ANTIMICROBIAL COATINGS BASED ON CHITOSAN DERIVATIVES AND QUATERNARY AMMONIUM SALTS FOR PACKAGING PAPER APPLICATIONS

P. NECHITA,[*] E. BOBU,[**] G. PARFENE,[***] R. M. DINICĂ,[***] and T. BĂLAN[**]

[*]*Dunărea de Jos University of Galaţi, Faculty of Engineering and Agronomy, Călăraşi 29, 810019, Braila, Romania*
[**] *"Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection, Mangeron 73, 700050, Iasi, Romania*
[***]*Dunărea de Jos University of Galaţi, Faculty of Sciences and Envrironmental Protection, Domneasca 111, Galati, Romania*
✉ *Corresponding author: P. Nechita, pnechita@yahoo.com*

Conventional food packaging systems are designed to act as passive barriers, protecting food against the surrounding environment, while active food packaging systems are designed to interact with surrounding medium in order to prevent harmful effects, like microbial contamination. In this paper, two (bio)active compounds – quaternary ammonium salts (QAS) and quaternary chitosan (QCh), were evaluated as potential materials to be used in formulations of antimicrobial coating for packaging paper applications. Composite particulates of QAS: ZnO at different ratios were applied on paper surface as water dispersions in a single layer, while QCh water solution of constant concentration was applied by multi-layer coating (1-3 layers). The antimicrobial activity of paper samples was evaluated against the gram-positive bacteria and against fungi, respectively. The QAS immobilized on ZnO particulates showed good antimicrobial activity against both gram-positive bacteria and fungi, which increased with QAS:ZnO ratio. The QCh films proved to be very effective for bacterial inhibition and paper strength improvement, but less effective against fungi.

Keywords: food packaging paper, antimicrobial activity, paper coating, quaternary ammonium salts, chitosan

INTRODUCTION

Antimicrobial properties are desired for many paper grades, such as tissue papers, hospital papers, office papers, wall papers and packaging papers. In recent years, the researchers' attention has been focused on special compounds as antimicrobial materials or biomarkers, which can be used for functionalizing fibre based products. These functionalised products (active papers) can have wide application, from protection and safety (purification of water and air, security against biological and chemical terrorism), diagnostics of health, food safety and quality testing for microbial contamination, as well as counterfeit prevention.[1,2,3]

Antimicrobially active paper can be obtained by the immobilization of antimicrobial agents on cellulose fibres before paper forming or by the incorporation into surface coating films. Antibacterial films can contain an *antimicrobial agent that migrates* to the surface of the food and kills pathogens, or can be based on bioactive compounds that are effective against surface growth of microorganisms, *without migration*.[4] This last type of films could be characterized by *contact-active antibacterial surfaces*, which is now an expanding research field. Whereas conventional antibacterial surfaces continuously leach biocides into their surroundings, the contact-active surfaces inhibit the growth of microorganisms only upon interaction with the surface. This type of surface is constructed having the antibacterial agent, usually a hydrophobic modified cationic polymer, irreversibly attached to the surface, which gives several benefits: first, the surface activity remains intact during the material life-span, without release of toxic compounds; second, the general antibacterial mechanism targets the outer bacterial cell envelope and minimizes the risk of emerging resistance.[5,6,7]

Antimicrobial materials

The development of antimicrobial paper implies the identification of efficient (bio)active materials and techniques for their embedding and immobilization into the internal structure or onto the surface of paper. From a general perspective, there is a large variety of antimicrobial materials, inorganic and organic compounds, as well as bio-molecules (like enzymes used in biosensor paper). However, only a limited number of the chemicals with antimicrobial properties have been investigated for antimicrobial paper so far.

