DETOXIFICATION OF MERCURY BY CHITOSAN

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Abstract

Chitosan, deacetylated chitin, is a polymer of 2-deoxy-2-amino glucose. Chitosan has the property of chelation of metals and the quantity of metal ions adsorbed varies considerably with different metals. Of the different metal ions, mercury shows maximum adsorption by chitosan. The largest consumer of mercury is the Chlor-alkali industry. It is followed by the industry producing electrical apparatus and the agricultural industry. Sewage effluent sometimes contains upto 10 times the level of mercury in natural water. Chitosan is found to bind mercury efficiently. The elimination of mercury by chitosan can be achieved by direct addition to solution or by percolation through a column of chitosan. The potential role of chitosan in the detoxification of water is discussed.

Key words : Chitin, chitosan, chelation, adsorption, detoxification.

Introduction

Man is exposed to various forms and levels of toxic contaminants of foods and water. Mercury is a potential contaminant of foods and water. Mercury is omnipresent in the environment : in air, water and soil-naturally and by human activities. In industrialized as well as upcoming neo-industrialized nations, huge quantities of effluents containing mercury are being expelled by mercury based industries, thus contributing to the pollution of the environment. Removal of mercury from effluents poses serious problems to industries. This problem could be solved using metal binding agents. The permitted level of mercury in drinking water is below 0.001 mg/litre (<1 ng/ml.) (WHO, 1978) (1).

The efficient removal of toxic metal ions from water, is an important and widely studied research area. A number of technologies have developed over the years to remove toxic metal ions from water (2). Filtration is very cheap and effective for removing particulate and other insolubles. Chitosan, has a number of properties indicating its potential application in waste water treatment.

Chitosan has been recognized as a scavenger of metal ions. This property is derived from the amine function in the C-2 position of the glucose ring. Chitosan

has the ability to chelate metal ions. The quantity of metal ions bound to chitosan is different with different metals (3,4,5,6). Chitosan is capable of binding the toxic metal mercury (7). Hence it can be used for the removal of mercury from industrial waste water (8). The ability of chitosan to bind mercury at different concentrations of mercury and at different particle size of chitosan has been studied in the present work, to assess the possible use of chitosan as a natural fiber to reduce the toxicity of mercury in water and waste water.

Materials and Methods

Chitosan was prepared by a method developed by Madhavan *et. al* (1974) (9). It was pulverized to different particle sizes in a pulverizer. A stock solution containing 1 g Mercury/ml was prepared by dissolving mercuric chloride (Analytical Reagent Grade) in distilled water and the experimental solutions were prepared by dilution of the stock solution.

(a) Percolation Method :-

Glass columns of internal diameter 0.8 cm. were packed with 1 gm. chitosan of particle size 0.5 mm. 100 ml solutions containing 10 ppm, 100 ppm and 500 ppm of Hg^{++} respectively were passed through these columns packed with chitosan at a flow rate of two minutes to collect each eluant fraction of 80 drops (4 ml). The fractions were collected using a fraction collector model LKB 2112 RediRac, Bromma, Sweden. The pH of mercuric chloride solution was 7. The temperature was 30°C. The residual mercury in the eluate was estimated using a mercury analyzer (MA 5800 A, electronic corporation of India).

(b) Direct addition method :-

One gram of chitosan was added to 100 ml solutions of different definite concentrations of mercury and residual mercury level was estimated after gently stirring for 50 minutes in a flask with the help of a magnetic stirrer in each case.

Chitosan of different particle sizes were used for a comparative evaluation of the efficiency of binding mercury. Calculated the percentage of mercury bound on chitosan. Results were expressed as : (a) mg of Hg^{2+} bound to 100 mg of chitosan (b) mg of Hg^{2+} bound from 100 mg of Hg^{2+} in the solution.

Table 1 : Rate of adsorption of mercury on Chitosan at different concentrations of mercury [Percolation]

Time of collection of eluant (min)	Concentration of Hg^{++} = 10 ppm		Concentration of Hg^{++} = 100 ppm		Concentration of Hg^{++} = 500 ppm	
	mg of Hg^{++} bound to 100 mg of chitosan average ± standard error of mean	% of Hg^{++} in the medium which gets bound on chitosan	mg of Hg^{++} bound to 100 mg of chitosan average ± standard error of mean	% of Hg^{++} in the medium which gets bound on chitosan	mg of Hg^{++} bound to 100 mg of chitosan average ± standard error of mean	% of Hg^{++} in the medium which gets bound on chitosan
2	0.003998 ± 3.33E-07	3.99	0.039960 ± 1.00E-05	3.99	0.199970 ± 6.00E-06	3.99
4	0.007998 ± 1.92E-07	7.99	0.079970 ± 6.67E-06	7.99	0.399950 ± 6.67E-06	7.99
6	0.011991 ± 3.33E-07	11.99	0.119930 ± 1.00E-05	11.99	0.599927 ± 8.39E-06	11.99
8	0.015980 ± 3.33E-06	15.98	0.159880 ± 6.67E-06	15.98	0.799900 ± 1.00E-05	15.99
10	0.019961 ± 5.09E-07	19.96	0.199773 ± 3.85E-06	19.97	0.999860 ± 1.00E-05	19.99
12	0.023944 ± 3.33E-07	23.94	0.239580 ± 1.00E-05	23.96	1.199600 ± 1.20E-04	23.99
14	0.027925 ± 6.94E-07	27.92	0.279167 ± 5.09E-06	27.92	1.399533 ± 1.34E-04	27.99
16	0.031912 ± 6.94E-07	31.91	0.318760 ± 1.00E-05	31.88	1.599467 ± 8.39E-05	31.99
18	0.035893 ± 6.94E-07	35.89	0.356523 ± 1.07E-05	35.69	1.799367 ± 8.39E-05	35.99
20	0.039874 ± 5.09E-07	39.87	0.393220 ± 6.67E-06	39.32	1.999360 ± 1.35E-04	39.99
22	0.043850 ± 8.82E-06	43.85	0.425020 ± 6.67E-06	42.50	2.198900 ± 3.33E-05	43.98
24	0.047857 ± 5.09E-06	47.85	0.457137 ± 1.07E-05	45.71	2.397831 ± 6.94E-05	47.96
26	0.051836 ± 3.85E-07	51.83	0.489280 ± 6.67E-06	48.92	2.594967 ± 1.68E-04	51.89
28	0.055821 ± 5.09E-07	55.82	0.521063 ± 1.90E-05	52.11	2.790060 ± 1.20E-05	55.80
30	0.055808 ± 6.67E-07	59.80	0.552060 ± 1.00E-05	55.21	2.983600 ± 1.76E-04	59.67
32	0.063795 ± 1.17E-06	63.79	0.583040 ± 1.33E-05	58.30	3.175620 ± 1.20E-05	63.51
34	0.067782 ± 6.67E-07	67.78	0.623047 ± 1.02E-05	62.30	3.360040 ± 1.02E-05	67.20
36	0.071766 ± 1.92E-07	71.76	0.654023 ± 5.09E-06	65.40	3.542733 ± 1.02E-04	70.85
38	0.075745 ± 1.15E-06	75.74	0.685003 ± 5.09E-06	68.50	3.726467 ± 1.02E-04	74.53
40	0.079731 ± 5.77E-07	79.73	0.715647 ± 1.87E-04	71.56	3.909133 ± 1.67E-04	78.18
42	0.080375 ± 1.92E-03	80.37	0.746963 ± 8.39E-06	74.69	4.091967 ± 1.02E-04	81.84
44	0.087686 ± 1.17E-06	87.68	0.777940 ± 1.33E-05	77.79	4.274700 ± 6.67E-05	85.49
46	0.091658 ± 5.09E-67	91.65	0.807280 ± 6.67E-06	80.73	4.457500 ± 1.00E-04	89.15
48	0.095632 ± 8.39E-07	95.63	0.834160 ± 1.00E-05	83.42	4.638600 ± 1.33E-04	92.77
50	0.099603 ± 6.94E-07	99.60	0.861040 ± 1.33E-05	86.10	4.818197 ± 1.02E-05	96.36

Rate of adsorption of mercury on Chitosan at different time Intervals (Percolation)

