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Synthesis of Completely Deacetylated Chitosan for Enhanced Anticandidal Action

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ABSTRACT

The study optimizes efficient synthesis of completely deacetylated chitosan or Ultra High Chitosan (UHC), establishing its enhanced physicochemical properties and using it to target Fluconazole resistant *Candida sps*. UHC and chitosan with 85% of degree of deacetylation (DD%) were synthesized using 2.5M Sodium Hydroxides (NaOH)at temperature ranging from 150-200°C. The biomaterial with varying DD% was tested for its anti-candidal activity which included MIC, MBC, inhibition of virulence factors and growth. Chitosan with >99DD% was produced using 5 folds lesser concentration and 4 fold lower volume of NaOH, lowering the wash water by 70%. When used as Fluconazole resistant anticandidal polymer, it exhibited 100% germ tube and 25% of biofilm inhibition. It also completely inhibited cell duplication within six hour.Synthesis of UHC with enhanced anti-candidal activity and physicochemical properties like FBC and WBC expands the application of this multi-faceted biopolymer.

Keywords: Ultra-High Chitosan, *Candida sps*., Degree of Deacetylation.

INTRODUCTION

Chitosan is one of the polycationic biopolymers that could find extensive applications in healthcare. It has been investigated as an antimicrobial material against algae, bacteria, yeasts and fungi in different forms like solutions, films and composites.Chitosan based scaffold materials have been used in controlled drug release(Bharathi et al., 2022). The schaffolds of chitosan for the delivery of TetracyclinHCl, Ofloxacin, Doxycyclin, Natamycin, Ciprofloxacin hydrochloride, metronidazole benzoate, Ornidazole, e-aminocaproic acid are the recently studied(Atia et al., 2022).

Chitosan itself has an antimicrobial potential due to its cationic nature. The factors like positive charge density, molecular weight, physical state of chitosan, pH, gram nature of bacteria affects the antimicrobial activity. The positively charged polymer is capable of interacting with negatively charged microorganisms inhibiting their growth and proliferation(Ardean et al., 2021). Of the varied emerging antifungal therapies, the use of chitosan with Fluconazole by Wei-Hsuan et al. 2020, seem to be promising against drug resistant Candidal strains. Low molecular weight chitosan with >75 DD% and at pH of four is also reported to have anticandidal activity. Increased degree of deacetylation is directly proportional to cationic charges of polymer which in turn enhances antimicrobial activity. Literature suggests, molecular weight as well as DD% affects this activity (Avelelas et al., 2019). Studies on protonation of amino groups of chitosan followed by its further quaterisation have also been reported to have excellent antibacterial and antifungal activities(Ardean et al., 2021). Studies correlating the DD% and zeta potential to anticandidal activities are however lacking and so are the effective and environmentally friendly methods of producing completely deacetylated chitosan or UHC i.e with DD% > 95%.

Opportunistic pathogens like *Candida* have developed resistance owing to unnecessary, prolonged exposure to antifungals, uncontrolled use of steroids and delay in effective antifungal therapy (CDC, 2022). According to World Health organizations, antimicrobial resistance is one of the top ten global public health concerns. Besides being a part of the body's natural flora, this opportunist is known to be present on inanimate surfaces including shower curtains, stethoscopes, door handle, and medical equipment etc. (Kim et al., 2021)Patients on mechanical ventilators are at a greater risk of developing Candidal infections (Jean et al., 2019). The symptoms of Candidal infections range from a white coloured thrush on nose, mouth, lungs, stomach to more invasive forms that include fever, abdominal pain, urinary tract infection, fall in blood pressure etc. (CDC, 2020).Candidal biofilm as well as the germ tube formation have been the key factors considered while treating the infections (Carola et al., 2022).

The present work effectively addresses the issues, paving way for scale up in production of UHC as well as widening its applications in the field of smart packaging, cholesterol binding, food preservation etc. UHC was synthesized using a new chemical method using less consumables and energy. The biopolymer thus synthesised was found to have superior physicochemical properties like zeta potential, Fat Binding Capacity (FBC) and Water Binding Capacity (WBC) and % solubility. The reduction in the volumes of wash water produced after deacetylation was achieved by substrate size reduction. As at industrial level, treatment of wash water adds more energy consumption in the whole process.