Inorganic antimicrobial compounds

Among mineral materials that have potential applications for antibacterial packaging, the following have been reported to present antibacterial activity: metal oxides such as titanium dioxide (TiO_2), zinc oxide (ZnO) and magnesium oxide (MgO), the bioactivity being attributed to the generation of reactive oxygen species – ROS; metallic ions such as silver, copper and zinc; silica and nanoclays.[3] Frequently, mineral compounds are used as nanostructured materials, which could be retained in paper during wet forming, or could be loaded into a polymer to obtain a nano-composite for surface application.[3]

Organic antimicrobial compounds

The organic antimicrobial compounds, which are thought to be most suitable for their incorporation into packaging paper include: bio-based chemicals, like polyphenolic compounds, which were proved to have antioxidant activity and chelating properties; chitosan and chitosan derivatives, which have antifungal and antibacterial activity and may serve as potential inclusion matrices of nanoscale antimicrobial particles;[8] synthetic compounds – triclosan ((2,4,4'-trichloro-2'-hydroxydiphenyl ether), quaternary ammonium salts, some classes of antibiotics based on aminoglycosides, as well as polymers with quaternary ammonium groups and biguanide groups;[8,9] enzymes (e.g. lysozyme) and bacteriocins (e.g. nisin) were also studied for packaging application.[10]

Quaternary ammonium salts are a versatile class of compounds having a wide range of interesting properties and continuing to receive increasing attention mainly because they can be easily functionalized at the nitrogen heterocyclic atom in order to obtain species of useful compounds for biological and industrial applications,[8,11] as for example cosmetics, pharmaceuticals, gene delivery, polymerization. Selected salts named viologens (4,4'-bipyridinium salts) also develop electrochromic properties. Due to their broad specificity of antimicrobial activity, N-heterocyclic quaternary ammonium salts represent some of the most used antiseptics and disinfectants.

Chitosan is a biopolymer with unique features; namely: it is a single cationic bio-polymer, containing hydroxyl groups, which are able to develop hydrogen bonds like cellulose fibres; it has the ability to form films with antimicrobial properties; it is biodegradable and non-toxic.[12,13] Unquestionably, one of the more interesting properties of chitosan is its ability to form films. Chitosan can be dissolved under slightly acidic aqueous conditions and cast into membranes or films with good mechanical strength and permeability. Chitosan films have been used in combination with synthetic polymers to produce packaging films for the quality preservation of a variety of food products. For example, films based on chitosan-PEO proved good antimicrobial effect against *E. coli*.[14]

Current trends in functional coating aim to develop green solutions for barrier properties to allow substitution of synthetic polymers with biopolymers originated from naturally renewable resources, like chitosan and other polysaccharides. Biopolymer-based films and coatings can act as efficient vehicles for incorporating various functional additives, including antimicrobials and antioxidants.[15, 16] However, despite extensive research over the last decade on the use of chitosan as papermaking additive, there are no significant applications yet. One of the main barriers to these applications is the lack of water solubility of chitosan in neutral/alkaline environment.[17,18] The solutions to obtain soluble derivatives are based on the chemical modification of chitosan *via* reactions of its primary amino function at C-2 and two hydroxyl functionalities. Amino functionality provides chemical reactions, such as acetylation, alkylation, quaternization, grafting and chelation of metals, while the hydroxyl functional groups also give various reactions, such as O-acetylation (e.g. O-carboxymethyl chitosan), H-bonding with polar atoms, grafting etc.[19, 20]

Considering the global interest in green solutions in food packaging, the present study is aimed as a preliminary investigation on the (bio)active quaternary ammonium salts (QAS) and quaternary chitosan (QCh) as potential compounds in developing contact-active antibacterial paper.

EXPERIMENTAL

Materials

Quaternary ammonium salts (QAS)

In this study, the 4,4'-bispyridinium diquatenary ammonium salts derived from 4,4'-bipyridine were synthesized and tested at lab scale. The synthesis is based on the alkylation reaction of the pyridine derivatives with reactive halogenated compounds (Figure 1). The reactions occurred in anhydrous solvents, which appears to be the most convenient method among those reported in the literature.[8,21] The cationic charge of quaternary nitrogen and alkyl chain confers high antimicrobial activity to these compounds.

In order to be applied on paper surface, the quaternary salts were immobilised on zinc oxide (ZnO) particulates at different QAS:ZnO ratio (Table 1). The powder of composite particulates was dispersed in distilled water at constant concentration in solids (60%). Then, dispersions with about 40% solids content and with different QAS concentrations were obtained by varying the QAS:ZnO ratio.

