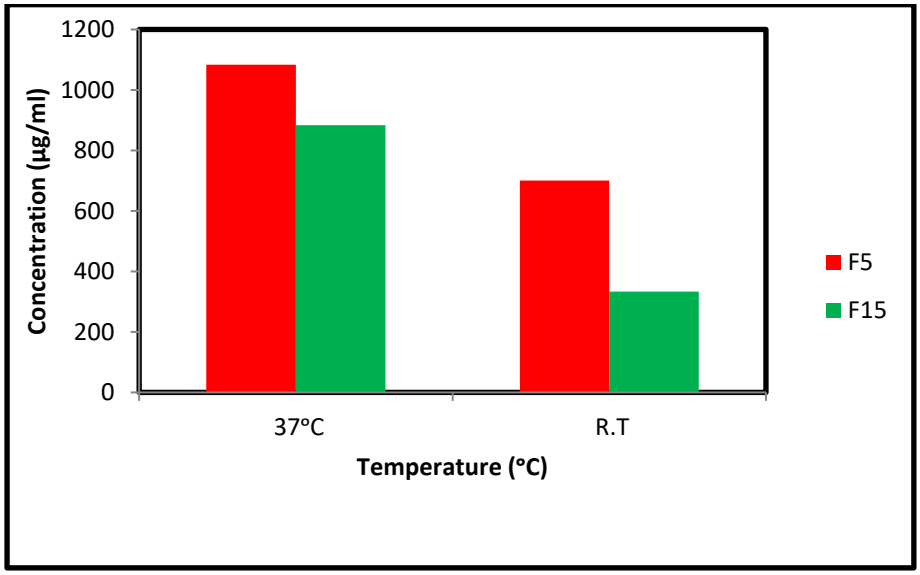

Figure 13: Result of Effect of Temperature on Chitosan production

3.5.3. EFFECT OF NITROGEN SOURCES ON CHITOSAN

In this study the effect of Nitrogen Sources on Chitosan production was observed by using Nitrogen Sources such as Peptone, Beef extract, Yeast extract. It was noted that the sample F5 gave best Chitosan production with Beef Extract whereas according to research [14], best Chitosan production was observed with Yeast extract, while in other study report [16], researcher observed that the production of chitosan was more when urea was used as a nitrogen source. In the study report [19], researchers observed the best production of chitosan with soyabean meal. In the study report [20] also researcher observed that the production of chitosan was more when yeast extract as a nitrogen source.

