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Chitosan from *Rhizopus stolonifer*: Process optimization, structural characterization and selective antimicrobial activity

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ABSTRACT:

Introduction: This study explores the optimization of growth conditions and the extraction, characterization and antimicrobial potential of chitosan derived from *Rhizopus stolonifer*, a filamentous fungus. *R. stolonifer* exhibited optimal biomass accumulation by the 5th day of cultivation (0.53g), after which growth plateaued; indicating the onset of the stationary phase. Maximum biomass was achieved at pH 6.0 and 30°C, with glucose as the most effective carbon sources, resulting to the highest fungal dry weight (0.483g). **Methodology**: Chitosan extraction was performed using modified method yielding maximum 81.4 mg on 7th day, with a calculated deacetylation degree of 72.53%, confirmed with FTIR spectroscopy through characteristic absorption bands at 1651 cm^{-1} and between 1032-1153cm^{-1}. **Results**: The extracted chitosan showed concentration depending antimicrobial activity; showing strong inhibition effects against *Bacillus cereus* and *Candida albicans* (16mm at 1000 µg), moderate activities against *Streptococcus mutans* (11 mm) and limited or not activity against Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. **Conclusion:** This result highlights suitability of *R. stolonifer* as an efficient and sustainable source of high quality chitosan, with promising use in agriculture, food preservation, also biomedicine specially for its selective microbial activity toward Gram-positive bacteria and fungi.

KEYWORDS: Chitosan, extraction, FTIR, antimicrobial properties, optimization

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1. INTRODUCTION

Fungi are employed in many different applications, such as baking and brewing and they are important in both ancient and contemporary biotechnological processes. [1] They also recycle organic materials by acting like decomposers. Fungi have many advantages, including the ability to synthesize various medication, alcohol, enzyme, organic acids and antibiotics. [2] The two most prevalent natural polysaccharides are chitin and cellulose. Fungal cell wall is the main source of the chitin that gives Ascomycetes, Zygomycetes, Basidiomycetes and Deuteromycetes their shape, strength and support. [3, 4] According to Garcia-Rubio et al 2020, [5] the key threadlike polymer in the cell layer of most fungi was chitin.

Chitin is made from a straight chain of 1- to 4-linked 2-acetamido-2-deoxy-β-D-glucopyranose units and doesn't dissolve well in regular fluid. [6, 7] For to be used in different application, it is often changed chemically. Chitosan, made by partly removing acetyle group from chitin is the most common changed form. Chitin is strong nitrogen-rich material that is usually white and hard. It acts as an internal support for invertebrate animals which does not have backbone and constitutes the tough outer shell of insect, crabs, lobsters and shrimp. [8] It is difficult to solubilize in most of solvents and chemically inert similar to that of the cellulose. But chitosan the partly deacetylated chitin as shown in fig 1 and has got a lot interest for its usage in biotech, farming, health care, also food due to it breaks down natural, works good in body and has helpful bio activity. [9] Since it could be better than current sources, more attention has put on getting chitin and chitosan from fungi. Crustacean leftovers which depending on season and location due to fishing, can be tricky to obtain. [10] On the other hand, fungal hyphae mats are easy to grow using fermentation regardless of time or area. [11, 12] Also, removing minerals doesn't really need when working fungal material, since fungal mycelia have much less non-organic stuff compared to crab and shrimp shell. [13]

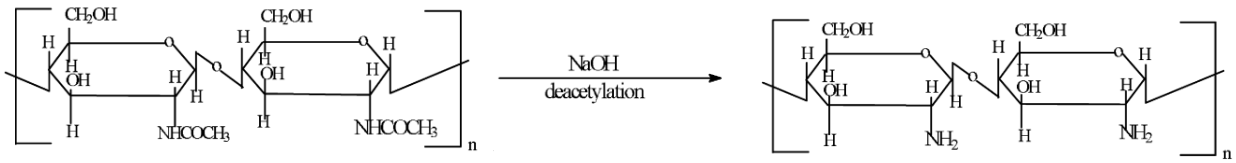

Fig 1. Chitosan is the deacetylated product of chitin

The manufacturing of advance, profit generating good could offer the industry a lucrative avenue given the substantial volume of fungal residue materials that has collected in the fermentation and mushroom-making industries and the cost associated with treating the trash. [14] Chitosan has a widespread quality's, including the capacity to form films, metal chelation, solution viscosity, polyelectrolyte behavior, solubility in different medium and unique optical and structural traits. [15] Typical amine reactions occur in it the most important being N-acylation and Schiff's reaction. [16] While cellulose glucans are difficult for obtain, chitosan glucans are easy to obtain under mild conditions. An