Quaternary chitosan (QCh)

Main features of the quaternary chitosan are the water solubility under neutral pH and cationic charge density over the whole pH range. Comparatively with chitosan, the QCh exhibits higher antimicrobial activity due to the quaternary nitrogen and alkyl groups. Furthermore, the QCh presents high affinity for cellulose fibre surface and has H-bonding potential, which can improve the strength properties of treated paper.[22]

The quaternary chitosan (Figure 2) was obtained by the O- and N-acylation of chitosan, provided by Sigma Aldrich (MW = $7.56 \cdot 10^5$ g/mol, DA = 20.8%) with (3-chlor-2-hydroxipropyl)-trimethyl-ammonium chloride (Quat-188) under alkaline conditions, following the method described in the literature.[23] A high substitution degree of 85% was obtained by using an adequate chitosan:Quat-188 ratio, established after preliminary tests with variable ratios. The QCh obtained by this method showed good water solubility under neutral pH and high cationic charge density under neutral and alkaline pH (Table 2).

Paper substrate for coating tests

The base paper for testing coating formulas was obtained in the laboratory on a Rapid Köthen at the basis weight of 50 ± 2 g/m^2; the paper stock was prepared as water suspension of the bleached hardwood and softwood kraft pulps (hardwood:softwood = 70:30), which were refined separately in a Valley Hollander at 30 °SR, without filler or any chemical additives.

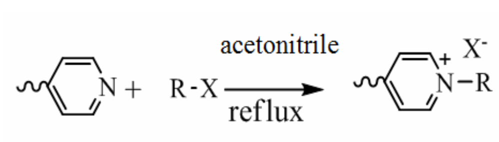


Figure 1: Synthesis of 4,4'-bispyridinium diquatenary salts

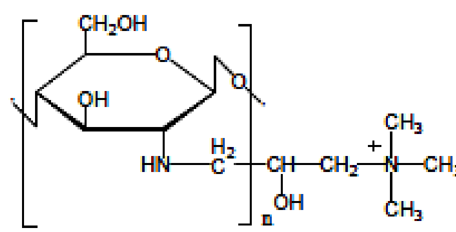


Figure 2: Quaternary chitosan

Table 1
Composition of particulate dispersion

Sample	0	1	2	3	4	5	6
QAS p to 100 ZnO p	0.00	0.05	0.06	0.07	0.08	0.09	0.1

Table 2
Solubility and cationic charge density of QCh, comparatively with native chitosan

Test/sample	Solution pH		
	pH = 4 (acetic acid 0.1 M)	pH = 7 (distilled water)	pH = 10 (water/Na_2CO_3)
Solubility (max. conc. in g/L):			
- Native chitosan	10 g/L, 50 C	Insoluble	Insoluble
- Quaternary chitosan (QCh)	N/A	5 g/L, 20 C	N/A

Cationic charge density (µeq/g):			
- Native chitosan	4960	1720	0
- Quaternary chitosan (QCh)	3540	3420	2340

Figure 3: Antimicrobial activity tests

Methods

Application of functional coating on paper surface

The dispersions of the QAS:ZnO particulates with different compositions (Table 1) and quaternary chitosan solution were applied on the paper surface using a Meyer rod laboratory coating system. The QAS:ZnO composite formulas were coated as a single layer of 15 ± 1 g/m^2 weight on a single paper side. The QCh water solution was applied first as 1, 2 and 3 layers in order to obtain different coating weights for bacterial growth tests; other samples were prepared with two successive layers at constant coating weight of 1 ± 0.05 g/m^2 on each paper side for both bacterial and fungal growth tests. After applying the coating formula, the paper sheets were dried first in the air (24 h) and then on a Rapid Köthen dryer for 5 min.

Assessment of antimicrobial activity

Both series of coated paper samples were tested against gram-positive bacteria and fungi. The gram-negative bacteria were not considered in this study, because it is well known that cationic compounds with quaternary nitrogen present good activity against these microorganisms due a strong electrostatic interaction of the cationic charge with the anionic charge on the cell surface of bacteria.[24] However, the hydrophobic cell surface, as in the case of gram-positive bacteria, is an important factor to consider in developing antibacterial surfaces.[25] In the case of chitosan derivatives, it is supposed that the link between the derivative molecule and the cell surface is achieved by lipoteichoic acid (LTA), allowing it to disrupt membrane functions by hydrophobic interactions.[26]

The QAS coated samples were evaluated against gram-positive bacteria – *Bacillus subtilis* and *Sarcina lutea*, and against fungi – *Geothricum candidum*. The antimicrobial activity tests were performed on Petri dishes with microorganism dispersion. The pre-cooled and fluid culture medium is added over this dispersion. After the solidification of culture medium, the sterile discs of paper samples were introduced and stored for about 72 h at 37 °C in the case of bacterial tests and for 120 h at 25 °C in the case of fungal tests. The inhibition area around the discs was registered (Figure 3).

