Graft copolymerization of methyl acrylate on chitosan: Initiated by ceric ammonium nitrate as the initiator-characterization and antimicrobial activity

Manoj Pati[1] and P. L. Nayak*[1&2]

[1]Eastern Academy of Science & Technology, Dept. of Chemistry and Environmental Engineering, Bhubaneswar, Odisha, India
[2]Foundation Research, Naya Bazar, Cuttack, Odisha, India

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ABSTRACT

Methyl acrylate was grafted onto chitosan by using the ceric ammonium nitrate as the initiator. The effect of initiator concentration, monomer concentration, time & temperature on % G and % E were studied. The grafted samples were characterized using FTIR, SEM, TGA methods. Evidence of grafting was confirmed by FTIR spectroscopy. The morphology evaluated by SEM. The thermal analysis indicated the different stages of the degradation of the grafted copolymer. The antibacterial and antifungal activities of the grafted polymer have also been investigated. The grafting compound was applied onto chitosan initiated by ceric ion at 1 wt. % add and was found to have a good antimicrobial and antifungal performance.

*Keywords***:** Chitosan, Methyl acrylate, Graft copolymerization, Antibacterial, Antifungal.

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INTRODUCTION

Chitosan is a linear polysaccharide derived from chitin, the second most abundant organic compounds only next to cellulose in the nature. Chitin can be found in the shells of marine invertebrates (crabs, crustaceans, etc.), fungi, insects and yeasts. Depending on the source, it generally functions as an exoskeleton, providing structural integrity, commonly embedded in a matrix of proteins, minerals and at times various other polysaccharides. Chitin is a homopolymer comprised of 2-acetamido-2-deoxy-β-D-glucopyranose units; however, some units exist in the deacetylated form as 2-amino-2-deoxy- β -D-glucopyranose. When chitin is deacetylated to at least 50%, it is referred to as chitosan, in other words this is essentially the N-deacetylated derivative of chitin (chemical structure is provided below in Figure 1A, where x = 1-y, y being the proportion of acetylated repetition units).

Fig-1 Structure of Chitosan

R = H or COCH₃
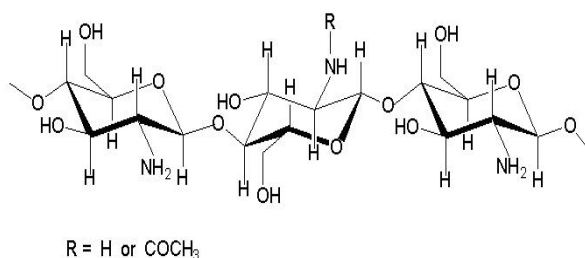
Chitosan has many interesting biological and chemical properties. The excellent features such as biocompatibility, ecologically safe biodegradability (degradation products of chitosan are non-toxic, non-immunogenic and non-carcinogenic) and low toxicity with versatile biological activities (chitosan has antimicrobial activity and low

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immunogenicity) recommends this biopolymer for applications in biomedicine [1-5]. In addition, chitosan is very efficient for interaction with the anionic solutes including dyes in acidic solutions due to the reactive groups, such as –OH and –NH$_2$. This property has been widely used for the removal of the water-soluble dyes, as an alternative to the conventional sorbents and flocculants [6,7]. However, due to inter- and intramolecular H-bonding, chitosan is only soluble in few dilute acid solutions (depending on the molecular weight), which limits its applications. As a result, many attempts have been made in chemical modification of chitosan, aiming at improving its water solubility. Among these an ideal way is the graft modification of chitosan [8-11]. The main advantage in the grafting efforts is the high degree of functionality of chitosan - the molecule backbone contains two hydroxyl groups and one primary amine group per repeat unit. The active primary amino groups on the molecule being reactive provide sites for the attachment of different side groups employing mild reaction conditions. In this way versatile materials based on chitosan with specific functionality can be obtained.