estimated 300 or more usages for chitin, chitosan and its derivatives exist, demonstrating their adaptability and potential across a wide range of industries. [17] The physico-chemical and biological characteristic of chitosan provides multiple potentials uses in field such as cosmetics, agriculture, food, biomedicine textiles and the refining of industrial effluents. [18] The reason chitosan has much more versatility than chitin for different usage is because it contains NH2 group. Because the core amino group and the primary and second hydroxyl groups is reactive, chitosan can be easily derivatised. [19] The first derivation of practically important was partially α-hydroxyethylated chitin or glycol chitin. It's a special biopolymer kind with advantage property like antibacterial activity, biodegradability and biocompatibility, which makes it a promising option for new functional material. Since chitosan is the only pseudo-natural cationic polymer; it is using in many different fields. [20]

Chitosan is considering as ideal medicinal excipient due its many benefit properties. It has versatile applications in drug delivery. [21] Chitosan based nanoparticle can effectively solubilize a wide range of hydrophobic drug, improve their bioavailability and extend their movement time in bloodstream making them suitable for targeted drug delivery. [22] It has been clear that biocompatible nanoparticle preferentially concentrates in solid tumors when given systemically due to their increased permeability and retention (EPR) effect. Numerous techniques and use for various chitosan base nanoparticles have been discovered in the recent years. [23] The current work focus on ionic gelation technique for manufacture of chitosan nanoparticle and screening fungus for fungal chitosan productions. The antibacterial property of chitosan nanoparticles was also examined.

2. MATERIALS AND METHOD

Fungal Culture

Rhizopus stolonifer was incubated on potato dextrose agar plate at 28 °C for seven days. A loopfull of active growing mycelia was inculcated in a 250 mL Erlenmeyer flask, containing 50 mL potato dextrose broth and keeped at 28 °C for 48 hours at 150rpm to obtain an uniform inoculum.

Determination of Optimum Growth Period

R. stolonifer were cultured in potato dextrose broth and was monitored over 8 days period to find out optimum time for max biomass. The culture maintained at pH 4.5 and temperature of 25° C. At regular intervals, flask having culture was sampled, filtered using Whatman filter papers, dried in 70 °C and weighted immediately to determine dry biomass without moisture interfere.

pH and Temperature

To identify optimum pH for fungal growth, culture was grow in potato dextrose broth (PDB) with initial pH adjusted between 4 to 10 using NaOH and HCl. The effect of pH on fungal biomass were evaluated by measuring the dry weight of mycelium after incubations. Similarlly, to determine the ideal temperature for fungal proliferations, the cultures was incubated at various temperature between 20 °C - 45°C and biomass measurements was used to asses which temperature supports highest growth.

Carbon Source

To study the outcome of various carbon sources on fungal fermentation, experiment was done in 500 mL Erlenmeyer flask filled with 300 mL of modified growth medium. The medium were added with different carbon sources like

fructose, starch, glucose, glycerol, lactose, sucrose and maltose to see their influence on fungal growth and metabolite production. A 6% (v/v) inoculum from pre-cultured fungal suspension was add into each flask. The cultures incubated at 28 °C for 96 hours on rotary shaker running at 150rpm to give proper mixing and air.

Chitosan Extraction

Fungal cultures were maintained on PDA slants and sub-cultured on Czapek-Dox agar (CDA) plates then transferred to Czapek-Dox broth (CDB) and incubated at 25 °C. Harvesting mycelia involved vacuum filtration, distilled water washing and freeze drying. Five gram of dried mycelia were ground, soaked for 12 hours in 100 millilitres of 2 M NaOH, then incubated for 13 hours at 45 degrees celcius. The alkali-insoluble mass (AIM) was collected by centrifugation (5 min at 16,000× g), washed with distilled water and 95 % ethanol and lyophilized. To extract chitosan, AIM was stirred in 2% acetic acid (200 mL / g) at 25°C for 1 hour. After that, the supernatant was attuned to pH 8.5 -9.0 using 2 N NaOH to precipitate chitosan. The pellet was centrifuge, washed (distilled water and 95% ethanol) and freeze dried. [24]