The QCh coated samples were tested against gram-positive bacteria (*Bacillus subtilis*) and against fungi (*Penicillium sp*.). The inhibition of bacterial growth was evaluated according to the standard SR EN ISO 846/2000. The work method consists in the application of sterilized paper coupons on the culture medium (TSBA) placed in Petri dishes and their inoculation with a 1-µL aliquot of standard bacterial suspension, which was diluted and centrifuged previously. The level of bacterial growth was evaluated after 48 h of incubation at 37 °C. The antifungal activity of the QCh coating was evaluated by two methods: a) by spraying the sterilized paper coupons with *Pencillium* conidia suspension and their placing on artificial culture medium (Sabouraud); b) the flooding of culture medium with *Pencillium* conidia suspension (0.5 mL per Petri dish) and placing paper coupons on its surface. After 168 h incubation (25 ºC), fungal growth was assessed by sample observation under a stereomicroscope (20x).

RESULTS AND DISCUSSION

Inhibition of microbial growth on paper coated with QAS:ZnO composites

With the objective of assessing the antibacterial activity of nitrogen heterocyclic compounds, in the experimental programme, the paper samples functionalized with 4,4'bispiridinium salts were compared with both paper samples covered only with ZnO dispersions and untreated paper (reference). This approach is based on the fact that inorganic materials, such as silver, copper, titanium oxide and zinc oxide

(ZnO) show antimicrobial activity against different microorganisms.[27]

In comparison with the reference, the paper samples treated only with ZnO dispersion show no significant antibacterial activity, but a relative good antifungal effect. The samples with QAS present a good activity against the gram-positive bacteria (*Bacillus subtilis* and *Sarcina lutea*) and a very good activity against the fungi (*Geothricum candidum*). The addition of QAS to the ZnO suspension improved substantially the antimicrobial effect on both bacteria and fungi, even at a very small ratio of 0.05p QAS/100p (Table 3).

Figure 4 evidences the inhibition areas for the two gram-positive bacteria and for fungi of the samples with the smallest contents of the QAS, in comparison with the reference paper (without coating) and with the sample coated only with ZnO dispersion. One can observe that an increase of the QAS content above 0.06p does not bring substantial improvements of antimicrobial activity.

Inhibition of microbial growth on paper surface coated with quaternary chitosan (QCh)