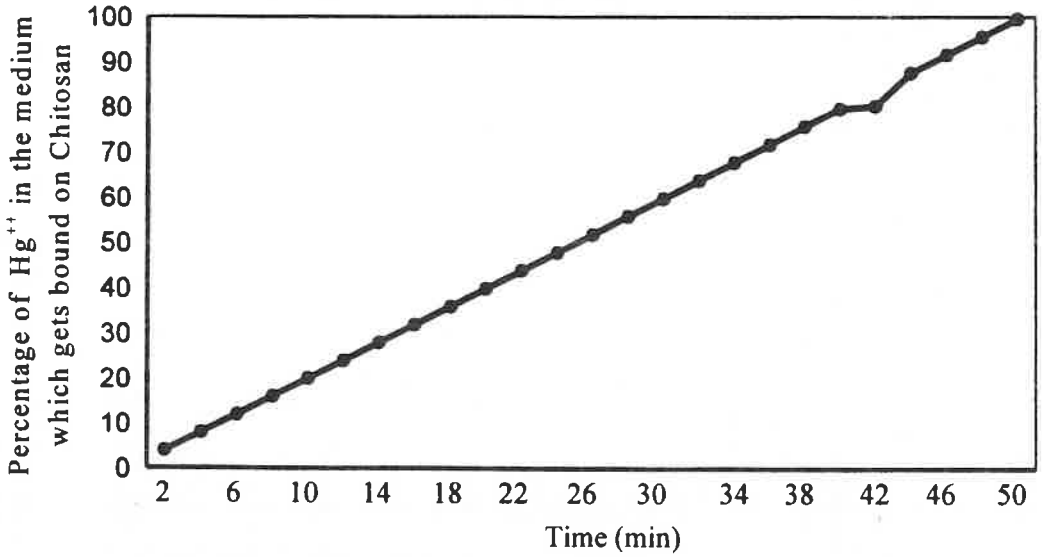

Fig 1

Concentration of mercury = 10 ppm

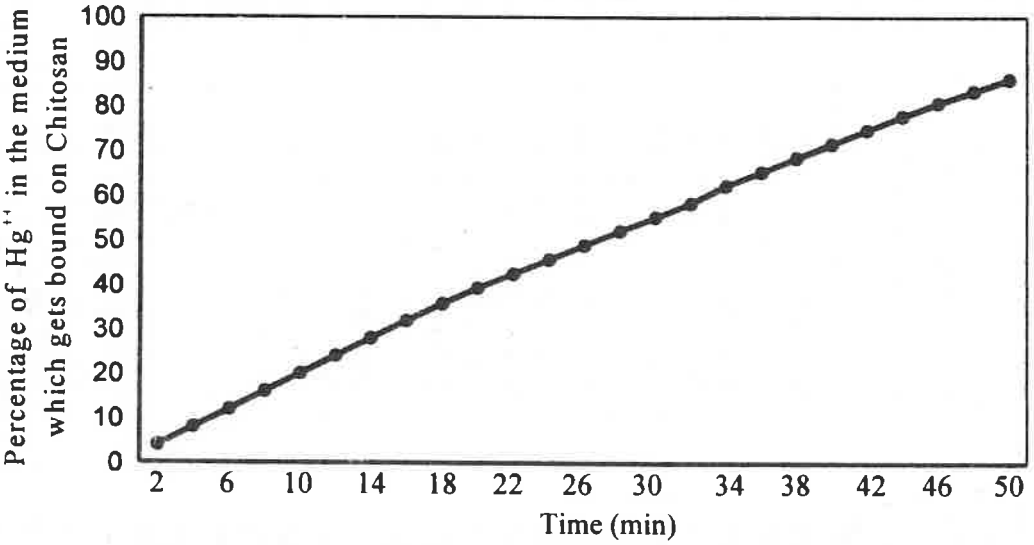

Fig 2

Concentration of mercury = 100 ppm

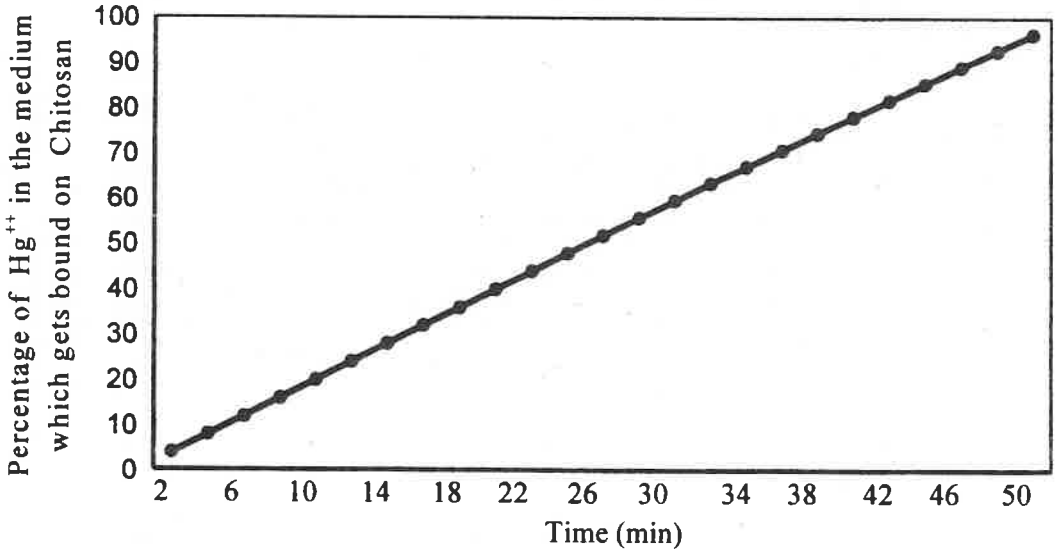

Fig 3

Concentration of mercury = 500 ppm

Temperature = 30°C, pH = 7 ; Weight of Chitosan = 1 gm

Rate of adsorption of mercury on Chitosan at different concentrations of Hg^{++} (Percolation)

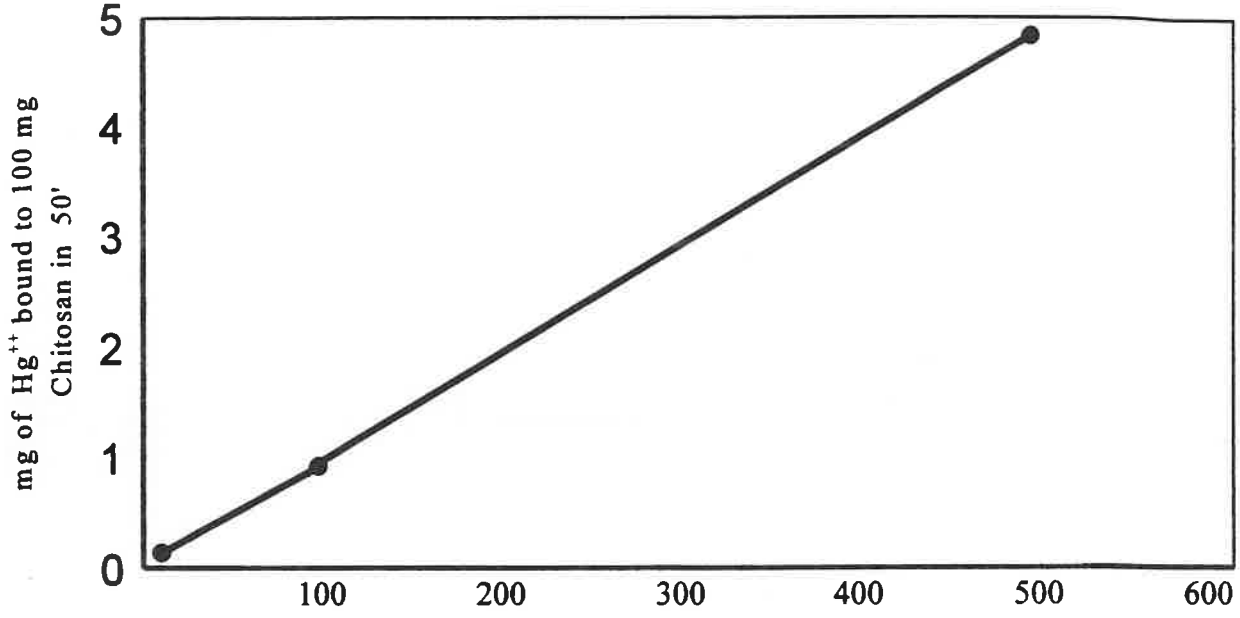

Fig 4

Temperature = 30°C, pH = 7 ; Weight of Chitosan = 1 gm

Rate of adsorption of mercury on Chitosan at different concentrations of Hg^{++}

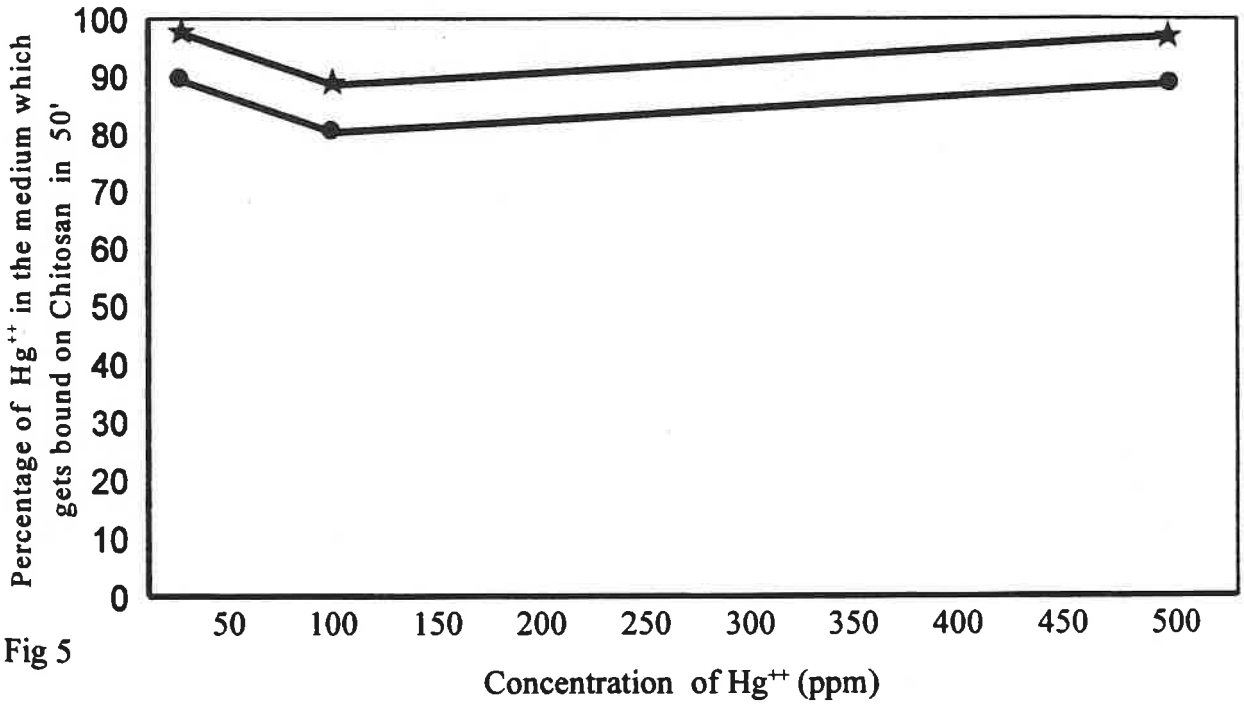

Fig 5

Temperature = 30°C, pH = 7 ; Weight of Chitosan = 1 gm

Rate of adsorption of mercury on Chitosan at different particle sizes (Percolation)