Fourier Transform Infra Red (FTIR) spectroscopic Analysis of Chitosan

The FTIR analysis of the prepared chitosan was accomplish following the procedure. In this method, 1% (w/v) chitosan solution was prepare using 1% acetic acid and allowed to dry. The resulting dried powder was finely ground and thoroughly mixed with potassium bromide (KBr) and then pressed into pellet. The FTIR spectra was documented using Thermo Nicolet Avatar 370 spectrophotometer covering a frequency range of 400-4000 cm^{-1}.[25]

Antimicrobial activity

The antimicrobial activity of chitosan extract was evaluated using disc diffusion method against a range of microbial strain. [26] The tested organism include *Bacillus cereus* ATCC 6633, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 27736, *Candida albicans* ATCC 90028, *Streptococcus mutans* and *Pseudomonas aeruginosa*. Sterile discs containing the chitosan extract and standard antibiotic ciprofloxacin discs were placed on agar plates that inoculated with each microorganism and plate were incubated under proper condition. The test for antimicrobial activity was performed in triplicates. Zone of inhibitions was measured to determine antimicrobial effectivity of chitosan extract against each strains.

Statistical analysis

The zone of inhibition was calculated as Mean±SD. The values were evaluated for the significant difference using one way ANOVA using Graphpad prism (version 9) at P-value < 0.05.

3. RESULTS

Optimization of Growth and Biomass Accumulation of *Rhizopus stolonifer*

R. stolonifer showed steady increase in biomass from day 1 (0.13 g) to Day 5 (0.53 g), indicating period of active growth. After day 5, the biomass slightly decline and then stabilised, suggesting onset of stationary phase. These results indicates that optimal period for harvesting maximum biomass is on or before 5th day of cultivation. The optimal incubation period is needed in industrial application where *R. stolonifer* is used for enzyme production, organic acid synthesis, or biotransformation process (Figure 2 and Figure 3).

Fig 2. Microscopic Identification of *Rhizopus stolonifer*

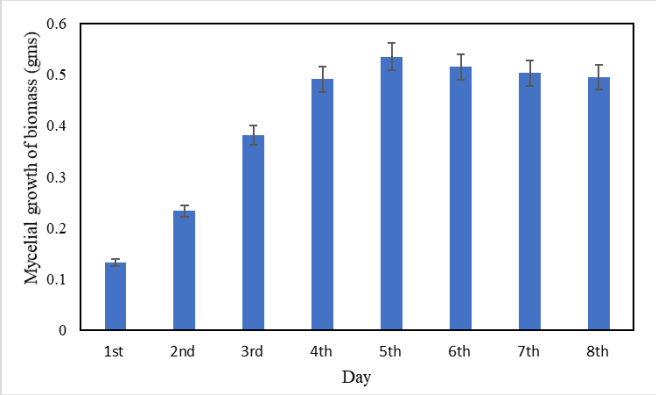

Fig 3. Growth of *Rhizopus stolonifera* biomass at different intervals (days)

Effect of pH

pH plays a major role in growth of the fungi also extracting chitosan. Maintain the best pH level in fermentation medium yield the best growth rate. Thus, the present study reveals the maximum growth of *R. stolonifer* was recorded at pH 6.0 as dry weight of 0.503g, according to results of pH experiments (Figure 4).

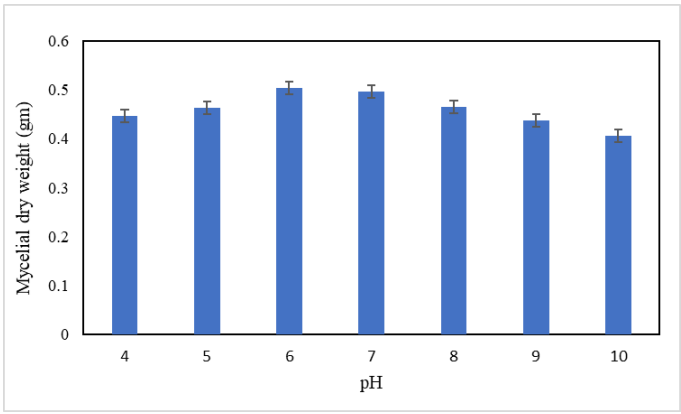

Fig 4: Growth of *Rhizopus stolonifer* at different pH

Effect of Temperature

In Figure 5, the result of different temperature on the growth of *R. stolonifer* at 20, 25, 30, 35, 40 and 45°C was investigated by inoculating a 3-mm disc of mycelium to 50 ml of Czapex Dox Broth. The optimum growth of dry weight of fungus was obtained at 30°C. The maximum yield was detected at 30°C.