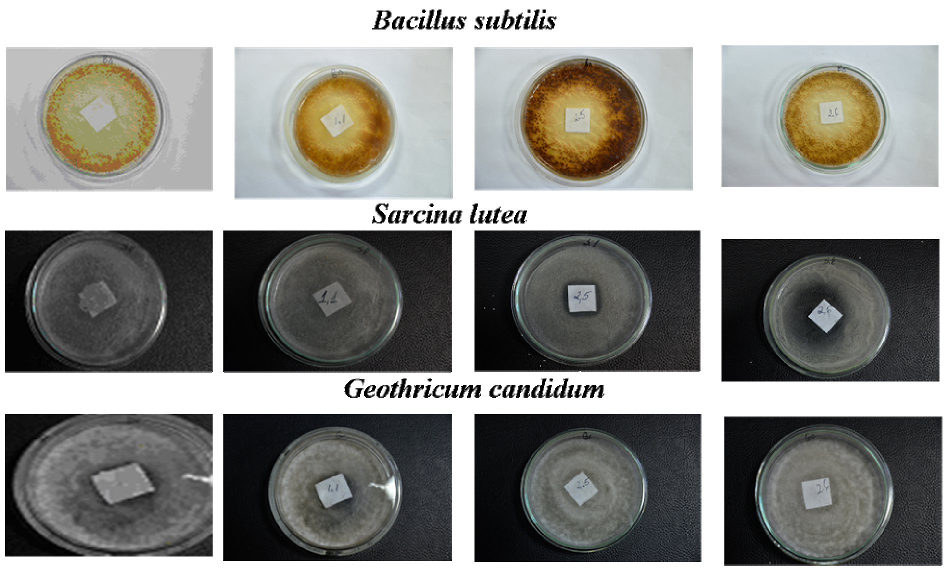
Gram-positive bacterial inhibition

Paper samples coated with 1, 2 and 3 fine layers of QCh and different coating weights (Table 4) were evaluated for the inhibition effect of gram-positive bacterial growth (*Bacillus subtilis*). Figure 5 shows the bacterial growth on the paper surface is inhibited by the QCh films and the effect depends on the coating weight. One can observe that a coating weight under 1 g/m^2/side (Q1 and Q2 samples) produces only a slight inhibition of bacterial growth. However, a much higher coating weight than 1 g/m^2/side does not appear necessary, since a total inhibition of *Bacillus subtilis* is achieved by the sample Q/Q with a coating weight of 1 g/m^2.

Table 3
Growth inhibition effect of paper coated with composite formulas

Sample: coating formula	*Bacillus subtilis*			*Sarcina lutea*			*Geothricum candidum*			
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	120 h
	Inhibition effect									
A 1.1: 100pZnO	-	-	+	-	-	-	-	+	++	++
A2.5: 100pZnO + 0.05p QAS	-	+	++	+	+	+	-	+	++	+++
A2.6: 100pZnO + 0.06p QAS	-	+	++	+	+	+	-	+	++	+++
A2.7: 100pZnO + 0.07p QAS	-	+	++	+	+	+	-	+	++	+++
A2.8: 100pZnO + 0.08p QAS	+	+	++	+	+	+	-	+	++	+++
A2.9: 100pZnO + 0.09p QAS	+	+	++	+	+	+	-	+	++	+++
A2.10: 100pZnO + 0.1p QAS	+	+	++	+	+	+	-	+	++	+++

"-" absence of inhibition; "+" small inhibition; "++" moderate inhibition; "+++" – high inhibition



a) Reference b) 100 ZnO c) 100 ZnO +0.05QAS d) 100 ZnO +0.06QAS

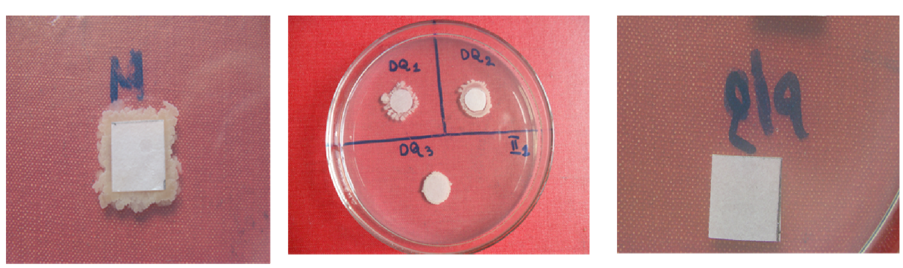
Figure 4: Inhibition effect of QAS:ZnO composite particulates against bacteria (*Bacillus subtilis, Sarcina lutea*) and fungi (*Geothricum candidum*)

Table 4
Coating weight of QCh layers applied on paper surface

Sample symbol	M	DQ 1 layer	DQ 2 layers	DQ 3 layers	Q/Q 2 layers
Coating weight, g/m^2/side	0 (reference)	0.46	0.85	1.19	1.02

Table 5
Fungal inhibition of multilayer coated paper with QCh

Paper sample/coating weight (g/m^2)	Paper covering (%) Assessment method	
	Spraying	Flooding
Reference (M)	100	>35
DQ1 / 0.46	90	27
DQ2 / 0.85	79	16
DQ3 / 1.19	58	8
Q/Q / 1.02	60	10



Reference | Samples with 1-3 layers, different coating weight | Samples with 2 layers, coating weight~1 g/m^2

Figure 5: Inhibition of *Bacillus subtilis* growth by QCh coatings of different weights