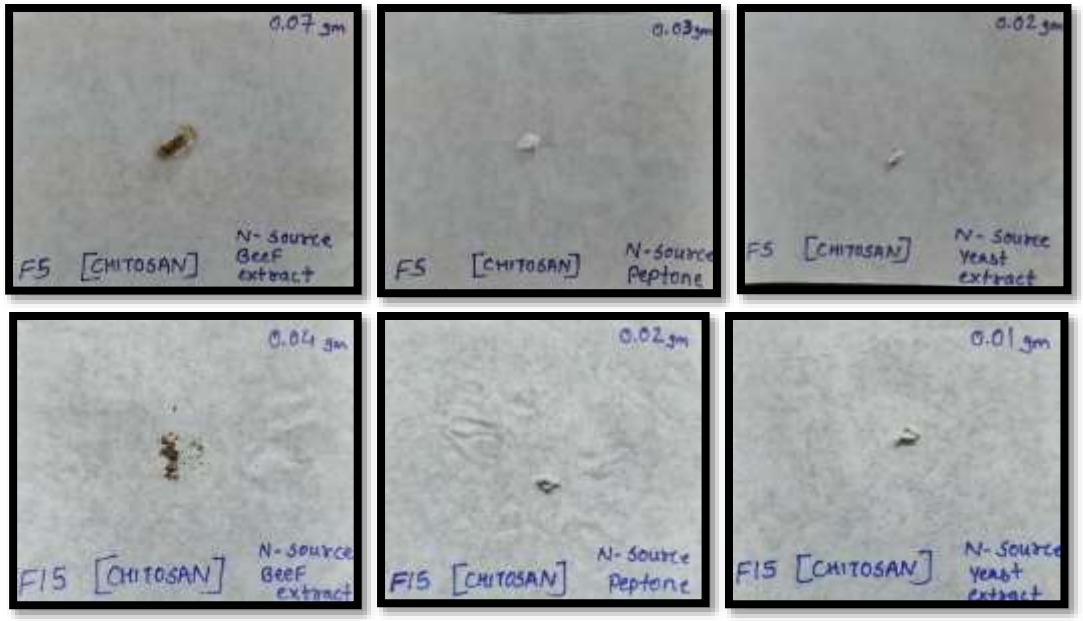

Figure 14: Result of Effect of Nitrogen sources on Chitosan production

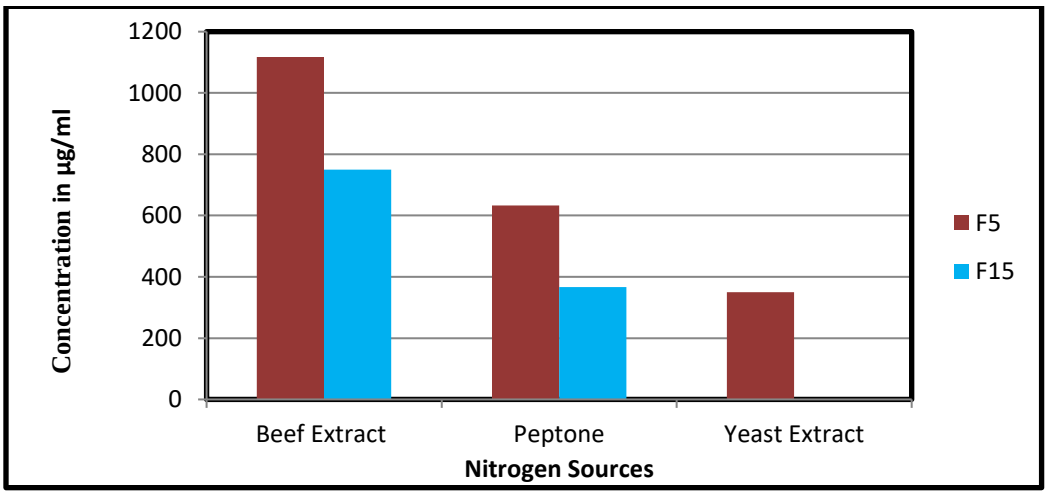

Figure 15: Result of Effect of Nitrogen sources on Chitosan production

3.6. FTIR (FOURIER TRANSFORM INFRARED SPECTROSCOPY) ANALYSIS

Fourier Transform Infrared Spectroscopy is a technique employed for the identification of the functional groups present in the sample. FTIR spectra of 2 different Chitosan produced by 2 fungal isolates was performed. Absorption band corresponding to functional groups were observed. For Chitosan sample F5, the FTIR spectra showed 14 peaks. The 2 major peaks at ~3283.15 cm^{-1} and ~1655.75 cm^{-1} were observed. The peak at ~3283 cm-1 confirmed hydroxyl group, where as the peak at ~1655.75 confirmed the amide band. The other peaks were obtained at ~2929 cm^{-1}, ~2363.56 cm^{-1}, 2345.75 cm^{-1}, 1736.09 cm^{-1}. The peak at ~1377.19 confirmed the presence of CH_3 group and the peak at ~1545 cm^{-1} corresponds to the N-H deformation of amide group. For Chitosan sample F15, the FTIR spectra showed 12 peaks. The 2 major peaks were obtained at ~3424.04 cm^{-1} and 1653.47 cm^{-1} were observed. The peak at ~3424.04 cm-1 confirmed the presence of hydroxyl group, where as the peak at ~1653.47 cm^{-1} confirmed the amide band. The other peaks were obtained at 2926.04 cm^{-1}, 2854.57 cm^{-1}, 2362.62 cm^{-1}, 2345.07 cm. The peak at ~1398.32 confirmed the presence of CH_3 group and the peak at ~1559.54 corresponds to the N-H deformation of amide group whereas according to the report [7], the FTIR spectra showed 10 peaks. The 2 major peaks at ~3451.2 cm^{-1} and ~1638cm^{-1} were observed. The peaks at ~3451.2 cm^{-1} confirmed the hydroxyl group, where as the peak at

~1638 cm^{-1} confirmed the amide band. The other peaks were obtained at ~2368 cm^{-1}, ~2234 cm^{-1}, ~1019 cm^{-1}. The peak at ~1418 cm^{-1} confirmed the presence of CH3 group and the peak at ~1560 cm^{-1} corresponds to the N-H deformation of amide group. The information from the respective wave number confirmed that the sample were of Chitosan.