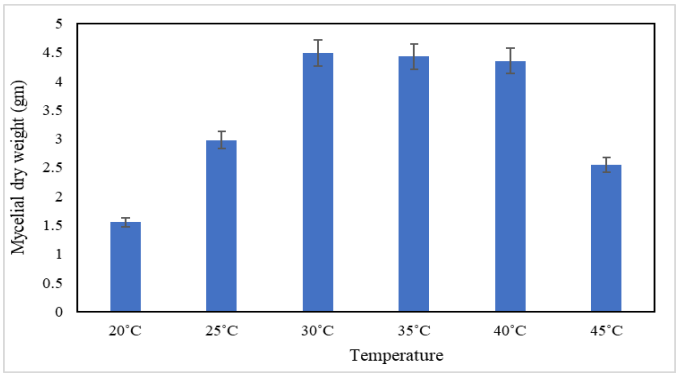

Fig 5 Growth of *Rhizopus stolonifer* at different temperature

Effect of Carbon Sources

The result of various carbon sources on growth of *R. stolonifer* was experimented with in Czapex Dox Broth (Figure 6). The test fungus was grown in 50 mL. CDB medium amended with fructose, starch, glucose, glycerol, lactose, sucrose and maltose in a 250 mL flask for 8 days. The maximum dry weight and maximum

growth in glucose amended medium with dry weight of 0.483 g were observed.

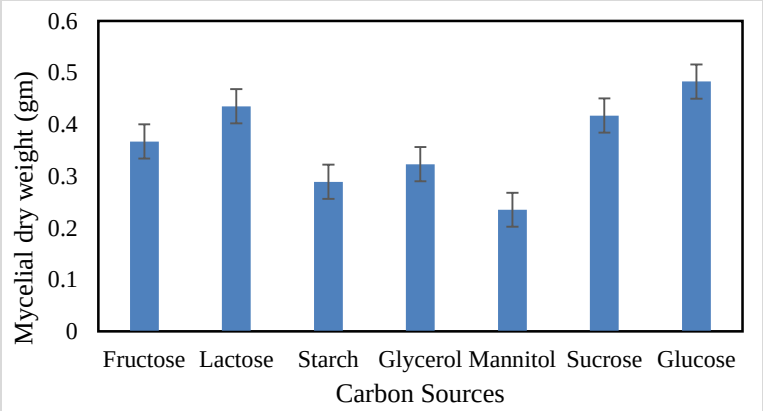

Fig 6: Growth of *Rhizopus stolonifer* with different carbon sources

Extraction and characterization of chitosan from *Rhizopus stolonifer*

The chitosan extraction from *R. stolonifer* was carried out by method of Fatima et al., (2021). [27] The results were found to be steady increase in chitosan production from day 2 (26.2 mg) to 7th day (81.4 mg), with highest yield observed on the 8th day at 80.5 mg (Table 1). Chitosan was extracted from *R. stolonifer* cultivated in Czapek Dox Broth (CDB) medium supplemented with malt extract. From 1 gram of dried fungal biomass, a chitosan yield of 7.62% was obtained. The extracted chitosan appeared as white flakes and was subjected to FTIR for structural characterization (Figure 7 and Figure 8). The characteristic absorption bands at 1651 cm-1, which corresponded to the amide I band (C=O stretching) and between 1032 and 1153 cm-1, which highlighted the C-O-C stretching vibrations in the polysaccharide backbone. The absorbance ratio method was used to calculate the degree of acetylation (DA) by comparing absorbance at 1655 cm-1 (amide I) to that at 3450 cm-1 (O-H stretching), and the results of the extracted chitosan showing high degree of deacetylation, i.e. DA value of 72.53%.