The influence of the number of layers and coating weight on the antibacterial effect of QCh could be explained by the uniformity of paper surface coverage, which depends on the amount of polymer applied on the surface unit and migration of the polymer solution into the paper substrate. Having in view that the QCh water solution (concentration of 5 g/L) has a relatively low viscosity, the first layer migrates fast into the porous structure of the paper substrate and thus, it will be only spotted with polymer. The migration rate decreases for the second and third layer (this was confirmed by a lower polymer uptake at subsequent layers) and the coverage area increases. However, it was observed that film uniformity was also impaired by a too low migration of the third layer and slow drying rate. For this reason, a number of samples were prepared by applying two layers of approximately constant coating weight (Q/Q), using different polymer concentrations, as established by preliminary tests. The total inhibition of bacterial growth in the case of the Q/Q samples allows us to conclude that the optimum coating weight for bacterial inhibition is around 1 g/m^2, applied in two successive layers. Of course, this conclusion is related to the paper substrate used in these experiments, because the interactions between the waterborne coating formula and the paper are strongly influenced by the physical and chemical properties of the paper.

Inhibition of fungal growth

The results concerning fungal growth on paper surface coated with QCh are presented in Table 4 for both assessment methods applied in this study, spraying of paper surface and respectively, flooding of culture medium with *Pencillium* conidia suspension. In Table 5, the inhibition effect is expressed as percentage of paper surface covered with fungi. Like in the case of bacterial inhibition, the efficiency increased with coating weight and maximum inhibition was reached for a coating weight higher than 1 g/m^2. The results differ for the two assessment methods, but it is clear that there is no total inhibition. In the case of the flooding method, the inhibition zone, created around coated paper coupons, was also evaluated (Figure 6).

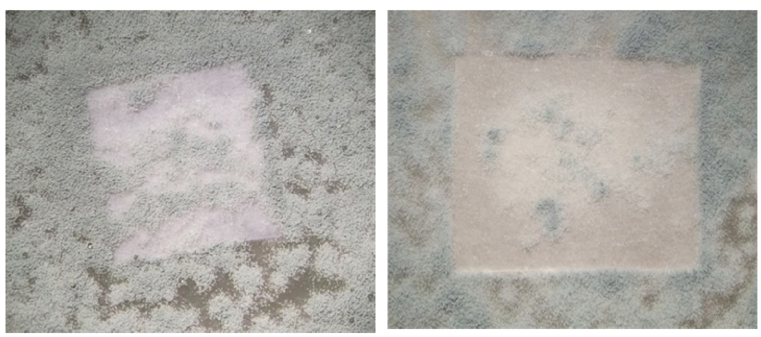


Figure 6: Stereomicroscope detail (20x) of fungal growth on reference and QCh coated paper

CONCLUSION

Two organic compounds with quaternary nitrogen functionality – quaternary ammonium salts (QAS) and quaternary chitosan derivative (QCh) – were obtained and evaluated individually as bioactive materials for the development of antimicrobial papers.

The QAS, applied on paper after immobilisation on zinc oxide particulates at different QAS:ZnO ratio, demonstrated a high inhibition effect against both bacteria and fungi. Very low QAS:ZnO ratios of 5-6 $x10^{-4}$ can achieve a high level of inhibition of both bacteria and fungi tested. The antifungal activity of QAS is supplemented slightly by ZnO particulates.

The QCh waterborne coating formulas have shown a high potential for total inhibition of the gram-positive bacteria, but a moderate antifungal activity. However, this limitation of the QCh as antimicrobial material could be compensated by its potential to form films with high mechanical strength and barrier properties important for paper packaging.[23] Therefore, having in view that the immobilisation of QAS:ZnO composites on paper surface requires a polymer to bind the particulates together and anchor them on the substrate, the QCh appears as an adequate candidate for this function.

All in all, the results of this study can be considered a promising starting point aimed at developing innovative active paper for food packaging based on a combination of multifunctional chitosan derivative and antimicrobial quaternary ammonium salts.