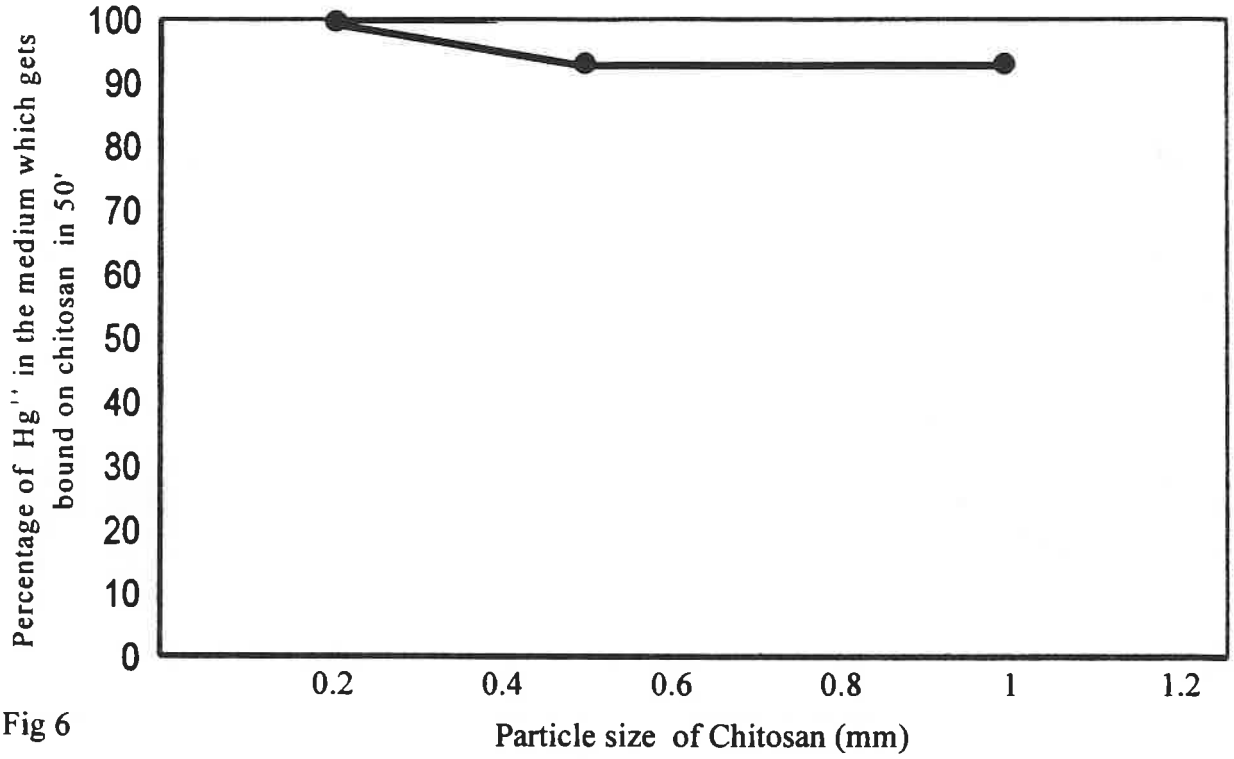

Fig 6

Concentration of mercury = 500 ppm
Temperature = 30°C, pH = 7 ; Weight of Chitosan = 1 gm

Rate of adsorption of mercury on Chitosan at different particle sizes (direct addition)

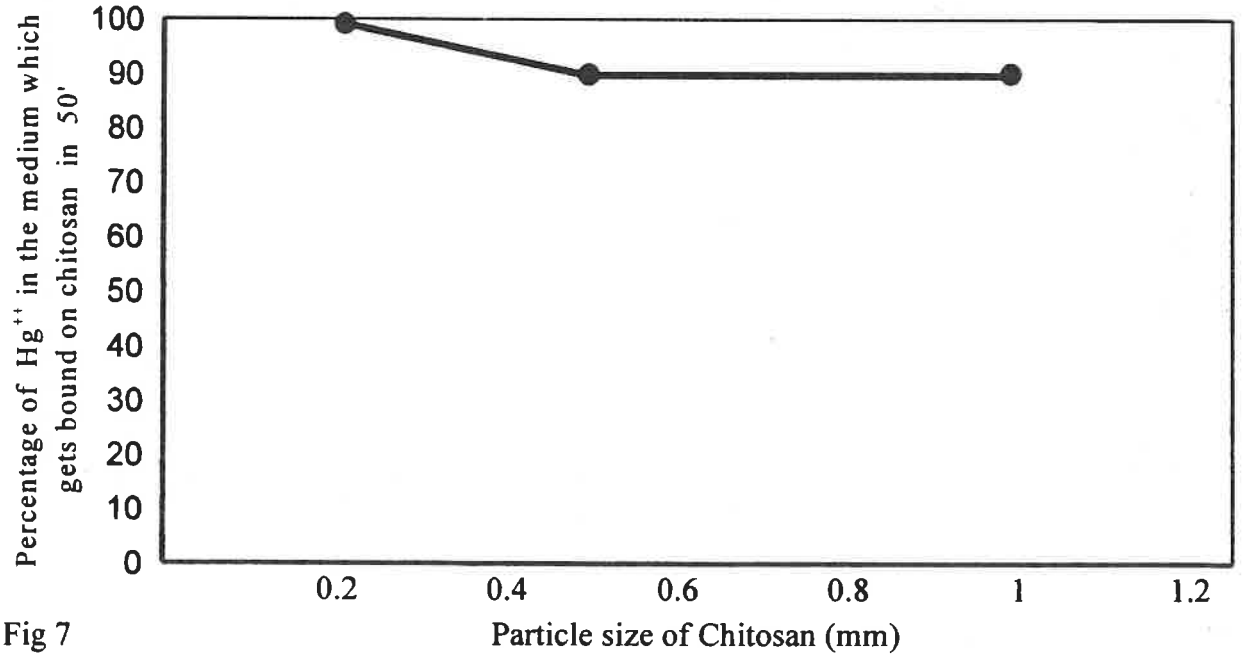

Fig 7

Concentration of Hg^{++} = 500 ppm
Temperature = 30°C, pH = 7 ; Weight of Chitosan = 1 gm

Results and Discussion

(a) Percolation method

The result of the analysis involving percolation of solutions containing Hg^{++} through a column of chitosan is presented in Table 1 and in figures, 1, 2 and 3. When 100 ml of 10 ppm mercury solution was passed through a column of 1 gm of chitosan, binding of mercury by chitosan was 99.6% in 50 minutes. Initially the binding was slow and with increased time of elution, there was considerable binding of mercury by Chitosan. But when the concentration of mercury was increased to 100 ppm and 500 ppm, the weight of mercury bound to the same weight (100 mg) of chitosan was increased. The result of the analysis is presented in figure 4. It was observed that when the concentration of mercury in the medium was increased, the binding of mercury by same quantity of chitosan was also increased.

(b) Direct addition method

The result obtained from direct addition of chitosan to mercury solution is presented in Figure 5. When the process is conducted in a direct addition manner, 1 gm chitosan was agitated with 100 ml solution, containing 10 ppm, 100 ppm and 500 ppm of mercury respectively. Milligram of mercury bound to 100 mg of chitosan at the 50th minute is plotted against the concentration of mercury. Fig. 6 shows the results of percolation of Hg^{++} while Fig. 7 shows the results of direct addition of chitosan of mesh size 60 (0.25 mm), 35 (0.5 mm) and 18 (1 mm) mesh. In both cases the concentration of mercury was 500 ppm. It was observed that when the particle size is reduced, percentage binding by Hg^{++} increased. Particle size was found to influence the binding of mercury on chitosan in accordance with earlier observations (Muzzarelli, 1971) (10).

Compared to direct addition method percolation method is more effective in detoxification of mercury by chitosan.

Conclusion

The results confirm the strong and efficient binding of mercury on chitosan. Hence chitosan can be recommended as an efficient natural fibre (adsorbent) to reduce the toxicity of metals in water and waste water. The experiment also shows that chitosan can be used for the complete removal of mercuric salts from water and from industrial effluents. Depending upon the initial concentration of mercury the quantity of chitosan and the number and duration of treatment can be adjusted to achieve complete removal of mercury from contaminated water or industrial effluents.

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WASTE WATER TREATMENT WITH CHITOSAN IN A PAPER RECYCLING PLANT

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Abstract: The feasibility of applying chitosan (η=50m Pa.s, Degree of deacetylation=79%) prepared from shrimps shells collected in Bahía Blanca estuary was assessed in this study for the waste water clarification in a paper recycling plant. A series of batch coagulation-flocculation test equipment (jar testing) was done to improve optimal dosage, temperature, effect of pH and rate of stirring. The characterization of the waste water before and after treatment with chitosan were performed. All results for the optimal dosage of chitosan indicated decreases in turbidity of 90-99 %, COD and BOD of 50-60 %, total coliforms (95-98 %) and almost 100% of fiber. No change of pH and conductivity was observed. This study showed the efficiency of chitosan (a natural cationic agent), instead of others usually used ones, for the treatment of this kind of wastes.

1. INTRODUCTION

This study deals with the treatment of the waste water produced by paper recycling plant (production of about 100 tn/day of paper for corrugated cardboard and 80 m^3 / day of water consumption). Although the plant is in a suburban area at a middle-sized city; effluents carrying on fine organic particles and fiber represents an environmental problem for surrounding people.

Chitosan as coagulant-flocculant has been assessed for the treatment of different water system [1,2,3,4]. Particularly, the adsorption of chitosan and water-soluble chitosan derivatives on cellulose has been studied by several authors primarily because of the possible utilization of chitosan in paper making [5].

Cellulose acquires a negative surface charge on immersion in water and hence electrostatic interaction between chitosan and the cellulose substrate would be expected to play an important role in the adsorption mechanism. Being the chitosan the unic natural cationic polyelectrolite, is an useful tool for the coagulation-flocculation process of this type of effluent.

Different dosages and conditions of chitosan and chitosan-bentonite were essayed in this work in order to evaluate the feasibility of applying this biopolymer on the clarifying of this waste water. In all cases chitosan was that obtained by the authors, having 50 mPa.s of viscosity and 79% of degree of deacetylation.

2. MATERIALS AND METHODS

2.1. Chitin obtention

Chitin was isolated from shrimps shells (*Artemesia longinaris*) collected in Bahía Blanca estuary. First homogenization was carried out and the product was rinsed in order to remove the soluble organics.

Alkaline treatment (NaOH 10% w/v - 24 hours) and further acid one (HCl 10% v/v - 10 minutes) were done. The product was rinsed until pH=7 (±0,1).

2.2. Chitosan obtention.

The chitosan was prepared by alkaline and thermic treatment of chitin with NaOH 70% (w/v) at 136 °C for 1 hour. In every steps the product was rinsed several times by deionized water until neutrality.

2.3. Jar testing

Series of batch coagulation-flocculation tests were done to improve optimal dosage for the clarification of the effluent. A conventional jar test apparatus was used in the experiment according to ASTM Standards [6]. Quality controls of the effluent before and after treatment with chitosan at the obtained optimal dosage were performed. The measurements of Turbidity, COD, BOD, Conductivity and pH were made according to Standard Methods [7], Fiber by the automatic Weend method and Totals coliforms by plate count in agar VRB (Violet Red Bile Agar) after 24 hours at 37 °C. Subsequently the variation of Turbidity vs. Temperature, pH and rate of stirring was assessed, working again at the optimal dosage.