Grafting vinyl monomers onto chitosan [10, 11] is one of the most effective methods to improve the performances of chitosan without sacrificing its properties and also is a challenging field of research with unlimited future prospects. Vinyl graft copolymerization can be described as the modification of a preexisting polymer chain (trunk polymer).

Graft copolymers are synthesized to improve physicochemical properties of synthetic/natural polymers for applications in agriculture, biomedicine and other fields. Different studies have been published on the grafting copolymerization of chitosan with various vinyl monomers like acryonitrile [12], methyl methacrylate [13], Acrylamide [14], acrylic acid [15], using cerium ammonium nitrate as redox initiators.

In this context, the present study is focused on the copolymerization of chitosan with different vinyl monomers via surfactant-free emulsion copolymerization (SFEP) using potassium persulfate as initiator. The SFEP technique is one of the most important methods for the synthesis of polymer beads with controlled size [16-21]. Polymeric colloidal micro- and nanoparticles possessing an extremely large surface area are attractive candidates as carrier vehicles for bioactive substances, such as therapeutic drugs, proteins, genes, or enzymes. For such applications, colloidal particles prepared from biocompatible and biodegradable polymers are desirable. .

In the present research program, we wish to report the graft copolymerization of Methyl Acrylate onto chitosan using ceric ammonium nitrate as the initiator. The graft co- polymerization was studied by varying the initiator, time, temperature and concentration of monomer. The grafted polymers were characterized by SEM, FTIR and TGA studies. The antibacterial and antifungal activities of the grafted samples have been reported.

MATERIALS AND METHODS

Materials
Chitosan (CS) (Degree of Deacetylation = 95% determined by 1H-NMR and Molecular Weight 13.45×104 Da) was purchased from India Sea Foods, Kerela, India. Methyl Acrylate and other chemicals were used as analytical grade and purchased from Sigma Aldrich Company.

2.1 Graft Copolymerization
A chitosan aqueous solution of 2 wt % was prepared by dissolving 20 g of chitosan powder in 1000 mL of acetic acid solution (1%, v/v). After chitosan was dissolved, the solutions were filtered with cheesecloth by vacuum aspiration to remove foam and any undissolved impurities. The ceric ammonium nitrate in 0.5 M nitric acid solution was then loaded into the reactor under continuous stirring. Then a known weight of Methyl Acrylate was also injected into the reactor. The reaction was assumed to have started at the moment the monomer was injected. The grafting reaction was carried out under nitrogen atmosphere in a 500 mL, four-necked flask equipped with a reflux condenser, a stirrer, dropping funnel, and a gas inlet system immersed in a constant temperature water bath. In a typical reaction, Chitosan (0.006–0.025 mol; 1–4 g) was dispersed in a definite volume of water with constant stirring and bubbling of a slow stream of nitrogen for 30 min at the desired temperature (20–40^{0}C). After 30 min, a freshly prepared 10 mL solution of CAN (0.02–0.06 mol, 0.11–0.33 g) in nitric acid (0.1–0.4N) was added and stirred for 10 min. Nitrogen gas was continuously passed through the reaction mixture and AN (0.091–0.152 mol, 6–10 mL) was added. In all the reactions, total volume of the reaction was kept constant. The grafting reaction was carried out for varying time intervals (1–4 h). The zero time of the reaction was at the time of monomer addition. After completion of the reaction, the reaction mixture was immediately poured into methanol in the ratio of 1 : 5 of material to liquor for precipitation. The precipitated product was recovered by centrifugation and washed with pure methanol (2×50 mL). The crude copolymer thus obtained was dried till constant weight under vacuum (7.6 mm Hg) for 24 h at 40^{0}C. The dried product was extracted with dimethylformamide for 48 h and washed with methanol to remove the homopolymer (polyacrylonitrile). The grafted Chitosan (Chitosan-g-MA) was dried to a constant weight

under vacuum (7.6 mm Hg) for 24 h at 40^0C. The percentage grafting (%G) and percentage grafting efficiency (%GE) were determined from the increase in the weight of Chitosan after grafting in the following manner:

$$G \% = \frac{\text{Weight of Polymer Grafted}}{\text{Initial Graft of Back bone}} \times 100$$

$$E \% = \frac{\text{Weight of Polymer Grafted}}{\text{Weight of Polymer grafted}} \times 100 + \text{Weight of Homopolymer}$$

