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Extraction of Chitosan from Basidiomycetes species and analysis of its antimicrobial property

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ABSTRACT

Basidiomycetes, commonly known as a fruiting fungus, is considered as a natural chitosan producer. This study optimized chitosan production by already isolated *Coprinopsis*-S1strain. and evaluated its antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Listeria monocytogenes*, *Psuedomonas aeruginosa*, common food borne pathogens. The organism has given the best result, 48.5 mg/L of chitosan production under an optimized condition of pH 6, temperature 25°C, 1% of maltose as a carbon and 1% of Yeast extract as a nitrogen source under submerged fermentation condition. The extract has given the best antimicrobial activity against *Listeria monocytogenes* with a maximum zone of inhibition in combination with nisin in a ratio of 1:1 of 24.6 mm, but it had not shown activity against *Bacillus subtilis* showing its antimicrobial property towards Gram negative bacteria rather than the Gram-positive ones.

Keywords: Chitosan, Basidiomycetes, Antimicrobial Activity

INTRODUCTION

Chitosan, a linear polysaccharide, with randomly arranged acetylated and deacetylated glucosamine units. It is water insoluble and weakly soluble in organic acid solution [1]. It has attracted many for exploring their application in edible food packaging of food materials because of its natural availability and antimicrobial property. Chitosan has also been reported to offer other benefits like anti-tumor and hypo-cholesterolemic functions. Due to antimicrobial activity of chitosan, it is used against a range of foodborne fungi, yeast, bacteria. Apart from its antimicrobial property, it also been reported to improve storage ability of perishable food by using in conjecture with modifying food packaging which can

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decrease the loss by transpiration. One major factor for choosing chitosan is for the growing consumer for food safety and its preservation without chemical preservations. Chitosan received US FDA approval for GRAS status as a food additive and its application in food system [1].

In recent years chitosan application in food and medical sector has gained a huge success because of its ability to get biodegraded, biocompatibility, non-toxigenic and a broad spectrum of anti-microbial activity. Chitosan is highly basic in nature which confers its uniqueness among the rest of the polysaccharides in comparison to others like cellulose, dextrans, alginic acids, where some are either acidic or neutral. Many functional properties are associated with chitosan like polyoxysalt formation, film formation, metal chelation and optical or structural isomerism, etc. Chemically chitosan is also a linear polyamine, possessing reactive amino group (-NH_2) and hydroxyl groups (-OH). It has the chelating ability for many transitional metal ions [2]. Its solubility depends on the degree of deacetylation, molecular weight, distribution of acetyl groups and nature of acids used for protonation on it.

It is biocompatible for being a natural polymer, non-toxic to human and safe. It binds with mammalian and microbial cells. It has also shown regenerative capacity on connective gum tissues. It has also been reported to help in the formation of osteoblast cells, responsible for bone formation. It helps in hemostasis, can act as fungistatic agent [2]. Its functionality as an antimicrobial agent against food various food borne pathogens like *Escherichia coli*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* [4] has set its demand in the food industries. The present study thereby explored the various conditions under which the chitosan could be produced the best by a already isolated basidiomycetous species, from the natural environment and to check its antimicrobial property.

MATERIALS AND METHODS:

Procurement of sub-culturing of the culture:

The already isolated basidiomycetous strain *Coprinopsis cinerea* strain BAF Ccult 4361-S1 (Coprinopsis-S1) and the food borne standard organisms were procured form the laboratory of Lovely Professional University, Microbiology department, Punjab, India. The culture was sub-cultured and maintained for further usage on wheat bran agar plates and slants by following standard protocol of fungus isolation and purification, at 28ºC and then at 4ºC, respectively [15].

Optimization of medium for chitosan production by *Coprinopsis*-S1 strain:

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The optimization was performed by considering one factor analysis method. The factors that were taken into consideration were pH, temperature, carbon source and nitrogen source. All the experiments were performed in triplicate and standard deviation was applied to reduce the error in the obtained result.

Optimization of pH:

For the determination of optimum pH for the high growth of culture, in three Erlenmeyer flask 50mL of wheat bran broth was prepared and pH was maintained at 4, 5, 6. In all flasks 3 plugs were inoculated from the culture plate of white rot fungi using sterile cork borer. The flasks were incubated at 28˚C for 4-5 days in shaking incubator. After the incubation period, the fungal mass was separated from the medium and dried at 50˚C till constant dry weight was obtained [6].