2.4 Working solutions

The tests were performed by adding solution of chitosan 1% (w/v) in acetic acid 1% (v/v). The use of bentonite as coagulant-aid was essayed at 2% (w/v) concentration.

3. RESULTS AND DISCUSSION

Firstly the optimal dosages of chitosan and chitosan-bentonite were assessed. Working only with chitosan it was 85-115 $mg.L^{-1}$, and the further addition of bentonite (100 $mg.L^{-1}$) as coagulant-aid yielded up an optimal concentration of 85-90 $mg.L^{-1}$ of chitosan. For instance, for a particular wastewater having an initial turbidity of 754 UNT, the optimal dosage graph is shown in Fig. 1.

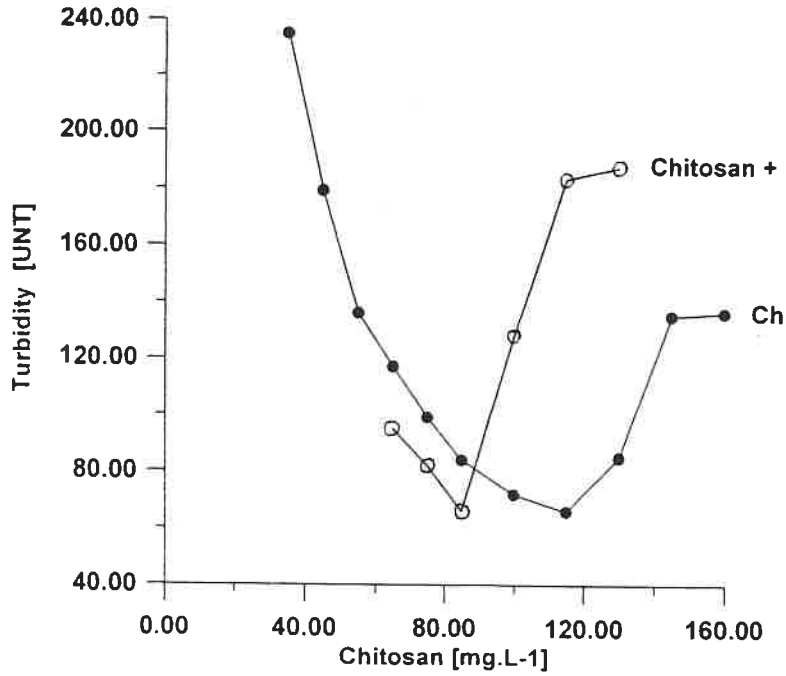

Fig. 1. Residual turbidities of supernatants after treatment with chitosan and chitosan-bentonite.

Fig.2 presents the results of the action of 15 seconds of different stirring velocities on the final turbidity after 15 min. of settling, working at optimal concentration of chitosan

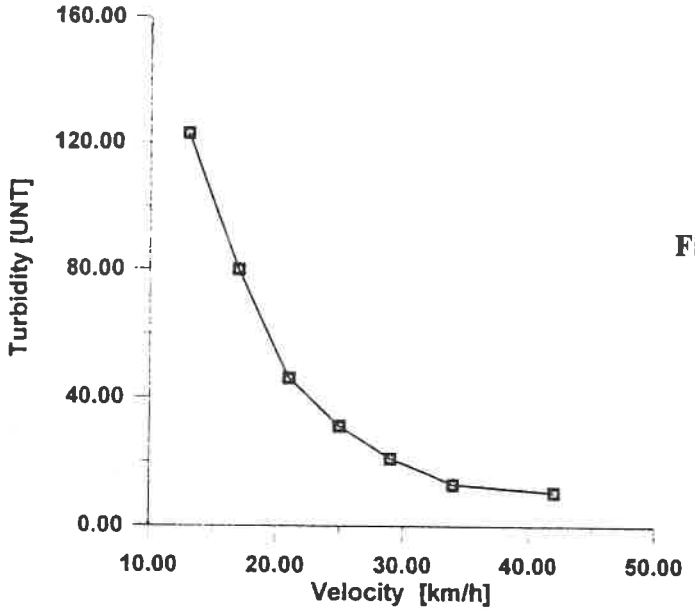

Fig. 2. Velocity of stirring and residual turbidities of supernatants after treatment.

Similarly, the behavior of the treatment at different temperatures and pH was studied, as presented in Fig. 3 and 4 respectively.

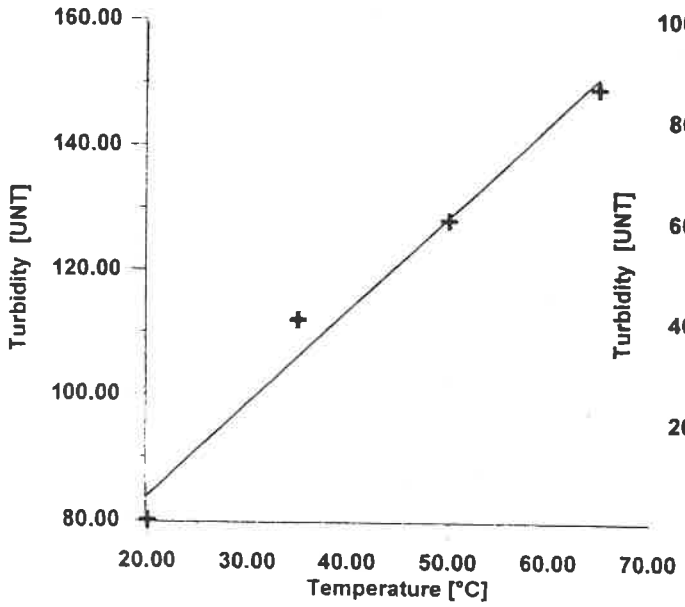

Fig. 3. Residual turbidities of supernatants after treatment at temperatures ranging between 20 y 65°C.

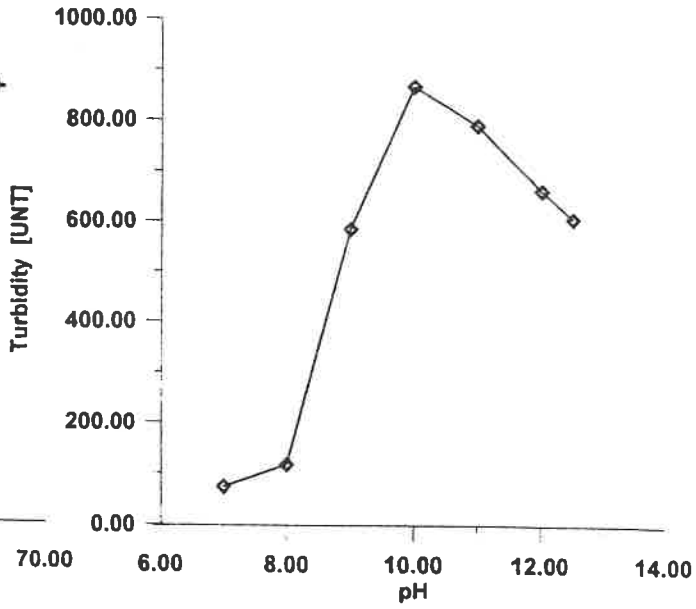

Fig. 4. Residual turbidities of supernatants after treatment at different pH values.

Values of the measured parameters before and after chitosan treatment, and percentage of disminution are shown in Table 1.

Turbidity [UNT]			
Initial	final	optimal dosage [mg chitosan.L^{-1}]	% dism
754,00	66,00	115	91,25
532,00	58,00	110	89,10
7816,00	39,40	85	99,50

COD [mg.L^{-1}]		
initial	final	% dism
3420,00	1644,00	51,93
3360,00	1596,00	52,50
4544,00	2116,00	53,43

BOD [mg.L^{-1}]		
initial	final	% dism
1394	672,00	51,79
1206	492,00	59,20

Total coliforms [UFC.L^{-1}]		
initial	final	% dism
2,1E+9	100,0E+6	95,12
3,5E+9	80,0E+6	97,73

Fiber [g.L^{-1}]		
initial	final	% dism
0,50	< L.D.	~ 100
0,85	< L.D.	~ 100

Table 1. Initial and final values of the measured parameters and percentage of disminution after chitosan treatment.

4. CONCLUSIONS

The chitosan optimal dosage for the waste water clarification in a paper recycling plant at working conditions was 85 - 105 mg/L. Efficiency does not increase by the use of bentonite as coagulant-aid, but its utilization allows lesser chitosan concentrations.

At the chitosan optimal dosage :
- the increase of stirring velocity yields a sharp disminution in turbidity,
- between 20 and 65 °C, final turbidity rises linearly with temperature,
- at pH values higher than 8, chitosan does not behaves as a good clarifying agent.

This study shows the efficiency of the use of chitosan and chitosan-bentonite pair for the waste water clarification in a paper recycling plant, giving reductions of 90-99% of turbidity, 50-60 % of COD and BOD, 95-98% of total coliforms and almost 100% of fiber.

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CHITIN AND CHITOSAN BIOSORBENTS FOR RADIONUCLIDES AND HEAVY METALS

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Abstract

Our studies on sorption properties of chitin and chitosan fungal sorbents "MYCOTON" are of ecological significance and promising for solving problems on liquidation of the consequences of the accident at the Chernobyl Nuclear Power Plant and for purification of industrial discharges of radionuclids and heavy metals. Special attention being paid to trans-uranium elements (U, Pu, Am, Cm) and caesium isotopes. Sorption properties are investigated both on model systems and on technological wastes of Chernobyl NPP, Khmelnitsky NPP, Mangishlak NPP and polluted natural objects. Sorbents are stable in solutions with the concentration of salts up to 450 g/l, high content of surface-active substances and oil products. The sorbent "MYCOTON-Cs" provides no less than a 1000-fold decrease in the level activity of liquid radio-active wastes from Chernobyl and Khmelnitsky NPP. Chitin sorbent with ferromagnetic properties "MYCOTON-M" permit purifying bottom sediments of water bodies and arable soils from heavy metals and radionuclides.

Keywords : Chitin, chitosan, fungi, sorbent, liquid wastes.

Materials and methods

"MYCOTON" is the name of new fibrous chitin-containing materials made of cell walls of the fungi (1). "MYCOTON" is produced from fruit bodies of the *Higher Basidiomycetes* (mainly species of the *Aphyllophorales*). The natural and cultivated fungi are good for production of the chitin-containing materials.