The UHC thus produced rendered benefits as an effective water soluble antifungal enabling the 100% inhibition of Candidal growth within 6hr, complete germ tube inhibition and 25% of biofilm inhibition. The study stands apart since the anticandidal activity of chitosan was boosted merely by modifying its method of synthesis, without additions of any antifungals or derivatization of this polycationic polymer.

MATERIALS AND METHODS

Materials

A clinical isolate of *Candida* spp. was procured from a hospital repository in Mumbai and was identified by standard methods. Chitin flakes with DD% of >50, purchased from Himedia, India pulverized using a grinder and sieved through a 0.5mm pore size metal sieve to obtain uniformly sized flakes that were further used for the study. Chitosan with DD% of >75, Sabouraud agar and broth, and Fetal Bovine Serum (FBS) were purchased from Himedia. NaOH pellets were purchased from SdFine chemicals. All the chemicals were of analytical grade. All experiments were conducted in triplicates.

Modified method of deacetylation

Chitin particles with 0.2mm size were treated with concentrations ranging from 2.5-7.5M NaOH with a ratio of 1:5 (w/v). These were then exposed to varying temperatures in a hot air oven between 150-200°C till NaOH recrystallized. This was then crushed in a mortar and pestle, washed with DW so as to obtain a pH of 7-7.5 and dried in a hot air oven at 60°C for 4hr.

Characterization

Acid-base titration for quantitative estimation of DD%

DD% was determined by acid-base titration. Chitosan was dissolved in 0.3N HCl at a 0.1:4 (w/v) ratio, and the volume was made to 80mL with DW. This was titrated against 0.1N NaOH and a change in pH was recorded(Zhao et al., 2022). Graph of pH vs volume of NaOH was plotted and DD% was calculated according to the following formula,

$$DD\% = 203 * (V2 - V1) * \frac{N}{m} + 42 * (V2 - V1) * 100 \quad (1)$$

Where, V2 and V1 are the volumes of NaOH, indicating 2 inflection points.
N = NaOH normality in moles
m = Mass of polymer in gram
203 is Molar mass of acetyl monomer in g/mol
42 is the Difference in molecular weight of acetyl and deacetylated monomer (g/mol)

Confirmatory test using potassium iodide and sulphuric acid

The change in colour of the precipitate upon mixing with Potassium Iodide solution in the presence of 1% H^2SO^4 confirmed the production of chitosan(Gomaa, 2017).

Qualitative estimation: By FTIR analysis

Samples were analyzed using a 3000 Hyperion Microscope with a Vertex 80 FTIR system for the frequency range from 4000 to 400cm^{-1}.

Water and Fat binding capacity of chitosan

The Water Binding Capacity (WBC) and Fat Binding Capacity (FBC) were calculated according to(Rasweefali M,et al.2021),where 20 times more volume of DW or Soybean oil was added respectively per gram of chitosan. WBC and FBC were calculated after 30min of mixing.

$$WBC(\%) = \frac{Water\ bound\ (g)}{Initial\ weight(G)} * 100 \quad (2)$$

$$FBC(\%) = \frac{Fat\ bound\ (g)}{Initial\ chitosan\ weight(g)} * 100 \quad (3)$$

Correlation of characteristics and anti-candidalactivity of chitosan with varying DD%

Chitosan with varying DD% was studied for its anticandidal activity. Chitosan with >50%, >75%, and 100 DD% were tested for their solubility %, zeta potential, MIC, MBC, killing time, and inhibition of virulence factors.

Solubility %

Chitosan was placed in a pre-weighed centrifuge tube and dissolved in 1% acetic acid with a ratio of 1:100 (w/v) at 60°C for 1 hr. This was centrifuged at 5000 RPM for 10 min. The dissolved chitosan in the supernatant was removed, and the remaining pellet was dried at 60°C and weighed. The solubility was calculated by the following formula (Rasweefali M,et al. 2021).

$$Solubility(\%) = \frac{M1-M2}{M1-M0} * 100 (4)$$

Where,
M0 = Tube's initial weight
M1 = Tube and sample initial weight
M2 = Tube and sample final weight

Zeta potential

Zeta potential is an electrokinetic potential in colloidal systems in the interfacial double layer at the location of the slipping plane versus a point in the bulk fluid away from the interface.The charge density of chitosan with varying DD%, suspended in 0.2% acetic acid was determined by usingMalvern's zetasizer, nanoZS90.