Optimization of temperature:

For the determination of optimum temperature for the high growth of culture, in two Erlenmeyer flask 50 mL of wheat bran broth was prepared respectively and 3 plugs from culture plate was inoculated and incubated at 25ºC, 30ºC and 35ºC incubator for 4-5 days. After the incubation period, the fungal mass was separated from the medium and dried at 50ºC till constant dry weight was obtained [6].

Optimization of carbon source:

For the determination of optimum carbon source, in Erlenmeyer flask 50 mL, three different types of carbon sources were taken into consideration, glucose, maltose and fructose, at a concentration range of 0.5%, 0.75% and 1% in wheat bran broth. In each flask, 3 plugs from culture plate was inoculated using sterile cork borer. The flasks were incubated at 30ºC in a shaking incubator for a period of 7 days [6].

Optimization of nitrogen source:

For the determination of optimum nitrogen source, in Erlenmeyer flask 50 mL of wheat bran broth three different nitrogen sources were taken into consideration like, Yeast Extract, peptone and ammonium sulphate, each at a concentration range of 0.25%, 0.5% and 1%. To all the flasks, the nitrogen sources were added aseptically. In each flask, 3 plugs from culture plate was inoculated using sterile cork borer. The flasks were incubated at 30ºC in shaking incubator for a period of 7 days [6].

Production of chitosan under optimized condition:

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To produce chitosan, 200 mL in 500 mL Erlenmeyer flask of wheat bran broth was used. 3-4 plugs from the culture plate was inoculated in the flask and incubated for 7 days under optimized condition of pH, temperature, sugar and nitrogen source in BOD incubator. After the 7^{th} day of growth, the fungal mat was filtered using and washed with distilled water until all traces of wheat bran was removed [6].

Extraction of chitosan:

The fungal mat isolated from the production medium was dried at 50°C till constant weight was observed. The dried fungal mat was finely ground. The powder formed was suspended in 1 mol L^{-1} NaOH solution (1:30 w/v). The solution was autoclaved at 121°C for 15 minutes. The insoluble alkali fraction was centrifuged at 12000 g for 15 minutes. The pellets obtained were washed with distilled water and pH of solution was maintained to a neutral (7) and again centrifuged at 12000 g for 15 minutes.

The residues obtained were treated with 2 % acetic acid (1:40 w/v) and solution was kept in water bath at 96˚C for 8 hours. The obtained slurry was centrifuged at 12000 g for 15 minutes and the insoluble parts of acid were discarded. The pH of the supernatant fluid obtained was adjusted to 10 by using 2 mol l^{-1} of NaOH. The solution was again centrifuged at 12000 g for another 15 minutes and the precipitates of chitosan was washed with distilled water, then with 95% ethanol (1:20 w/v) and acetone (1:20 w/v), respectively. The precipitates were dried at 60°C till a constant weight was obtained [7].

Antimicrobial activity

The nutrient broth was prepared for individual strain and autoclaved. The broth was inoculated with strains and incubated at 37˚C for 24 hours. After 24 hours of incubation, antimicrobial activity was checked by agar-well diffusion method. Muller-Hinton agar was prepared and autoclaved. The media was poured in sterile petri plates and reference strains of *Escherichia coli (R-1), Bacillus substilis (R-2), Listeria monocytogenes (R-3), Klebsiella pneumonia (R-5)* were spread with the help of sterile glass spreader. With the help of sterile 1000 µL tip, 3 wells and the prepared solution was poured in the wells and incubated for 24 hours at 37˚C. Next day zone of inhibition was observed, and the diameter was measured of the inhibition zone. The antimicrobial activity was checked of extracted chitosan, pure nisin and a combination of chitosan and nisin (at a ratio of 1:1).

RESULT:

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Optimization for the growth of white rot fungi:

Optimization of pH

The maximum fungal biomass of 43.8± 0.76 was seen at pH 6 and the moderate growth was seen at pH 5 around 38.3± 0.87 of the fungal biomass was observed [Table 1].