A great number of chitin-containing material modifications have been developed for special purposes. Basic material mark is "MYCOTON-Ch". Chitin can be modified into chitosan. Mark of such material is "MYCOTON-Chs". To improve sorption capacity to caesium we have developed "MYCOTON-Cs". We have developed also sorbent "MYCOTON-M" having ferromagnetic properties. The combination of these

two sorbents has mark "MYCOTON-Cs-M". A paper like and nonwowen goods for filters have mark "MYCOTON-Ff" (2).

The chitin sorbents "MYCOTON" have been investigated in the Russian Research Center "Kurchatov Institute" and widely tested both on the natural polluted objects and at the industrial enterprises. Static and column methods of radionuclides extraction were used for experiments.

The distribution coefficients (K_d) were principal criteria of sorption properties and determined from relationship:

$$K_d = M_s / M_{aq} \times V_{aq} / P_s, \text{ where}$$

M_s and M_{aq} are the amount of metals in the solid and aqueous phases in arbitrary units, V_{aq} is the volume of the aqueous phase in *ml*; P_s is the mass of the sorbent in *g*.

The sorption properties were determined as a function of both the pH value and the concentration of some acids in the solution. Nitric, hydrochloric, phosphoric and acetic acids were considered. The exchange capacity of "MYCOTON" was determined spectrophotometrically, by X-ray electron spectrometry and with the help of radio-active tracers in saturating sorbent by a stable isotope of europium (2, 3).

Results and discussion

Physical and chemical properties. "MYCOTON" has a fine fibrous structure of hollow cylindrical fibres of 3 - 5 μ in diameter and in length up to several mm. The walls of fibres have 0,2 - 1 μ thickness and are of high strength and elasticity. They are stable to the action of UV- and penetrating radiation as well as to most of solvents. It does not loss its properties up to the temperature of 150° C.

"MYCOTON" has specific chitin X-ray reflection by the angles of diffraction 2 Θ = 9°, 13°, 19°30', 21°, 24°, 26°. Infra-red spectra has specific lines in 3400, 2930, 1650, 1560, 1380, 1080 cm^{-1} (4).

Fungal chitin is in the microfibrillous form. The geometrical surface of microfibrillas of "Mycoton" is more than 1000 m^2/g. It has good ion- and electron-exchange properties and strong chelating ability. "Mycoton" due to the presence of melanins has very high resistance to the action of alpha- and gamma-radiation. It does not lose its sorption properties for want of doses up to 100 Megarad.

Sorption properties. Sorbents "Mycoton" have revealed high sorption properties to actinides - Pa, U, Np, Pu, Am, Cm, lanthanides - Ce, Eu and other heavy metals - Bi, Pb, Hg, Cs, Cd, Sr, Mn, Cr. The investigations showed that chitin sorbent "MYCOTON-Ch" has a higher sorption capacity,

than chitosan "MYCOTON-Chs". The sorption maximum lies in the vicinity of pH 5 - 6 for all tested elements and in the area of relatively low pH the distribution coefficient of plutonium are even higher then those of uranium, americium and curium (Fig. 1). In the mineral acid medium (HCl, HNO_3, H_3PO_4) all dependences are of extreme character with a maximum in the vicinity of a 10^{-3} - 10^{-2} mol/l concentration (Fig. 2). The highest distribution coefficients (10^5) are observed in the acetic acid solutions. The exchange capacity on europium is 0.6 - 1,2 mg.-mol./g and about 240 mg/g on plutonium.

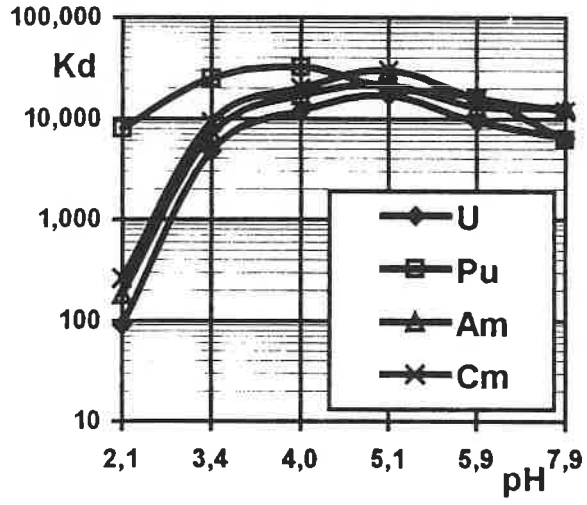

Fig. 1. The solution pH effect on the distribution coefficient of transuranium elements on chitin sorbent "Mycoton-Ch".

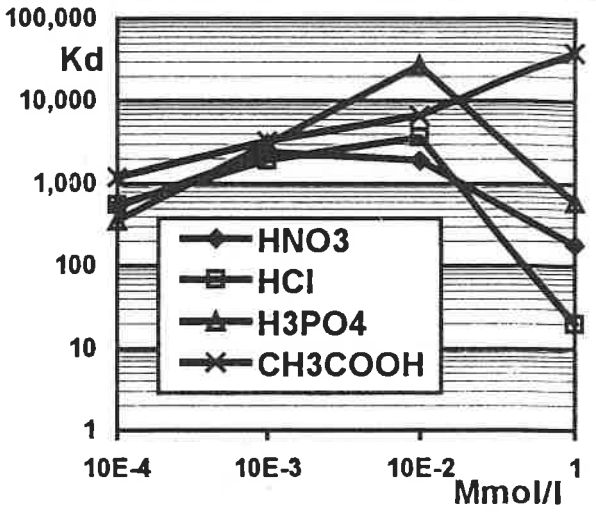

Fig. 2. The effect of acids on the distribution coefficients of plutonium ("Mycoton-Ch").

The kinetic of sorption characteristic for radionuclides are essentially different: 150 min are enough to set equilibrium for Pu and Am on "MYCOTON-Ch". The sorption for Cs on "MYCOTON-Cs" occurs more rapidly. The distribution coefficient of Cs reaches values of 10^4 for 5 min and then it increases within an hour up to 10^5. "MYCOTON" has high effectiveness for heavy metals. The distribution coefficient of most of them makes up 10^3 - 10^5. The Cl^-, NO_3^-, SO_4^{-2} and acetate-ions have small influence on the sorption.

The indifference of sorbents "MYCOTON" to ions of light metals (K, Na, Ca, Mg) is their very valuable quality. Due to this quality the chitin sorbents do not lose their properties in the concentrated salt solutions of different ionic composition. The content of salts may be 450 g/l. Besides, "MYCOTON" is tolerant to high concentrations of surface-active substances (5 g/l) and oil products (0.1 g/l).

Radio-active wastes decontamination. "MYCOTON" has been subjected to tests on radio-active wastes of the Chernobyl NPP. These wastes contain isotopes of caesium and cobalt and 317 g/l of various salts. It allows reducing the content of radio-active isotopes by 1000 times (Tab. 1).

This sorbent has also shown high ability on other types of liquid radio-active wastes of Khmelnitskiy NPP and Mangishlak NPP. The first contained up to 450 g/l of salts, out of them - 150 g/l of boron. The isotopes of caesium

Table 1

DECONTAMINATION OF EVAPORATION CONCENTRATES FROM *CHERNOBYL NPP* BY "MYCOTON-Cs"

NN wastes	Isotopes	Activity before decont., Ci/l	Activity after decont., Ci/l	Decont. coeff.
201/2	Co^{60}	$1.8 \cdot 10^{-7}$	$8.2 \cdot 10^{-9}$	22
	Cs^{134}	$3.8 \cdot 10^{-7}$	$< 10^{-10}$	> 3000
	Cs^{137}	$5.7 \cdot 10^{-6}$	$< 10^{-9}$	> 5000
	Σ	$6.2 \cdot 10^{-6}$	$8.2 \cdot 10^{-9}$	930
201/3	Co^{60}	$1.1 \cdot 10^{-6}$	$4.9 \cdot 10^{-8}$	26
	Cs^{134}	$2.8 \cdot 10^{-5}$	$3.1 \cdot 10^{-8}$	900
	Cs^{137}	$5.0 \cdot 10^{-4}$	$1.6 \cdot 10^{-8}$	> 30000
	Σ	$5.3 \cdot 10^{-4}$	$9.6 \cdot 10^{-8}$	> 5000

are extracted by "MYCOTON-Cs" up to the safe level, and for decontamination of cobalt additionally used the flocculation by chitosan was used additionally (Tab. 2). The wastes of Mangishlak NPP contained few salts and a degree of clearing from caesium reached very high indexes (Tab. 3)

Table 2

DECONTAMINATION OF THE EVAPORATION CONCENTRATES FROM *KHMELNITSKY NPP* BY "MYCOTON-Cs" + CHITOSAN

NN wastes	Isotopes	Activity before decont., Ci/l	Activity after decont., Ci/l	Decont. coeff.
20B02	Co^{60}	$2.5 \cdot 10^{-6}$	$3.4 \cdot 10^{-9}$	735
	Cs^{134}	$6.4 \cdot 10^{-5}$	$1.9 \cdot 10^{-9}$	33000
	Cs^{137}	$1.7 \cdot 10^{-4}$	$1.9 \cdot 10^{-8}$	8900
	Σ	$2.4 \cdot 10^{-4}$	$2.4 \cdot 10^{-8}$	10000

"MYCOTON-Cs" and "MYCOTON-Ch" were used for decontamination of radio-active water polluted as a result of the accident at the Chernobyl NPP. The first from them well extracts isotopes of caesium and trans-uranium elements. Second, practically not absorbs caesium also allows selectively to extract divided trans-uranium elements. Some results of these experiments are given in Tab. 4.