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

1mg/mL chitosan stock solution with DD% of >50, >75, and 100 was prepared in 0.5% acetic acid and antimicrobial activity was tested using concentrations from 0.01 to 1mg/mL. *Candida sps.* with a cell density of 10^4cells/mL was treated with chitosan for 24h at 37°C in sabouraud broth. Absorbance was measured at 600nm to observe the reduction in growth. The spread plate method was also performed to determine MBC on Sabouraud agar plates, and a colony count was taken after 24h of incubation at 37°C. A test containing only sabouraud broth was considered a positive control whereas Fluconazole of 1mg/mL was used for negative control(Darroudi et al., 2021).

Time kill assay

Active cells at a density of $3*10^4$cells/ml were exposed to Chitosan samples at a concentration of 0.06mg/mL, Fluconazole (1mg/mL) for control, and only Sabouraud broth for positive control. At an hour interval, growth was evaluated by spreading 100µL suspension on Sabouraud agar plate. The plates were incubated at 37°C for 24 hr and CFU count was taken(Ibrahim et al., 2021).

Germ Tube inhibition Assay

The Germ Tube Inhibition Assay is a screening test used to differentiate *Candida* spp. from other yeast. When *Candida* sps. is grown in human or sheep serum or Fetal Bovine Serum (FBS), it forms germ tubes, which can be detected as filamentous outgrowths extending from yeast like cells. The test included the use of 10^6cells/ml of *Candida* sps. A positive control containing 500µl of fluconazole (1mg/ml), a test including 500µl chitosan solutions (0.06mg/ml), a negative control with 500µl of sterile DW, and an acid control with 0.5% of acetic acid was also used to check the effect of acidic conditions on germ tube formation. These tubes were incubated for 2h. Each tube was then incubated at 37°C with 500µl of FBS for 3h to initiate germ tube formation(Gupta, Bisht, & Kelkar-Mane, 2019). Inhibition and formation of germ tubes were calculated according to the following equation:

$$\%Germ\ tube\ formation = \frac{No.of\ cells\ showing\ germ\ tube\ formation}{Total\ no.of\ cells} * 100 \quad (5)$$

$$\%Germ\ tube\ inhibition = 100 - \%\ germ\ tube\ formation \quad (6)$$

Biofilm quantification

Active *Candidal* cells were exposed to Chitosan with >50, >75, and 100DD% at a concentration of 0.06mg/ml. Fluconazole 1mg/ml was used for negative control. It was then incubated for a period of 24h, during which biofilm formation was allowed in the test tube. Briefly, 10^7cells/ml were allowed to adhere to the glass surface by incubation at 37°C for 24h. with test solutions at 150RPM, followed by washing with PBS (pH 7.4) three times to remove non-adherent cells and dried at 37°C for 20min. Biofilm was stainedusing 0.01% crystal violet for 30min., followed by washing with PBS to remove excess stain. The absorbed stain was eluted using 30% glacial acetic acid and measured at 550nm(Mudiar & Kelkar-Mane, 2018).

The absorbance of crystal violet eluted from a glass tube incubated without chitosan was considered a negative control. The absorbance was compared with the samples to calculate the percentage inhibition of biofilm using the formula:

$$\%Inhibition = \frac{Abs.control\ -Abs.test}{Abs.control} * 100 \quad (7)$$

Statistical analysis
All experiments were conducted in triplicate and mean, standard deviations were computed using Microsoft Excel.

RESULT AND DISCUSSION

Acid-base titration for quantitative estimation of DD%
Deacetylation is a NaOH demanding step and industrially 50-30% NaOH is currently being utilized. The DD% of chitosan thus obtained was76.23 ± 0.21%. The modified method proposed in this study utilized 7.5M, 5M and 2.5M NaOH for deacetylation giving chitosan with >99DD%. Lowering the temperatures to 150°C with 2.5M NaOH resulted in chitosan with 83-85DD% and reduction in the volumes of wash water (Table1).

Confirmatory test: By Potassium Iodide and Sulfuric acid method
Production of chitosan was confirmed by using Potassium Iodide and Sulphuric acid, wherein the test showed a dark purple precipitate of chitosan after the addition of Sulphuric acid.