Table 1: Estimation of fungal chitosan

S.No	No. of days	Dry mass of chitosan content (gms)	Total content of chitosan (mg)
1.	2nd	0.310	26.2
2.	3rd	0.456	39.3
3.	4th	0.595	50.9
4.	5th	0.704	64.9
5.	6th	0.826	78.6
6.	7th	0.873	81.4

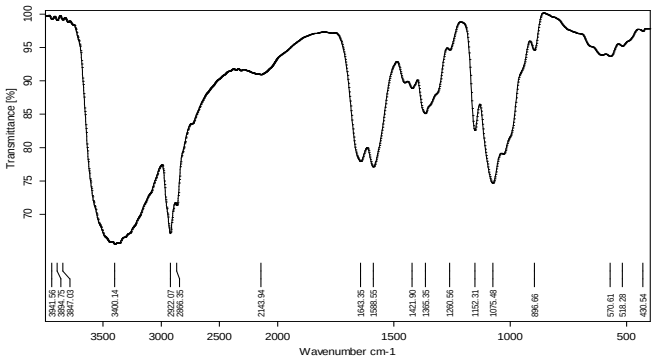

Fig 7: FTIR spectrum of chitosan standard (Sigma chem.)

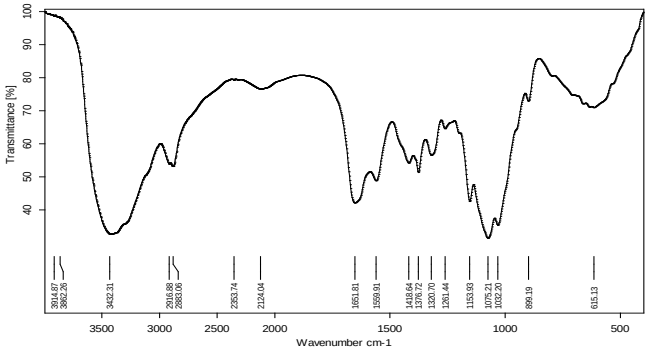

Fig 8: FTIR spectrum of extracted chitosan

Antimicrobial activity of the chitosan

The antimicrobial activity of the chitosan from *R. stolonifer* was evaluated against a test organism using varying concentrations (250 µg, 500 µg and 1000µg) and compared with the standard antibiotic ciprofloxacin as shown in the Table 2. The results were exhibited that chitosan had concentration-dependent inhibition effects, with the highest activity recorded at 1000 µg. *Bacillus cereus* and *Candida albicans* was showed significant sensitivity at 1000 µg, both having a zone of inhibition of 16 mm, which reduce to 6 mm at 500 µg and not present at 250 µg. Also, *Streptococcus mutans* didn't show any activity at 250 µg but had moderate sensitivity with zone of 11 mm and 7 mm at 1000 µg and 500 µg, respectively. While *Klebsiella pneumoniae* and *Escherichia coli* were totally resistant at every concentration tested, *Pseudomonas aeruginosa* had only a slight sensitivity (6 mm) at the highest concentration. The results of standard antibiotic disc Ciprofloxacin shows that maximum zone of inhibition was recorded against *B. cereus* (35±4mm), followed by *E.coli* (29±3mm), *K. pneumoniae* (26±3mm), *P.aeruginosa* (25±1.5mm) and *S. mutans* (17±1mm). However, no activity was recorded against *C. albicans.*

Table 2: Antimicrobial activity of the chitosan nanoparticles

Test organisms	**Sample Conc. (1000 µg)**	**Sample Conc. (500 µg)**	**Sample Conc. (250 µg)**	**Ciprofloxacin (standard 5 µg)**
Bacillus cereus **ATCC 6633**	16±2mm	6±1mm	Nil	35±4mm
Escherichia coli **ATCC 35218**	Nil	Nil	Nil	29±2mm
Klebsiella pneumonia **ATCC 27736**	Nil	Nil	Nil	26±3mm
Candida albicans ***ATCC 90028***	16±1.5mm	6±2mm	Nil	---
Streptococcus mutans	11±2mm	7±2mm	Nil	17±1mm
Pseudomonas aeroginosa	6±0.5mm	Nil	Nil	25±1.5mm

4. DISCUSSION

The present study investigated several factors that influence the growth and chitosan production potential of *R. stolonifer*, aiming to optimize the conditions for maximum biomass accumulation and bioactive compound yield. The growth curve of R. stolonifer showed a clear exponential phase from day 1 to day 5, with biomass increasing from 0.13 g to 0.53 g This trend indicates active fungal metabolism supported by enough nutrient availability, and favorable environmental condition, consistent with earlier observations in similar filamentous fungi [28, 29]. A slightly decline and then stabilization in biomass after day 5 suggest the transition to stationary phase, possibly due to nutrient

deplet or build-up of metabolic byproducts in the medium. [30] These finding imply that day 5 marks the best harvesting time for maximum biomass. This is important in industrial application like enzyme production organic acid synthesis, and biotransformation.