Table 3

DECONTAMINATION OF EVAPORATION CONCENTRATES FROM *MANGISHLAK NPP* BY "MYCOTON-Cs"

NN wastes	Isotopes	Activity before decont., Ci/l	Activity after decont., Ci/l	Decont. coeff.
Б-02-3	Co^{60}	$2.67 \cdot 10^{-6}$	$2.24 \cdot 10^{-6}$	1,2
	Cs^{137}	$5.97 \cdot 10^{-3}$	$1.78 \cdot 10^{-8}$	335000
	Σ	$6.0 \cdot 10^{-3}$	$2.25 \cdot 10^{-6}$	2600
10K	Co^{60}	$1.25 \cdot 10^{-6}$	$1.0 \cdot 10^{-10}$	12500
	Cs^{137}	$3.2 \cdot 10^{-6}$	$1.9 \cdot 10^{-8}$	170
	Σ	$4.45 \cdot 10^{-6}$	$1.9 \cdot 10^{-8}$	230

Table 4

DECONTAMINATION OF THE LIQUID RADIO-ACTIVE WASTES FROM ENCASEMENT *"U K R Y T I E" OF CHERNOBYL NPP* BY "MYCOTON-Cs"

NN wastes	Isotopes	Activity before decont., Ci/l	Activity after decont., Ci/l	Decont. coeff.
002	Co^{60}	$4.1 \cdot 10^{-9}$	-	
	Cs^{134}	$3.9 \cdot 10^{-6}$	$4.5 \cdot 10^{-9}$	900
	Cs^{137}	$7.9 \cdot 10^{-5}$	$2.0 \cdot 10^{-8}$	3900
	Σ	$8.3 \cdot 10^{-5}$	$2.8 \cdot 10^{-8}$	3000
0054	Co^{60}	$6.8 \cdot 10^{-7}$	$2.5 \cdot 10^{-8}$	27
	Cs^{134}	$2.5 \cdot 10^{-5}$	$2.5 \cdot 10^{-8}$	1000
	Cs^{137}	$4.8 \cdot 10^{-4}$	$1.6 \cdot 10^{-7}$	3000
	Σ	$5.1 \cdot 10^{-4}$	$2.1 \cdot 10^{-7}$	2400

Using sorbents "MYCOTON" it is possible to decrease the content of radionuclides in liquid radio-active wastes by 1.000 - 10.000 times and more. The exchange capacity enables decontamination up to 1000 volumes wastes by 1 volume of "MYCOTON". It is possible to receive a 200-fold decrease in volumes of radio-active wastes of the NPP which are subjected to burial as compared with the standard method. "MYCOTON" has a very low ash content and which may be burnt, thus increasing the compacting coefficient.

Ferromagnetic chitin sorbents. "MYCOTON-M" and "MYCOTON-Cs M" open new possibilities for decontamination of systems with liquid and solid phases as silts of natural reservoirs or polluted soils. "MYCOTON-M" is brought into such system, it absorbs ions of heavy metals and radionuclides. Then the used sorbent is separated by magnet.

Ferromagnetic sorbents "MYCOTON" were tested both in laboratory and on a natural objects. There were silts of the river Dnieper polluted with industrial wastes, silts of the

Chernobyl NPP channel and soil polluted with radionuclides after the Chernobyl NPP accident (2). Some results of these experiments are presented in Tab. 5.

Table 5

DECONTAMINATION OF BOTTOM SEDIMENTS FROM THE *DNIEPER* BY FERROMAGNETIC SORBENT "MYCOTON-M"

NN	Metal	Concentration, mg/kg		Decont., %
		before decont.	after decont.	
1	Mn	218.00	39.00	82
	Cd	0.52	0.00	100
	Pb	253.00	102.00	60
	Hg	44.00	0.00	100
2	Mn	922.00	266.00	71
	Cd	0.49	0.00	100
	Pb	19.00	0.00	100
	Hg	9.00	0.00	100

Our experiments also have shown possibilities of using ferromagnetic sorbents "MYCOTON" for purifying agricultural soils. The content of radionuclids and heavy metals in plants after soil restoration does not exceed the admissible level.

Conclusion

Chitin-containing sorbents "MYCOTON" have also broad perspectives for a solution of many other problems connected with purification of liquid wastes and water from various contaminations and for site restoration.

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REMOVAL OF PHENOLS FROM WASTEWATER BY AN ENZYME AND CHITOSAN

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ABSTRACT

Chitosan was found to act as an adsorbent and a coagulant for colored products by tyrosinase from phenols. The products are more easily removed by chitosan as a cationic polymer coagulant than an adsorbent. Phenols are not precipitated by coagulants, but their enzymatic reaction products are easily precipitated by chitosan. These results indicate that the combination of tyrosinase and chitosan is effective in removing carcinogenic phenols from an aqueous solution. In addition, the reduction rate of phenols was observed to be accelerated in the presence of chitosan.

INTRODUCTION

An enzymatic approach to the removal of toxic chemicals from industrial wastewater has attracted much interest recently. Tyrosinase catalyzes hydoxylation of monophenols with molecular oxygen to form *o*-hydroquinones, and then, dehydrogenation of the quinones occurs to form the corresponding *o*-benzoquinones. The benzoquinones undergo a nonenzymatic polymerization to yield water-insoluble substances. Peroxidase need costly hydrogen peroxide as oxidant, whereas tyrosinase utilized molecular oxygen. Tyrosinase dephenolization has an advantage in that there is no difference between the crude tyrosinase prepared from mushroom and highly purified commercial enzymes in the efficiency of dephenolization from water containing various phenols[1]. In our preliminary experiments, however, no precipitate have observed from polymerization of phenols by tyrosinase. This suggests that it is difficult to remove completely toxic phenols and their oxidation products by precipitation. Recently, chitosan has been shown to be especially effective for removal of quinones formed by the oxidation of phenols with tyrosinase[2,3,4].

In the present work, we demonstrate that treatment of phenols with tyrosinase, in the presence of chitosan as adsorbent and coagulant, is much more effective compared with tyrosinase alone.

MATERIALS AND METHODS

Chemicals

Tyrosinase (EC 1.14.18.1) was obtained commercially from Sigma Chemicals (St.Louis, MO) and had a specific activity of 3500 units/mg. Tyrosinase activity was determined from a change in optical density(A280 nm) in a reaction mixture containing L-tyrosine. Phenol were purchased from Wako Chemicals (Tokyo, Japan); *p*-chlorophenol, *p*-methoxyphenol, *p*-cresol, and catechol were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Cellulose (MN100, powder), chitin from crabshells (powder) and chitosan were obtained from Nacalai Tesque (Kyoto, Japan).

Incubation conditions analysis

All reactions with soluble tyrosinase were carried out at 25°C in 7 ml of 0.05 M phosphate buffer (pH 7.0) containing tyrosinase and each phenol (0.5 mM). Chitosan less than 150 μm was added to reaction solutions as an adsorbent. After a prescribed time, the sample was withdrawn and assayed for phenols, and the absorption spectra of reaction solutions were measured. In the case of a combination of tyrosinase and a coagulant, the coagulant was added to reaction solutions (pH 7.0) after an enzymatic reaction. and chitosan were dissolved at 0.5% concentration in 0.5% acetic acid, and added to the solutions.

The disappearance of phenols was monitored by high performance liquid chromatography using a Shimadzu LC-3A (Tokyo, Japan) combined with a spectrophotometer (Japan Spectroscopic UVIDEC 100-III, Tokyo Japan) and a integrator (Shimadzu C-R1B, Tokyo Japan). A reverse phase column, Shim-pak C18(5 u, 6 mm i.d. x 15 cm, Shimadzu), was used, and the mobile phase, flow rate 1.0 ml/min, consisted of methanol and water and the ratio was 1:1.

RESULTS AND DISCUSSION

Dephenolization by soluble tyrosinase

The extent of coloration of phenol or *p*-chlorophenol solution by incubation with tyrosinase, together with the reduction of each phenol are shown in Figure 1. The degree of coloration was given as the increase in the absorbance at 400 nm. The concentration of phenol and *p*-chlorophenol decreased with time, and 100% of *p*-chlorophenol was removed after 3 h and 100 % of phenol after 4 h. The coloration indicates the production of colored products from phenols. These results indicate that phenol was completely transformed, but the products formed were water soluble and not precipitate of phenolic polymer. To investigate the effect of the amount of enzyme, we added 20-fold the amount of tyrosinase (400 units/mL), but no precipitate was observed.

Removal of colored products by adsorption

To remove the colored products formed from phenol by tyrosinase, the use of chitin and chitosan was investigated. Removal efficiency was estimated from the change in absorbance at 400 nm (data not shown). The colored products were removed to a lesser extent by cellulose, but very rapidly by chitin and chitosan. The removal of the products from the solution by chitosan was higher than that by chitin. This is because of the difference of reactivity between an amino group and an acetylamino group for the colored products formed from phenol. Figure 2 shows the absorption spectra of reaction solutions

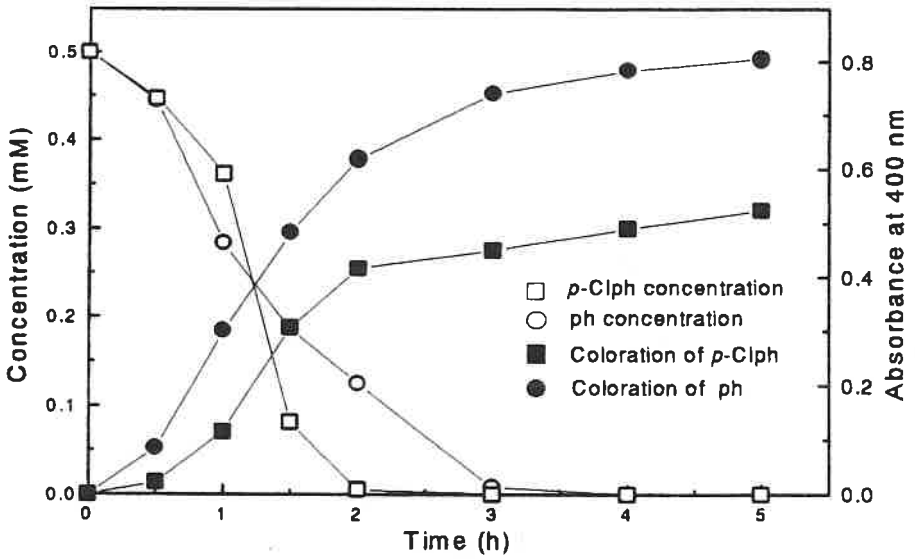

Figure 1. Coloration of reaction solution and reduction of phenol and *p*-chlorophenol by incubation with tyrosinase. Phenol: 0.5 mM; *p*-chlorophenol: 0.5mM; tyrosinase activity: 20 units/mL (pH7). Incubation time: 3h.