Qualitative estimation: By FTIR analysis
FTIR spectroscopy has been widely used to study the chitin and chitosan molecular interactions. Typical chitin and chitosan spectra showed 4 signature bands. A broad peak from 3000 to 3800cm^{-1} is indicative of O-H stretching and N-H stretching vibration. Broadness of the peak was related to the extent of hydrogen bonding. As oxygen pulls away the electrons, less energy was required for vibration and the peak appeared broader. Figure 1 shows the broadness of the peak at 3000-3600cm^{-1} decreased after deacetylation, which was indicative of a reduction in hydrogen bonding. A decrease in band intensities can be ordered from the samples A, B, D and C. As indicated by DD% of sample A, B and D were 100%, whereas sample C had 83-85DD%.

Band intensities at 1676cm^{-1}, 1637cm^{-1} and 1575cm^{-1} were strongly related to degree of deacetylation, as they were associated with C=O stretching vibration, prevalence of NH_2 group of amide I band and NH_2 bending vibration of amide II band respectively. After deacetylation, intensity at 1676cm^{-1} decreased and intensity of the 1575cm^{-1} band increased, indicating the prevalence of the NH_2 group. Sample A and B showed lowest intensities of band around 1676cm^{-1} indicating superior deacetylation than sample D and C. The intensity of the band around 1575cm^{-1} was lower in all the samples.

Bands between 929cm^{-1} to1298cm^{-1} corresponds to skeletal vibration or C-O-C deformation. All deacetylated samples showed decreased band intensity of C-O-C deformation. This can be attributed to the breaks in the inter-polymeric linkages leading to increased reactivity and thus wider applications.

The band observed around 560cm^{-1} was related to NH bending vibration, which was higher in samples A and B. Though sample D showed 100 DD% in acid base titration, the same was not supported by FTIR spectra.

Other observed bands at 2933cm^{-1} represented stretching vibrations of C-H (of CH_3 and CH_2). The absorption band around 1400cm^{-1} confirms the presence of CH_3 bending and CH_2 deformation in chitin. The fluctuation in the intensities observed around the abovementioned bands varied with the concentration of NaOH used. The abovementioned bands are considered the signature spectrum of chitosan. Supplementing the titration method of determining DD% with FTIR appears necessary when the spectra of the samples are compared.

Water and Fat Binding Capacity
The WBC of procured chitin flakes was 629%, whereas samples B showed 646%. Changes in WBC% are attributed to crystallinity, residual protein content, and the presence of salt-forming groups of polymers and temperature of deacetylation.

The FBC of untreated chitin was 400% and sample C showed 544%. FBC of sample C was found to be highest for 544%. The fat binding capacities of chitosan are higher than chitin and depend on physicochemical properties as well as molecular weight. The WBC and FBC of chitosan appear to increase prepared by modified method corroborate with there in18 and 19.

Correlation of characteristics and anti-candidalactivity of chitosan with varying DD%
Solubility%
UHC showed 91% solubility, whereas commercial chitosan showed 24% solubility and commercial chitin showed 7% solubility. Solubility of chitosan is dependent on free amino groups or DD%. The amino groups present in chitosan are protonated ($-NH_3^+$) and solubilize in acidic conditions further increasing electrostatic attractions to

negatively charged microbial surfaces. Chitosan solubilized in acidic conditions are more effective against *C. albicans* when compared with chitosan dispersed in water at same concentration(Qin et al., 2006).

Zeta Potential

Zeta potential values obtained for chitosan with varying degrees of D areas tabulated in the table 3. UHC reported zeta potential of 35.8mv, followed by >75DD% with 29mv >50DD% with 15.6mv and of zeta potential. High zeta potential is an indication of the good stability of the colloidal system.

Literature cites chitosan nanoparticles with zeta potential of 20.6mv±1.04 to have better antimicrobial action than those with 13.6mv±0.52(Alqahtani et al., 2020).

MIC and MBC determination

The anti-candidal activity of chitosan was carried out using a 24h old culture with a cell density of 10^4cell/ml. Chitosan with concentration range of 1 to 0.01mg/ml were used in the study. Chitosan samples with >50, >75, and 100 DD% showed 0.5, 0.25, and 0.25mg/ml of MICs, while MBCs were 0.5 and 0.25mg/ml, 0.06mg/ml respectively. The free amino groups of UHC, solubility, DD%, zeta potential are combined factors responsible for anticandidal activity. Similar results have been observed by Avelelas F, et al. where chitosan with 88.2DD% showed higher antimicrobial activity in comparison to 87.1DD% and 84.1DD%(Avelelas et al., 2019).