2.2 FTIR
The Fourier Transform Infrared Spectrum (FTIR) of grafted samples was measured in KBr pellets using a JASCO FTIR-5300 spectrophotometer in the range 4000–650 cm^{-1}.

2.3 SEM (Scanning Electron Microscope) Studies
Scanning electron microscopy images at 500 magnifications were obtained for Chitosan and Chitosan-g-MA using Zeiss EVO 40 EP Scanning Electron Microscope (Cambridge, England). The sample was laid on the aluminum stub using double-sided conducting adhesive tape and was sputter coated with gold.

2.4 Antibactarial Susceptibility Test
The disc diffusion method was used to screen the antibacterial activity. *In vitro* antibacterial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were pre- pared by pouring 15 ml of molten media into sterile Petri plates. The plates were allowed to solidify for 5 min and 0.1% inoculum (0.5 McFarland standard) suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. 50 μl concentration of test sample was loaded on 0.5 cm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured with trans-parent ruler in millimeter. For each bacterial strain, nega-tive controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diame- ter and the result obtained was tabulated) and Ampicillin (10 mcg/disc) were used.

2.5 Antifungal activity :
Further thegrafting compound were found to be highly toxic against clinically isolated fungal species. At a concentration of 50 μl grafting compound revealed a higher antifungal activity against C. albicans, Candida kefyr, Aspergillus niger whereas intermediated activity were showed against C. tropicalis, C. krusei, A. flavus, A. fumigatus. The inhibitory activities of all the grafting compound are reported in Table 6. The data results were compared with the standard antimicrobics of Ketoconazole (30 mg) and Itraconazole (30 mg).
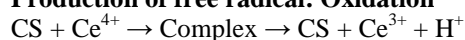
RESULTS AND DISCUSSION

CAN has been used extensively as the redox initiator for effecting grafting of a variety of vinyl monomers onto biopolymers. The formation of free radicals in Ce(IV)-treated biopolymers has been confirmed by electron spin resonance. The mechanism by which Ce(IV) interacts with biopolymer to form free radical involves the formation of a coordination complex between the Ce(IV) and the hydroxyl group of biopolymer. The Ce(IV)-biopolymer complex then disproportionates forming a free radical on the biopolymer chain and Ce(III).

Evidence for complex formation has been obtained by kinetic and spectrophotometric methods for the oxidation of various alcohols and substrates containing alcohol groups by Ce(IV) ions in perchloric and nitric acids. The postulated mechanism has been supported by the model compound studies of Ce(IV) oxidation of monohydric alcohols and 1,2-glycols and suggest that the C_2AC_3 glycol and the C_6 hydroxyl of an anhydro-D-glucose unit may be preferred sites for free-radical generation.
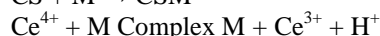
3.1 Determination of the Optimum Reaction Conditions
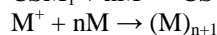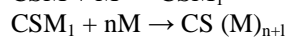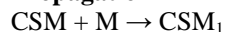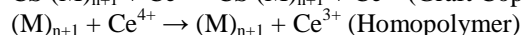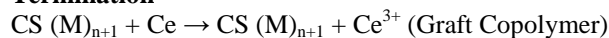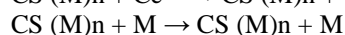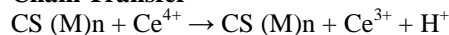To optimize the conditions for grafting of MA onto Chitosan, the concentration of nitric acid, free-radical initiator, monomer, Chitosan, time, and temperature were varied.