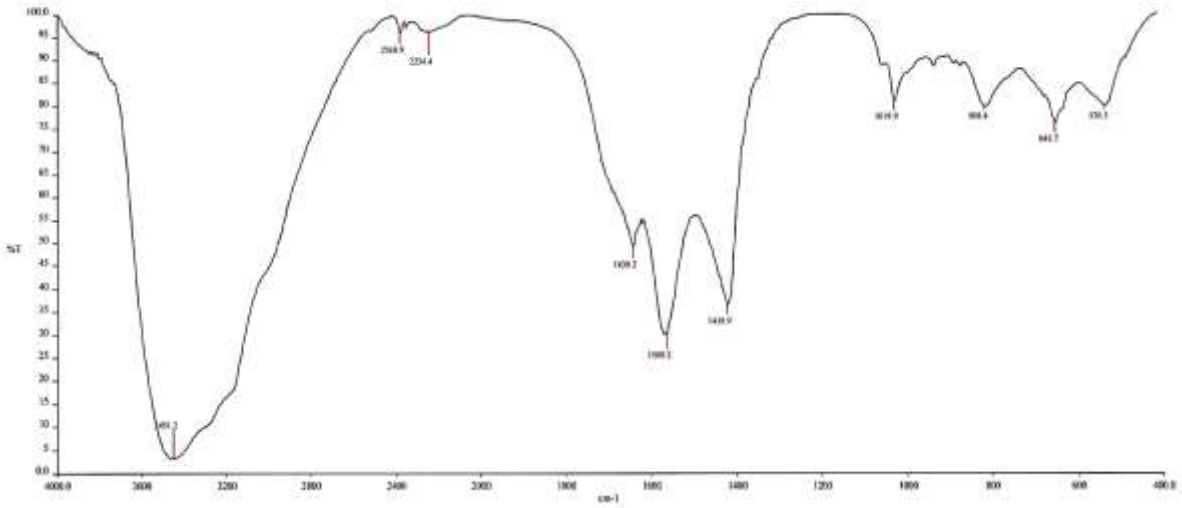

Figure 16: Graph of FTIR analysis of Chitosan

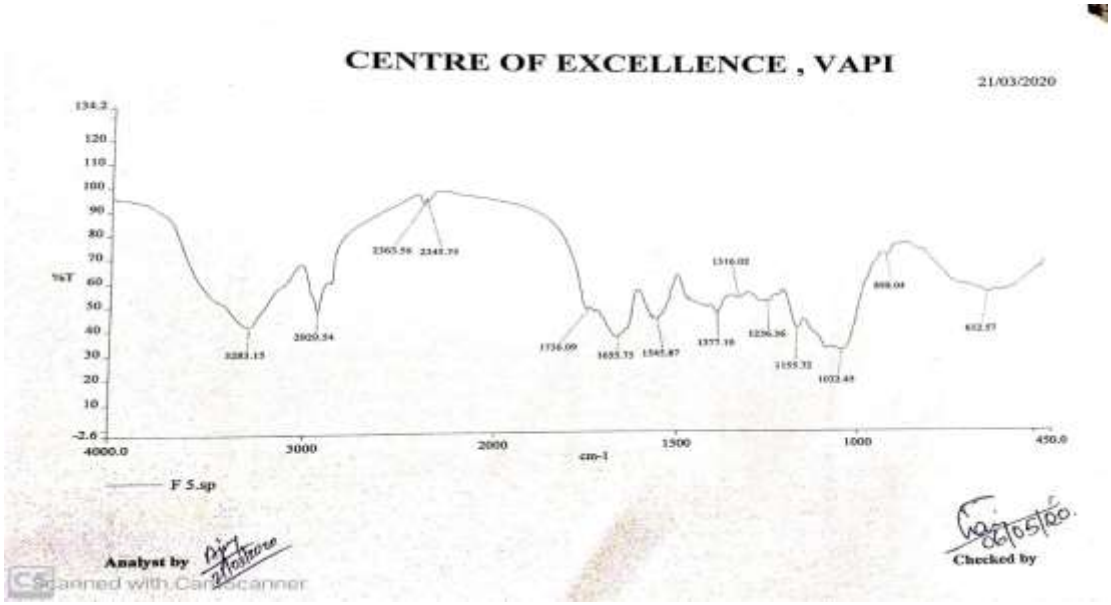

Figure 17: Graph of FTIR analysis of Chitosan sample F5

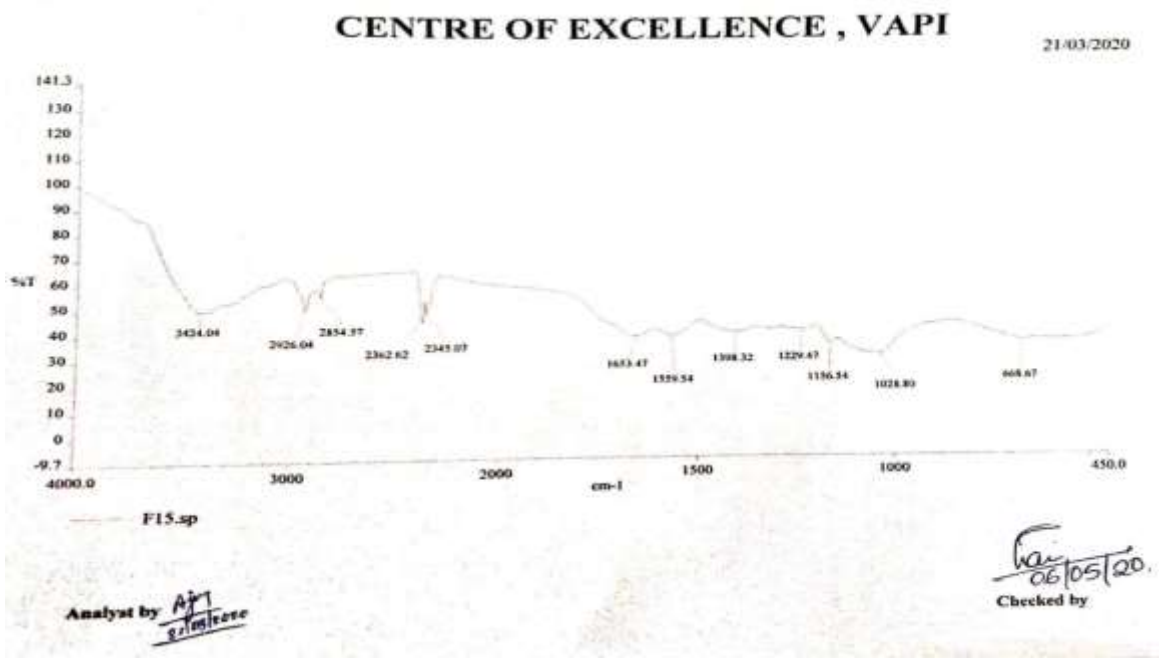

Figure 18: Graph of FTIR analysis of Chitosan sample F15

4. CONCLUSION

Finally it can be concluded that the *Aspergillus spp.* used in the above work are promising source of chitosan. The production and evaluation of chitosan obtained from *Aspergillusspp* was successfully done. Isolate F5 was the good Chitosan producer. It has an effective Antimicrobial and Antioxidant activity. Hence the chitosan obtained from the *Aspergillusspp.*could be utilized in the industries such as food, Pharmaceutical etc. By recent developments in pharmaceutical biotechnology, these species can be genetically manipulated and further optimized for getting better result and can also be adapted by industries in producing a quality and economical.

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