Table 1 : Optimization of pH for chitosan production

pH	4	5	6
Biomass (mg/L)	15.5± 1.05	38.3± 0.87	43.8± 0.76

Optimization of temperature:

At 25˚C, maximum growth was observed and the fungal dry biomass was 45.4 ± 0.25 gm/L. the moderate growth was observed at 30˚C and the weight of fungal dry biomass calculated was 30.3± 0.55 [Table 2]. The pH and temperature are the most important factor for the growth of the fungi. Fluctuation in any of this factor ceases the growth. pH is maintained for the structural balance of the fungi like its cell membrane, cell wall and its cytoplasmic content. It has been observed that acidic pH favors the growth of the fungi and also for the production of the metabolites. The mycelium of the basidiomycetes was found to be acidic in nature. The most common examples of basidiomycetes like *G. lucidum*, *L. tuberregium* and *Grifolafrondosa*, *P atroumbonata* and *Volvariella speciose* grows at acidic pH range 5.5 to 6.5 [6].

Table 2 : Optimization of temperature for chitosan production

Temperature(ºC)	25	30	35
Biomass (mg/L)	45.4 ± 0.25	30.3± 0.55	27.2± 0.35

Optimization of carbon source:

The maximum growth of fungi was seen when maltose, a disaccharide was added in the medium. The fungal dry biomass was found to be 46.8 ± 0.21 gm/L at a percentage of 1% w/v of maltose [Table 3]. the moderate growth was observed when fructose was added to the medium. The medium containing

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maltose was statistically significant ($p<0.05$) gave highest yield of the mycelial growth. Lai [8] stated that the glucose rich medium gave highest growth of the mycelial of *Lignosus rhinoceros.*

Table 3 : Optimization of carbon source for chitosan production

Sugar as C-source	Percentage concentration		
	0.5	0.75	1
Glucose	31.3± 0.15	28.5± 0.61	33.6± 0.18
Maltose	27.2± 0.29	37.1± 0.43	46.8 ± 0.21
Fructose	22.4± 0.86	26.5± 0.11	30.3± 0.27

Optimization of nitrogen source:

The maximum growth of fungi was observed when yeast extract was added to the medium at a concentration of 1%. The dry weight of fungal biomass was 48.5± 0.21 gm/L [Table 4]. The medium enriched with ammonium sulphate was statistically significant ($p<0.05$) and yield high amount of the mycelial growth. The report stated that basidiomycetes requires organic nitrogen source for the growth [8]. A report by Suberu showed that the highest growth of mycelia of *G. lucidum* was due to the presence of the maltose sugar as carbon source in the medium [9]. Another research showed the mycelial biomass of the *Psyathyerella atroumbonata* was high when glucose as carbon source was added to the medium [6]. Similarly, reports on the growth of the basidiomycetes such as *Lentinus tuberregium, G. frondosa, Agaricus blazei* and *Psathyerella atroumbonata* were high when yeast extract as nitrogen source was added to the medium [6]. It has been reported that the growth of the basidiomycetes depends on the complexity of the nitrogen source [10]

.**Table 4 : Optimization of nitrogen source for chitosan production**

Nitrogen source	Percentage concentration		
	0.5	1	1.5
Yeast Extract	34.2± 0.16	48.5± 0.21	36.6± 0.11
Peptone	28.6± 0.09	32.17± 0.41	22.3 ± 0.31
Ammonium Suphate	16.4± 0.86	28.5± 0.11	32.7± 0.29

Production and isolation of chitosan under optimized condition:

The isolated chitosan from various steps was amounted 3 grams from 4 flasks containing 200 mL of the wheat bran broth. Many authors documented the production of chitosan from different sources like bacteria and fungi. Under optimized condition the amount of chitosan produced from the isolate was