Production of free radical: Oxidation
$CS + Ce^{4+} \rightarrow Complex \rightarrow CS + Ce^{3+} + H^+$

Initiation
$CS + M \rightarrow CSM$
$Ce^{4+} + M$ Complex $M + Ce^{3+} + H^+$

Propagation

$CSM + M \rightarrow CSM_1$

$CSM_1 + nM \rightarrow CS\ (M)_{n+1}$

$M^+ + nM \rightarrow (M)_{n+1}$

Termination

$CS\ (M)_{n+1} + Ce \rightarrow CS\ (M)_{n+1} + Ce^{3+}$ (Graft Copolymer)

$(M)_{n+1} + Ce^{4+} \rightarrow (M)_{n+1} + Ce^{3+}$ (Homopolymer)

Chain Transfer

$CS\ (M)n + Ce^{4+} \rightarrow CS\ (M)n + Ce^{3+} + H^+$

$CS\ (M)n + M \rightarrow CS\ (M)n + M$

(Where CH, m, CH(M)$_{n+1}$ and (M)$_{n+1}$ represent chitosan, Methyl Acrylate, the graftcopolymer and homopolymer, respectively)

3.2 Effect of CAN (Ceric Ammonium Nitrate) Concentration

The effect of the concentration of the initiator Ce^{4+} on grafting of Methyl Acrylate. It was observed that the maximum percentage of grafting occurred at 5. 70 x10^{-3} M. A further increase in the Ce^{4+} concentration leads to a decrease in the grafting percentage of Methyl Acrylate. This could be explained by the fact that ceric ion at a higher concentration causes the termination of grafting polymeric chain growth since ceric ion is a very good terminator. Another factor which could contribute to a decrease in the grafting percentage at higher concentration of initiator is the increase the homopolymer formation, which competes with the grafting reaction for the available monomer
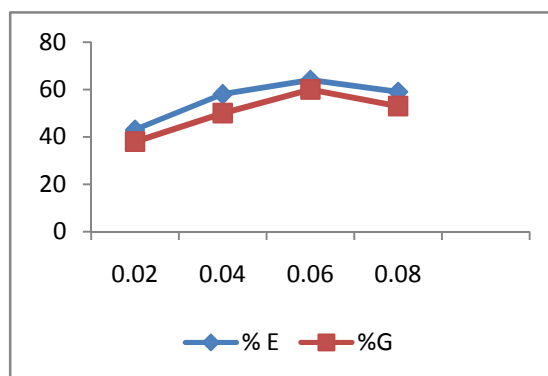

Fig-2. Effect of CAN Concentration on Chitosan/MA

3.3 Effect of monomer concentration

The effect of the Methyl Acrylate concentration on the graft yield obtained with chitosan is shown. An increase in the monomer concentration is accompanied by significant increase in grafting up to 0, 75M. However with the further increase in the concentration of monomer, grafting is found decrees. This could be ascribed to the substantial amount of polymer grafted on the subtract backbone, which inhibit the diffusion of ce^{4+} and the monomer into chitosan for further grafting.