Time kill assay

Within 4h, CFU count indicated 99% inhibition and at 6h, growthof *Candida*was completely inhibited by UHC at a concentration of 0.06mg/ml. On the other hand, chitosan with >75 DD% and >50 DD% did not show inhibition till 7hr of incubation. The slow decrease in CFU count in control could be attributed to the stressed population of cells due to their suspension in PBS (Figure 2). Recent references have also showned killing of *Candida* cells within 9hr by UHC compared to chitosan with 78.71DD%(Kim et al., 2022).

Germ Tube formation

Cells of *Candida* sp. were examined microscopically at 45X and the percentage of germ tube formation for each tube was calculated. 50 sequential cells were counted and cells showing constriction at the site of emergence were disregarded. UHC completely inhibited the germ tube formation whilechitosan with >75DD% inhibited 99% of germ tube formation whereas Chitosan with >50DD% showed 54% inhibition. Germ tube formation is putative virulence factor of *Candida spp*. and it can be completely inhibited by UHC and can thus be used for invasive infections.

Biofilm Quantification

Fluconazole used for positive control with a 1mg/ml concentration showed 27±0.5% of biofilm inhibition. Chitosan with >50, >75, and 100 DD% showed 23±4, 17±3, and 25±1% of biofilm inhibition DD% (Figure 3). Amin Zhang et al. showed enhanced anti-biofilm action by 88DD% than in comparison to 50DD% chitosan. Both the virulence factors were inhibited effectively by the UHC as compared to intermediate and low DD%. The standard error when samples with >50DD% and >75DD% was more due to inconsistent range of DD% of product.

CONCLUSION

The method proposed herein paves way for effective deacetylation of chitin with a potential to be scalable. The work also correlates the anticandidal activity to the degree of deacetylation and polycationic nature of chitosan. UHC synthesized had 2.08 fold lower MBC than studiesreported so far. The inhibition of virulence factors including germtube and biofilm formation followed by complete inhibition of candida within 6hrs of exposure by UHC without any additives or modifications has so far been not been reported. The enhanced physicochemical properties of UHC like the zeta potential, FBC, WBC enable its potential use in smart packaging and beyond.

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Table 1. Concentration and time required by modified method, DD% obtained and volumes of wash water generated

Sample code	Temperatures(^{0}C)	NaOH concentration (M)	DD%	Wash water (ml)
A	150-200	7.5	100	180-340
B	150-200	5	100	160-185
C	150-180	2.5	83-85	102-220
D	190-200		100	125-132
Standard Chitin	-	-	>50	-
Standard Chitosan	-	-	>75	-

Table 2.WBC and FBC of chitosan obtained after classical and modified method of deacetylation in comparison to references 20 and 21.

Sample	WBC %	FBC %
Standard chitin	629	400
Standard Chitosan		
Sample A	672	414
Sample B	515	410
Sample C	646	544
Sample D	266	433
1. (Noet al., 2000)	458-805	314-535
2. (Vanitha et al. 2018)	274-795	316-519

Table 3.Zeta potential (mV) of samples with >50, >75 and 100DD%

DD%	Zeta Potential (mV)
>50	15.6
>75	29
100	35.8

Table 4. MIC and MBC values of chitosan samples

Sample	MIC	MBC
50 DD %	0.5	0.5
75 DD %	0.25	0.25
100 DD %	0.25	0.06

Table 5.% Germ tube formation and inhibition after treatment of chitosan

Samples	% inhibition of germ tube
Positive control	0
Negative control	73
Acid control (0.5% Acetic acid)	56
>50DD%	54
>75DD%	99
100DD%	100