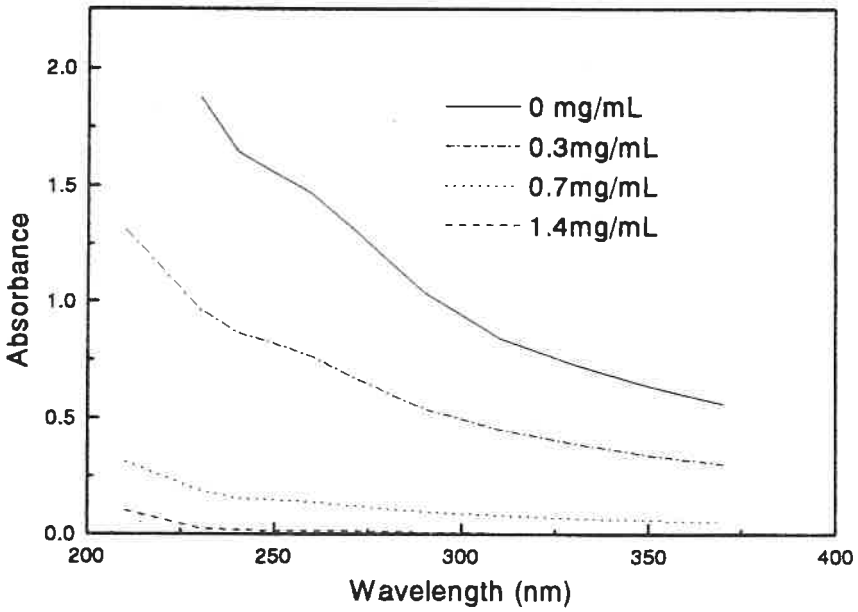

Figure 2. Absorption spectra of the reaction solution obtained by incubation of phenol with tyrosinase in the presence or absence of chitosan. Incubation conditions were as described in Figure 1.

of phenol oxidized by tyrosinase in the presence of chitosan. Chitosan, 1.4 mg/L, completely removed the colored substances formed from phenol, which could no longer be found in the solution. No removal of phenol was observed by chitosan alone. It is well known that quinone is easily attacked by a lone electron-pair of a nitrogen in an amino group in nucleophilic reaction to form a carbon-nitrogen bond[5]. Chitosan containing a free amino group is more reactive to quinone formed from phenol than chitin in which the group is acetylated, because of steric hindrance. Chitin is not necessary to deacetylate;

therefore, chitin may be more favorable in practical wastewater treatment in comparison with chitosan.

The results obtained from the treatment of substituted phenols by 20 units/mL tyrosinase, in the presence or absence of chitosan, are shown in Table 1 and Figure 3. The colored products from substituted phenols as well as phenol were removed by chitosan (Table 1). The reduction rate of phenol and *p*-methoxyphenol by tyrosinase was found to be accelerated in the presence of chitosan (Fig.3). This may be because chitosan removed the reaction products which would inhibit tyrosinase activity. The same effect was observed for the oxidation of *p*-cresol and *p*-chlorophenol by tyrosinase. Sun et al.[2] reported that chitosan enhanced the reaction of cresol with tyrosinase.

Table 1. Effects of chitosan on removal of colored products formed from substituted phenol with tyrosinase.

Phenols	Absorbance at 400 nm	
	Absence of chitosan	Presence of chitosan
Phenol	0.460	0.000
p-Chlorophenol	0.227	0.001
p-Methoxyphenol	0.119	0.003
p-Cresol	0.131	0.002
Catechol	0.383	0.002

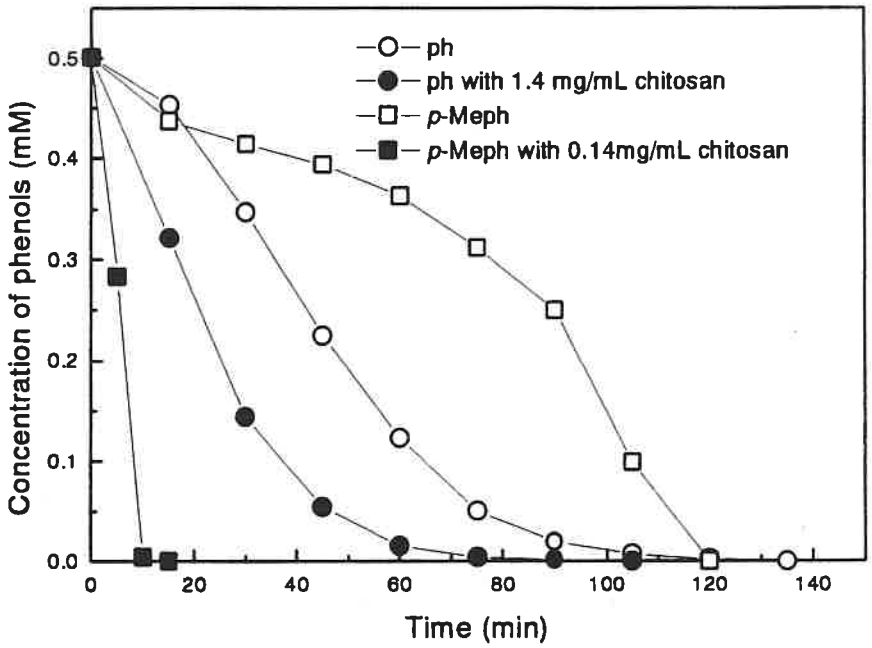

Figure 3. Effect of chitosan on removal of phenol and *p*-methoxyphenol with tyrosinase. Phenol: 0.5mM; *p*-methoxyphenol: 0.5mM; tyrosinase activity: 20 units/mL(pH7).

Even colored products were removed by chitosan, a small peak was observed at wavelengths under 260 nm (Fig. 2, 1.4 mg/L). This spectrum is similar to that of tyrosinase. From the quantitative assay of protein according to the procedure of Bearden[6], it was found

that about two thirds of the initial tyrosinase remained in the solution. Thus this method using soluble tyrosinase removed phenol and its colored reaction products, but some of enzyme remained in the solution.

Removal of colored products by coagulant

To remove the products by coagulation and flocculation, chitosan dissolved in 0.5 % acetic acid solution was added to reaction solutions. Chitosan is a cationic polymer coagulant used for wastewater treatment among other applications. Color removal of each enzymatic reaction solution of phenol and *p*-chlorophenol by chitosan is shown in Figure 4. The optimum dosage range of chitosan for both phenols was from 40 mg/L to 90 mg/L, and over 90 % of the color was removed. Figure 5 shows the removal of TOC and AOX from enzymatic reaction solution of *p*-chlorophenol by chitosan. In the optimum dosage about 90% of TOC and 100% of AOX were removed. This indicates that it is much more effective to use chitosan as a coagulant rather than an adsorbent.

Tyrosinase catalyzes hydroxylation of monophenols with molecular oxygen to form *o*-hydroquinones, and dehydrogenation of the quinones forms the corresponding *o*-benzoquinones[7]. This is also because ouinone is easily attacked by a lone electron-pair from a nitrogen in an amino group in a nucleophilic reaction to form a carbon-nitrogen bond[5]. Therefore, quinones formed by tyrosinase react with the amino group in chitosan or the coagulants, and flocculation occurs by intermolecular binding.

There is an optimum concentration range of chitosan for maximum flocculation. Thus when the dosage was less or more than that range, flocculation did not occur (Figure 2 and 3). The same has been reported on the effect of polymer concentration on flocculation of suspended solid in solution by Michaels[8], who suggested that, if excess polymer is present,

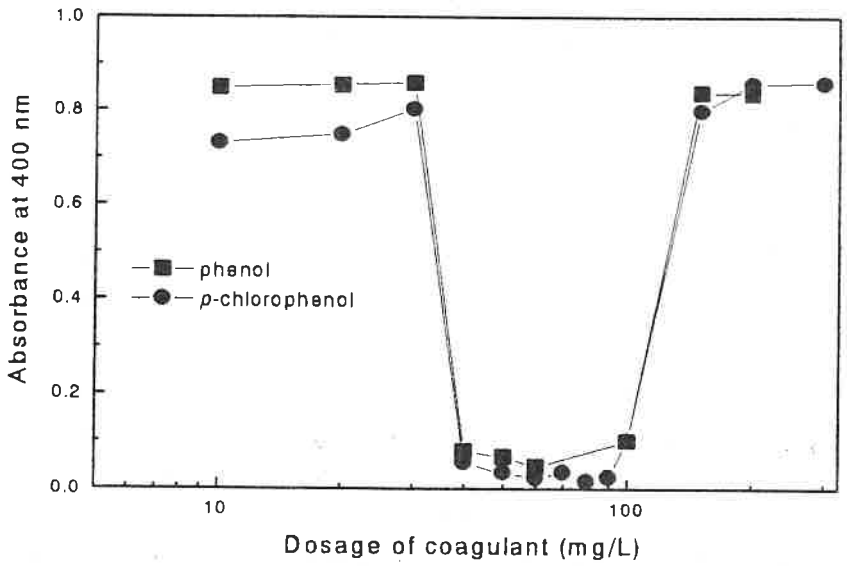

Figure 4. Color removal from reaction solutions of phenol and *p*-chlorophenol incubated by tyrosinase with chitosan. Incubation conditions were as described in Figure 1.

all adsorptive sites on the suspended particle surface must be occupied by adsorption of individual molecules, and "bridging" is minimized. The adsorbed polymer is strongly

hydrophilic and charged, thus causing the stabilization of colloidal dispersion. The observations in Figure 2 and 3 can be explained as follows: when there is an excess of coagulant in solution, because quinones react with individual coagulant molecules and cannot link between coagulant molecules, coagulation does not occur. The products thus cannot precipitate. On the other hand, in the case of an insufficiency of coagulant, the products cannot aggregate.

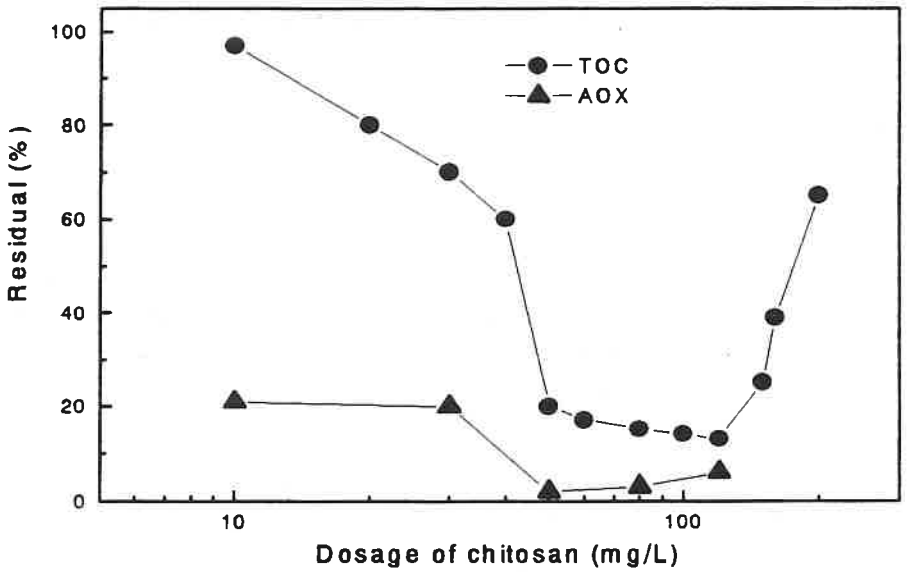

Figure 5. Effect of chitosan as a coagulant on removal of TOC and AOX from the reaction solution. Incubation conditions were as described in Figure 1.