3.4 Effect of Time

The effect of the reaction time on the percentage of grafting and grafting efficiency. The percentage of grafting was found to increase linearly with time and then approximately constant. The initial increase in the rate due to the increase in the number of grafting site, but this number remain constant with further increase of time.
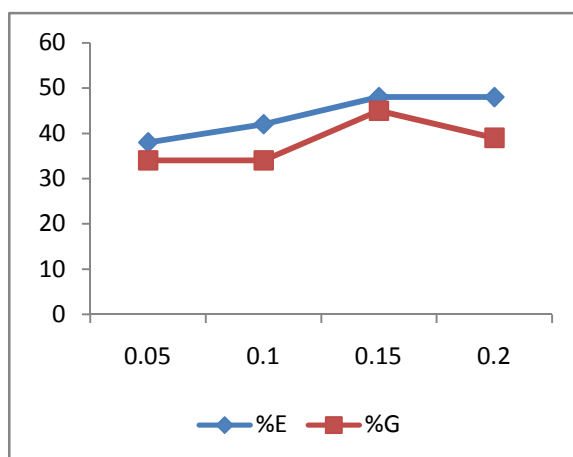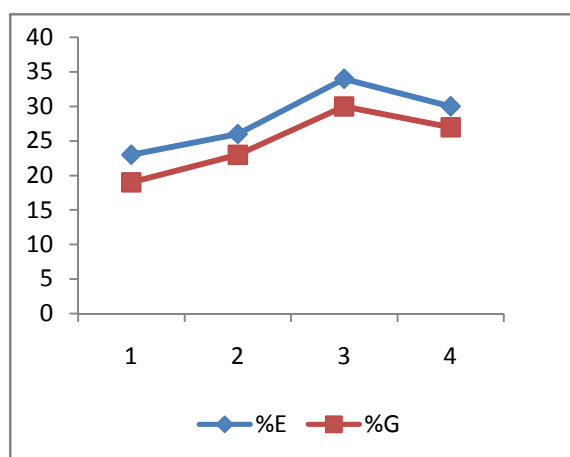
Fig-3 Effect of monomer concentration on Chitosan/MA

Fig-4 Effect of Time on Chitosan/MA

3.5 Effect of Temperature

The dependence of grafting yield on temperature in the range of 25°C – 52°C . The maximum grafting of Methyl Acrylate occurs at 35°C within 180min. A further increase in temperature reduces the percentage grafting. This is to be expected since at higher temperature various chain transform reaction are accelerated which leads to a decrease in the percentage of grafting on in other words the formation of more homopolymer.
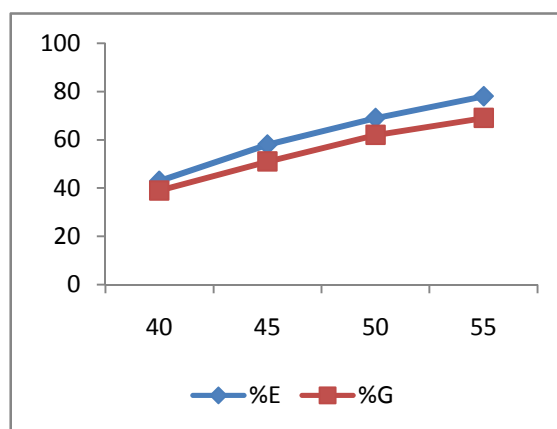

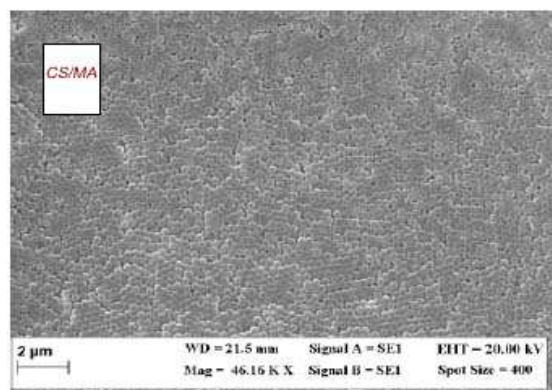**Fig-5 Effect of Temperature on Chitosan/MA**