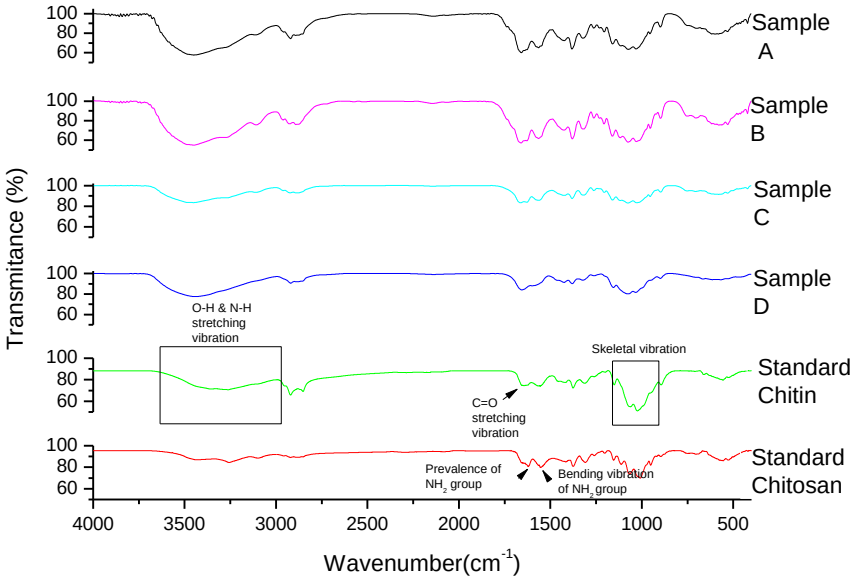

Fig. 1 FTIR spectra of chitin (>50DD%), Sample A, B, C and D.

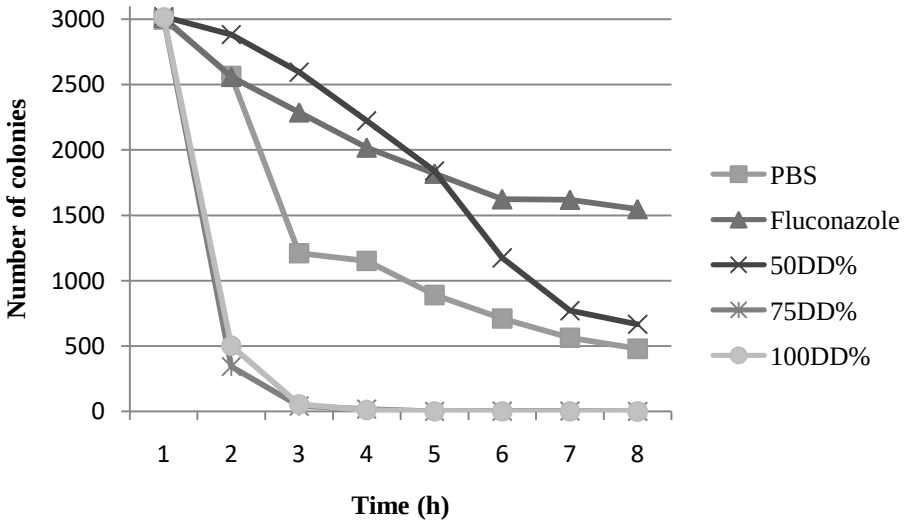

Fig. 2 Time kill assay of *Candidasps*. using chitosan with >50,>75 and 100DD%.

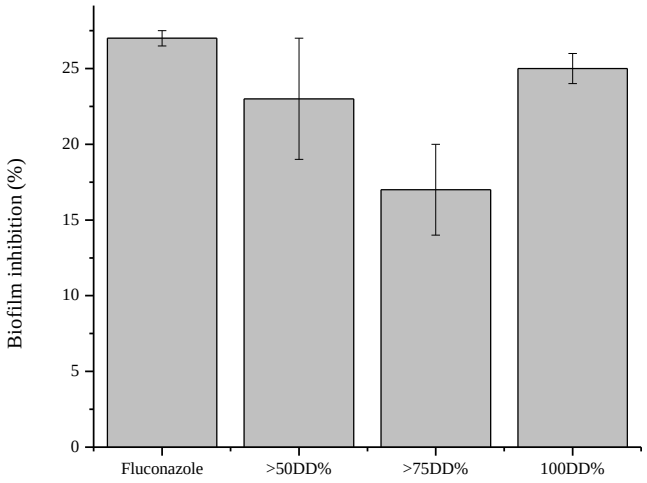

Fig. 3 Biofilm inhibition while positive control (Fluconazole), chitosan with >50, >75 and 100DD% were tested. Values are expressed in percentage biofilm inhibition. SD (n=3).