REFERENCES

[1] S. Aikio, S. Grönqvist, L. Hakola, E. Hurme, S. Jussila *et al*., Bioactive Paper and Fibre Products - Patent and Literary Survey, *VTT Working Papers 51*, Espoo Finland, 2006.
[2] G. Siqueira, J. Bras and A. Dufresne, *Polymer*, **2**, 728 (2010).
[3] M. Patel, "Developments in Antibacterial Paper", Pira International Ltd, 2009.
[4] C. Pérez-Pérez, C. Regalado-González, C. A. Rodríguez-Rodríguez, J. R. Barbosa-Rodríguez and F. Villaseñor-Ortega, in "Advances in Agricultural and Food Biotechnology", edited by R. G. Guevara-González and I. Torres-Pacheco1, Research Signpost, India, 2006, pp. 93-216.
[5] A. M. Bieser, J. C. Tiller, *Macromol. Biosci*., **11**, 526 (2011).
[6] B. B. Hsu, J. Ouyang, S. Y. Wong, P. T. Hammond, A. M. Klibanov, *Biotechnol. Lett*., **33**, 411 (2011).
[7] B. B. Hsu, S. Y. Wong, P. T. Hammond, J. Z. Chen, A. M. Klibanov, *Proc. Nat. Acad. Sci. USA*, **108**, 61 (2011).
[8] I. Baussanne, A. Bussière, S. Halder, C. Ganem-Elbaz, M. Ouberai *et al*., *J. Med. Chem*., **59**, 119 (2010).
[9] M. Kong, X. G. Chen, K. Xing, *Int. J. Food Microbiol*., **144**, 51 (2010).
[10] V. Coma, *Polímeros*, **23**, 287 (2013).
[11] K. Khaoula, A.T. Elmira, S. Desobry, *Compr. Rev. Food Sci. F*., **9**, 82 (2010).
[12] J. Dutta, S. Tripathi, P. Dutta, *Food Sci. Technol. Int*., **18**, 3 (2012).
[13] E. Bobu, F. Ciolacu, R. Parpalea, in *Procs. Workshop–Sustainable Management of Microbiological Activity in Papermaking*, Grenoble, 15-16 January, 2004, paper 11.
[14] S. Zivanovic, J. Li, P. M. Davidson, K. Kit, *Biomacromolecules*, **8**, 1505 (2007).
[15] A. M. C. Ribeiro, L. Barbassa and L. Dias de Melo, in "Antimicrobial Biomimetics, Biomimetic Based Applications", edited by Marko Cavrak, InTech, DOI: 10.5772/14400 (2011).
[16] H. Han and A. Gennadios, in "Innovations in Food Packaging", edited by J. H. Han, Elsevier Academic Press, London, 2005, pp. 239-262.
[17] P. Fatehi, L. Qian, R. Kititerakun, T. Rirksomboon and H. Xiao, *Tappi J*., **8**, 29 (2009).
[18] E. Bobu, R. Nicu, J. Desbriers, *Cellulose Chem. Technol*., **45**, 105 (2011).
[19] E. Bobu, R. Nicu, M. Lupei, F. Ciolacu, J. Desbriers, *Cellulose Chem. Technol*., **45**, 619 (2011).
[20] P. K. Dutta, S. Tripathi, G. K. Mehotra, J. Dutta, *Food Chem*., **114**, 1173 (2009).
[21] R. Dinică, B. Furdui, G. Bahrim, M. Demeunynck, *Rev. Roum. Chim*., **53**, 21 (2008).
[22] F. Ciolacu, T. Balan, R. Nicu and E. Bobu, in *Procs. International Symposium on Applied Interface Chemistry,* Munich, 25-26 February, 2014, Paper 10.
[23] W. Sajomsang, S. Tantayanon, V. Tangpasuthadol, W. H. Daly, *Carbohyd. Res*., **344**, 502 (2009).
[24] Y. C. Chung, Y. P. Su, C. C. Chen, G. Jia, H. L. Wang *et al*., *Acta Pharmacol*., **25**, 932 (2004).
[25] S. P. Stranda, T. Nordengenb, K. Otgaard, *Water Res*., **36**, 4745 (2002).
[26] D. Raafat, K. V. Bargen, A. Haas, H. G. Sah, *Appl. Environ. Microbiol*., **74**, 3764 (2008).
[27] P. J. Perez Espitia, N. F. F. Soares, J. S. dos Reis Coimbra, N. J. de Andrade, R. S. Cruz *et al*., *Food Bioprocess. Technol*., **5**, 1447 (2012).