Among the tested pH levels, pH 6.0 showed the highest biomass production (0.503 g), matching with findings reported for *Aspergillus niger, Aspergillus ochraceous* [31] and *Tuber koreanum*. [32] This pH may favor activity of key fungal enzymes and maintain membrane integrity, thus enhancing nutrient uptake and metabolic efficiency.

Temperature optimization showed 30°C as the optimal point for fungal growth, with maximum dry weight recorded at this condition. These results closely match earlier findings in related species, [33] although slight inter-strain variability is common due to differences in physiological tolerance and metabolic rates.

Glucose was the most effective carbon source for *R. stolonifer*, giving a maximum biomass of 0.483 g. The better growth in glucose-amended media is supported by its quick assimilation and role in boosting energy metabolism, as also seen in *Fusarium verticillioides* [34] and *Candida albicans*. [35] The results highlight the importance of easily metabolizable sugars in maximizing fungal biomass.

Chitosan extraction from *R. stolonifer* showed a steady increase in yield, peaking at 81.4 mg on day 7. This corresponds with the active growth phase where chitin builds-up in the fungal cell wall. The slight drop on Day 8 might be because of enzymatic degradation or early signs of nutrient exhaustion Compared to yields reported in *Mucor pseudolusitanicus* (up to 40.59 mg/g biomass), [36, 37] the performance of *R. stolonifer* is remarkable and highlights its potential as competitive fungal chitosan producer.

FTIR analysis confirmed the structural integrity of the extracted chitosan with characteristic absorption bands for amide I, and polysaccharide backbones. The degree of deacetylation (DA) was calculated as 72.53%, showing a high level of functional group conversion, and solubility as per methods described by previous researchers [38,39] This value falls within the reported ran ge for fungal chitosan (74-99%), [40,41] and is important for biological activity. The high DA supports the suitability of *R. stolonifer* derived chitosan for applications requiring bioactivity and solubility including biomedical and pharmaceutical fields. Also fungal-derived chitosan offers advantages over crustacean sources in terms of sustainability, reduced allergenicity, and freedom from seasonal harvesting. [42-44]

The antimicrobial activity of *R. stolonifer* chitosan demonstrated concentration dependent inhibition especially against Gram-positive bacteria (*Bacillus cereus, Streptococcus mutans*), and the yeast *Candida albicans*. Maximum inhibition was seen at 1000 µg/mL, with zones of inhibition up to 16 mm In contrast, Gram-negative bacteria (*Klebsiella pneumoniae Escherichia coli*) showed resistance while *Pseudomonas aeruginosa* had minimal sensitivity. Moreover, in comparison with the standard antibiotic ciprofloxacin the extracted chitosan has lower activity against all the bacteria tested. However, it possesses antifungal activity against

Candida albicans where the standard antibiotic ciprofloxacin lacks antifungal activity This pattern matches previous studies showing the electrostatic interactions between positively charged chitosan amino groups and negatively charged microbial cell walls. [45-47] The outer membrane of Gram-negative bacteria likely acts as a structural barrier explaining their lower susceptibility. [48, 51]

Overall, these findings support the potential of *R. stolonifer* derived chitosan as a natural and effective antimicrobial agent particularly in applications targeting Gram-positive pathogens, and fungal infections. The combined growth efficiency chitosan yield, high DA, and selective antimicrobial action confirm the suitability of *R. stolonifer* as a sustainable biotechnological resource.

5. CONCLUSION

This study shows that chitosan from *R. stolonifer* could be a reliable and sustainable biopolymer with good antibacterial property. The activity seems depends a lot on the concentration and the specific microbes involved, but overall, it's looks promising for things like food protection, pharma uses and maybe even in medical. Fungal chitosan giving benefits such as easy to grow, lower allergy risk and fit with vegetarian or diet limits when compare to usual sources like crustacean shells. So, fungal chitosan offers a strong alternate for making eco-friendly and effective antimicrobial agents.

Abbreviations:

AIM- Alkali Insoluble mass

CDA- Czapek-Dox Agar

CDB- Czapek-Dox broth

DA -Degree of acetylation

FTIR- Fourier Transform Infra-Red spectroscopy

gms- grams

HCl- Hydrochloric acid

mg- Milligram

µg- Microgram

mm- millimeter

NaOH- sodium Hydroxide

PDA- Potato dextrose agar

PDB- potato dextrose broth

SD- Standard Deviation

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Writing – original draft: SA, PAR

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