Fig-6 SEM Picture of Grafted Chitosan/MA

4. SCM Analysis of Grafting Compound :

It clearly exhibits the polysaccharide nature having varied particle sizes with rough surface. Even the polysaccharide seems completely scattered alongwith larger particles. A change in colour of the polysaccharide on grafting and the thick polymeric coating of MA on their surface alongwith grafting of MA such that all the gap between polysaccharide particles have been closed indicate the effect of grafting . It can be seen that individual polysaccharide molecules of Chitosan have joined through these surface coatings during grafting process.
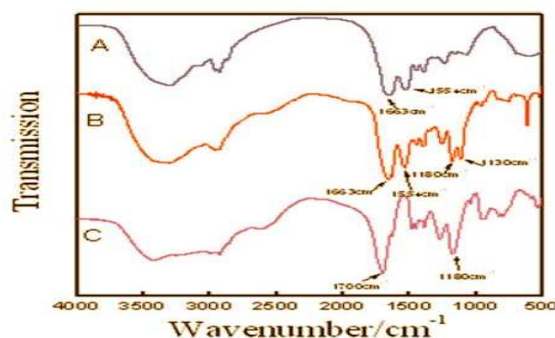

Fig-7. FTIR Spectra of pure chitosan grafted with MA (Monomer)

4.1 FTIR Studies

The FTIR spectra of the Chitosan, Chitosan-g-PMA, and the PMA homopolymer are shown in Figure 8. The most important absorption peaks for PMA were at 1700 and 1180 cm^{-1}. These peaks were caused by C=O stretching vibrations and C-O stretching vibrations, respectively. The well-characterized absorption peaks for CS were at 1663 and 1545 cm [-1]. They were caused by the carboxyl and amide groups of CS, respectively. This was in agreement with the experimental results of Wu and Zhang.[23] Compared with the spectra of PMA, CS, and the graft CS, the observed absorption peaks at 1663 and 1180 cm [-1] for the graft copolymer indicated that the MA had grafted onto CS.
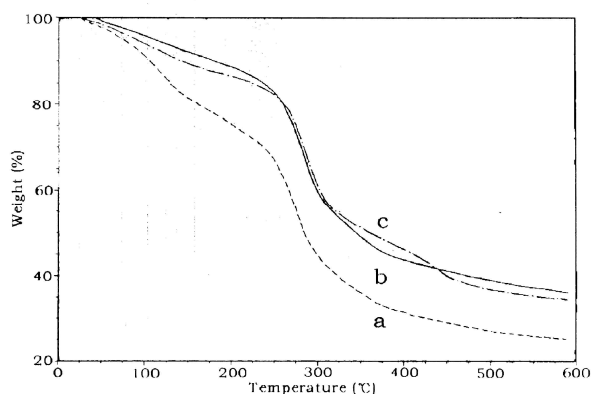

Fig-8. TGA curves of (a) the original chitosan, (b) the copolymer and (c) the TGA of chitosan-g-MA (Methyleacylate) graftcopolymer
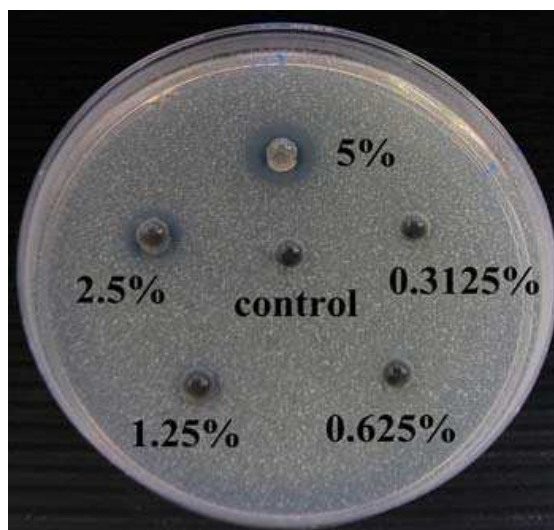
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Fig-9. Anti bacterial Activities of Grafted Chitosan-MA

Bacteria	MIC (mg/ml)
Escherichia coli	18.02±1.00
Enterobacter cloacae	23.02±1.07
Enterobacter cloacae	25.00±0.97
Enterococcus faecalis	29.75±1.84
Proteus vulgaris	29.09±1.78

4.2 TGA Studies :
The TGA of pure chitosan and chitosan-g-MA (Methylacrylate) is given in fig.-7. The grafting was also supported by thermogravimetric analysis.