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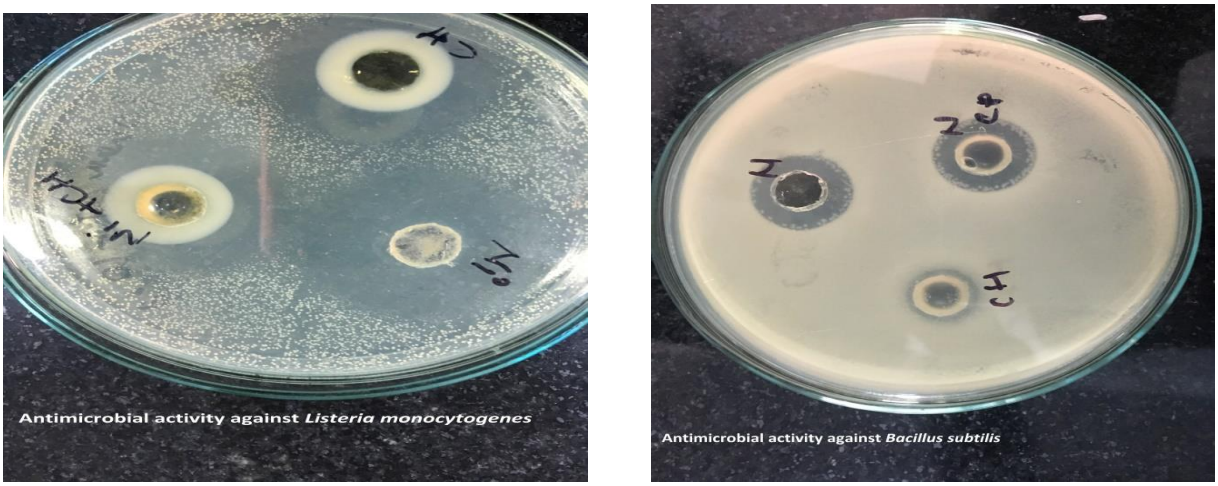
48.5 mg/L, showing the positive effect of maltose and yeast extract at a concentration of 1% w/v of each of the sources to be appropriate for the production at 25°C. Kaur reported the amount of the chitosan produced by *Serratia spp*. and *Bacillus spp*. were 100 mg per liter of the medium, 160 mg per liter of the medium respectively [11]. S. et al. 2014, reported that *Aspergillus niger* produced 160 mg of the chitosan under SMF conditions. Gharieb significantly reported that *Mucor rouxii* RCMB 015002 produced 35 mg of chitosan per mL of the medium; *Cunninghamella elegans* RCMB 012002 produced 29 mg of chitosan per mL of the medium, *Rhizopus spp*. produced 13 mg of chitosan per 50 mL of the medium [13]. Kumaresapillai and his colleagues reported 23.1 % of chitosan from *Aspergillus niger* MTCC 872, 25.2 % of chitosan from *Aspergillus niger* MTCC 1785 and 26.1 % of chitosan from *Aspergillus niger* MTCC 2208.

Antimicrobial activity:

Zone of inhibition of chitosan, nisin and chitosan-nisin was observed against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*. Among all the pathogens, gram positive bacteria showed cleared zone of inhibition. There was little effect of the chitosan, nisin and chitosan-nisin on gram negative bacteria [Image 1].



(a) (b)

Image 1: Antimicrobial activity of nisin (Ni), chitosan (CH) and chitosan-nisin (N CH) (a,b)

Highest inhibition zone of 28.6 mm diameter was seen in *Listeria monocytogenes* of nisin. Moderate zone of inhibition of nisin was seen in *Bacillus subitilis* and *Pseudomonas aeruginosa*. And the

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diameter of zone observed was 20.6 mm and 18.6 mm respectively. The least zone of inhibition of 12.3 mm diameter of nisin activity was seen against *Escherichia coli*.

In the case of chitosan activity, highest zone was observed against *Listeria monocytogenes* and the diameter of the inhibitory zone was 24.6 mm. Moderate inhibition zones was observed against *Escherichia coli* and *Pseudomonas aeruginosa* and the diameter of inhibitory zone observed was 15.3 and 15.3 respectively. The least zone of inhibition was seen against *Bacillus subtilis* of 11 mm in diameter. A comparative study was done by A. Shanmugam showed the antimicrobial activity of 75 % chitosan against *Escherichia coli* and *Pseudomonas aeruginosa* [15]. The diameter of zone of inhibition against both bacteria was 12 mm and 15 mm respectively. In the same study the 50 % concentration of chitosan showed 14 mm diameter of inhibition zone against *Pseudomonas aeruginosa* and with 25 % of chitosan concentration 13 mm diameter of inhibition zone was observed against *Pseudomonas aeruginosa*. Tetracycline was taken as control and 14 mm diameter of inhibiton zone was observed against *Pseudomonas aeruginosa*.

Lastly the highest zone of inhibition of chitosan-nisin solution was seen against *Listeria monocytogenes* and the diameter observed was 24.6 mm. the inhibitory zone of the solution against *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* was found to be 16 mm, 17 mm and 17.3 mm in diameter respectively.

Conclusion

The study there shows the effective nature of chitosan which can be further be used in various fields either in pharmaceutics or in food industries or in packaging industries as a potent antimicrobial agent which has shown good activity against Listeria species, a potent food borne pathogen.

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