5. Anti bacterial Activities of Grafted Chitosan MA :
A preliminary study has been carried out to compare the antibacterial activity of grafted chitosan hydrochloride film samples with that of chitosan. The study was carried out against *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus* using the inhibition zone method. The results are shown in **Table 1**. It was observed that grafting improved the antibacterial activity of chitosans. While the inhibition zone diameter for chitosan film ranged between 9 and 11 mm against indicated bacteria, the inhibition zone increased up to 17 mm (against *B. subtilis*) by grafting. Although the difference is not significant, activity of gram-positive bacteria seems to be more pronounced; increase in the inhibition zone diameter is 4 - 5 mm in gram-negative ones whereas it is 5 - 7 mm in gram-positive ones. Grafted samples showed an increas-ing antibacterial activity as the degree of grafting increased for all of gram-negative and gram positive bacterias; a minimum of 2 mm increase was observed consistently when the grafting percentage increased from 82.5% to 145%. Average film weight (thickness) also effected the degree of antimicrobial activity of both chitosan and grafted chitosan samples. And 3 - 4 mm in-crease was observed when the average compound weight was increased from 134 to 296.

Antibactarial activities of Grafting CS/MA Compound minimal inhibitory concentration (MIC) mg/ml.

5.1 Antifungal activity:
Table 2. Antifungal activities of Grafting compounds Minimal inhibitory concentration (MIC) mg/ml .Further the grafting compound was found to be highly toxic against clinically isolated fungal species. At a concentration of 50 μl grafting compound revealed a higher antifungal activity against C. albicans, Candida kefyr, Aspergillus niger whereas intermediated activity were showed against C. tropicalis, C. krusei, A. flavus, A. fumigatus. The inhibitory activities of the entire grafting compound are reported in Table 2. The data results were compared with the standard antimicrobics of Ketoconazole (30 mg) and Itraconazole (30 mg).
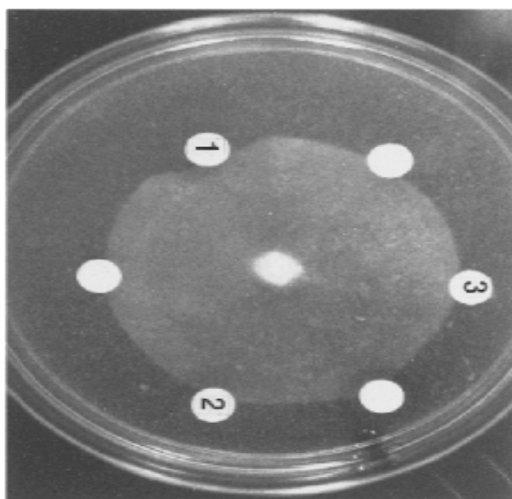
Fig.-10- Antifungal Activities of Grafted Chitosan-MA

Fungus	MIC (mg/ml)
C. albicans	12. 30±0. 03
C. tropicalis	14. 32±0. 19
C. krusei	15. 28±0. 02
C. Kefyr	11. 33±0. 24
A. flavus	18. 63±0. 34

Antifungal activities of Grafting CS/MA Compound minimal inhibitory concentration (MIC) mg/ml.

CONCLUSION

The graft copolymerization of MA onto Chitosan in aqueous medium was initiated effectively with CAN. The reaction conditions were optimized for grafting of MA onto Chitosan by varying the concentration of Chitosan, CAN, MA, HNO_3, polymerization time, and reaction temperature. The characterization of the grafted products by means of FTIR, scanning electron microscopy, and thermal analysis furnished evidence of grafting of MA onto Chitosan. Chitosan-MA has overall high thermal stability than pure Chitosan. This study has demonstrated that grafting compound showed a higher antibacterial and fungal activity. Grafting compound markedly inhibited the growth of most bacterial and fungal tested although their inhibitory effects differed.

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