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POSSIBILITY OF APPLICATION OF QUATERNARY CHITOSAN HAVING PENDANT GALACTOSE RESIDUES AS GENE DELIVERY TOOL

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Abstract

Chitosan is a non-toxic and biocompatible amino-type of polysaccharide. The quaternary chitosan can form polyelectrolyte complex with DNA. So, it is expected to be used as a carrier of DNA in gene delivery systems. However, chitosan itself has no recognizable moiety. On the other hand, it is well known that some kinds of saccharide play important roles in biological recognition on cellular surface. The "cluster effect" has been recently noted in biological recognition by carbohydrate-receptor binding in relation to cell-cell interaction. So, multi-antennary sugar chain is expected to be very effective in the application for cellular recognition devices. In order to achieve an effective gene delivery *via* receptor-mediated endocytosis, we designed *N,N,N*-trimethyl(TM)-chitosan/tetragalactose antenna conjugate (**TC-Gal4A**). The interaction between **TC-Gal4A** conjugate and RCA120 lectin was investigated as a simple model experiment by using a biosensor based on surface plasmon resonance. In order to investigate the specific delivery of DNA by **TC-Gal4A** conjugate, the expression of β-galactosidase activity by **TC-Gal4A**/DNA (pSVβGal plasmid) complex was evaluated in HepG2 cells. The conjugate showed the high binding affinity against RCA120 and the complex expressed the significant β-galactosidase activity.

Keywords: Chitosan, galactose, antenna, gene delivery, HepG2

It was suggested that the "cluster effect", an increase in the binding strength beyond that expected from the increase in galactose concentration, depended on the maximum spatial inter-galactose distance, the flexibility of the arm connecting galactosyl residue, and the branch point [1, 2]. So, multi-antennary sugar chain is expected to be very effective in the application for cellular recognition device.

Recently, the gene therapy involving the delivery of gene to target cells has become a topic in medical chemistry. The gene-transfer methods that adopt natural receptor-mediated endocytosis pathways for the delivery of DNA into target cells have been developed. Ligands for cellular receptors, such as transferrin [3, 4], viral proteins [5], insulin bound to albumin [6], and asialoorosomucoid [7, 8] have been used for the import of the DNA molecule. For this purpose, these ligands have been conjugated to DNA-binding compounds, such as a polycation and an intercalating agent. Incubating DNA with these protein conjugates generates ligand-coated DNA which can bind receptors to the cellular surface and is subsequently internalized.

Chitosan is a cationic natural polysaccharide which can form a polyelectrolyte complex with DNA. So, it is expected to be used as a carrier of DNA in gene delivery systems. However, chitosan itself has no recognizable moiety and has low solubility in water, except for when it is in an acidic condition. In order to achieve an efficient gene delivery *via* receptor-mediated endocytosis, we designed quaternary chitosan conjugates having galactose residues, *N,N,N*-trimethyl(TM)-chitosan/galactose conjugates [9], and quaternary chitosan conjugate having antennary galactose residues, TM-chitosan/tetragalactose antenna conjugate [10]. In this paper, we are concerned with the polyelectrolyte complex formation with DNA, the cellular recognition ability of these conjugates, and the specific gene delivery by the polycation-DNA complex. Moreover, we deal with the cluster effect of tetra-antennary galactose residues and the effect of local galactose concentration in these conjugates.

Materials and methods

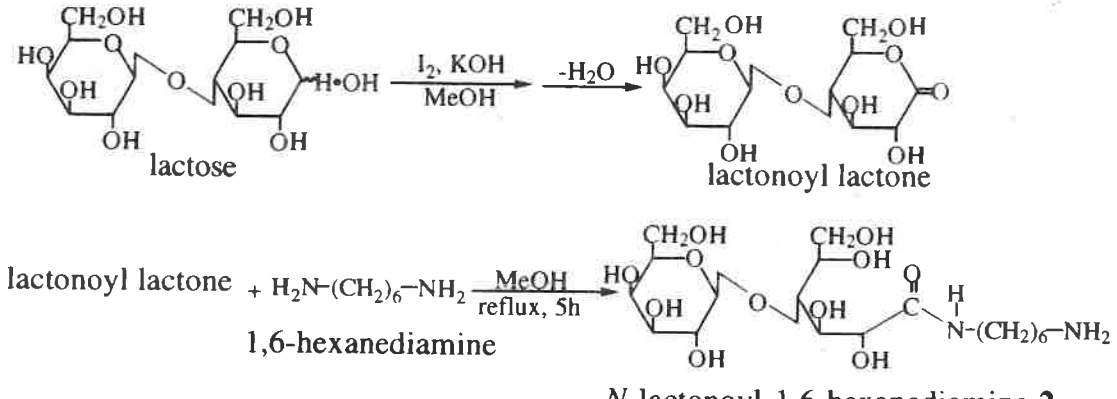
Chitosan was provided by Kimitsu Chemical Industries, Ltd. pSVluc and Maker 1 (Lambda phage DNA/Hind III digest) were purchased from Wako Pure Chemical Industries. Fluorescein isothiocyanate (FITC) labeled lectin from Abrus Precatorius agglutinin (APA) and biotin labeled lectin from Ricinus communis agglutinin-120 (RCA_{120}) were purchased from SIGMA Chemical Company, and pSVβGal was purchased from Promega. HepG2 and Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku Co.) containing 10 % heat-inactivated fetal calf serum (FCS) (HAZELETON Biologics, Inc.), 4 mM of L-glutamine, 36 mM of sodium bicarbonate, and 60 mg/l of kanamycin at 37°C in a humidified atmosphere containing 5 % CO_2 in air. The cells used in each test were cultured in 96-well flat-bottomed plates (CORNING Laboratory Sciences Company) in 200 ml of culture medium.

The synthesis of the 6-O-carboxymethyl(CM)-TM-chitosan (**1**) was prepared from chitosan according to the method described in the reference [11]. By changing the reaction condition, the ratio of monochloroacetic acid to TM-chitosan in the reaction, 6-O-carboxymethyl(CM)-TM-chitosan having two values of degree of substitution of carboxymethyl group per sugar unit (DCM), was synthesized.

The synthesis of the galactose residue unit was performed through three reaction steps shown in Scheme 1. Lactonoyl lactone was prepared from lactose according to the method described in the reference [12].

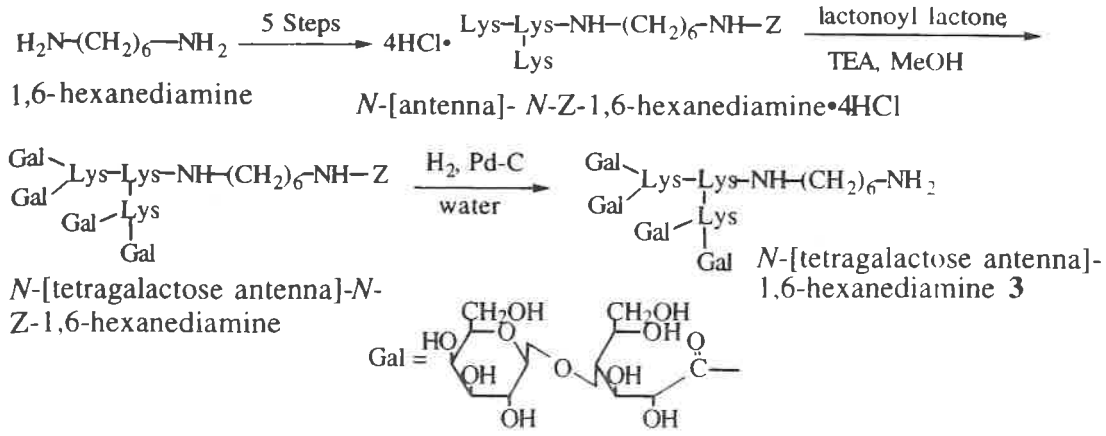
The synthesis of the tetragalactose antenna residue was performed as shown in Scheme 2. *N*-[Tetragalactose antenna]-1,6-hexanediamine (**3**) was prepared according to the following method. *N*-[Antenna]-*N*-Z-1,6-hexanediamine•4HCl (760mg, 973μmol), which was synthesized through five reaction steps by the method in reference [13], and lactonoyl lactone (1.60g, 4.71mmol) were dissolved in methanol. TEA (600μl, 4.29mmol) was added and refluxed for 5 h. This reaction mixture was evaporated and then reprecipitated from water and ethanol (963mg, 483μmol, yield : 49.6%). The *N*-[tetragalactose antenna]-*N*-Z-1,6-hexanediamine (424mg, 213μmol) was dissolved in water and hydrogenolyzed in the presence of palladium black (40mg) at room temperature for 24h. After removal of the catalyst by filtration, the filtrate was evaporated under reduced pressure. *N*-[Tetragalactose antenna]-1,6-hexanediamine was isolated by ion-exchange chromatography (SP-Sephadex, conditioning : water, eluent : 0.1M ammonium bicarbonate), and freeze-dried to be afforded as powder, 378mg, 202μmol (yield : 95.0%).

The synthesis of TM-chitosan/galactose conjugate was performed as shown in Scheme 3. In the case of TM-chitosan/galactose conjugate (degree of substitution of galactose group per sugar unit (DGal)= 5 mol%/sugar unit : **TC-Gal5**), **1** (DCM = 5 mol%/sugar unit, 0.2 g, 842 μmol/sugar, 42 μmol/COOH) was dissolved in DMF/water (1:1 v/v) and WSC (15 mg, 79 μmol)



N-lactonoyl-1,6-hexanediamine **2**

Scheme 1. Synthesis of galactose residue.



Scheme 2 . Synthesis of tetragalactose antenna.

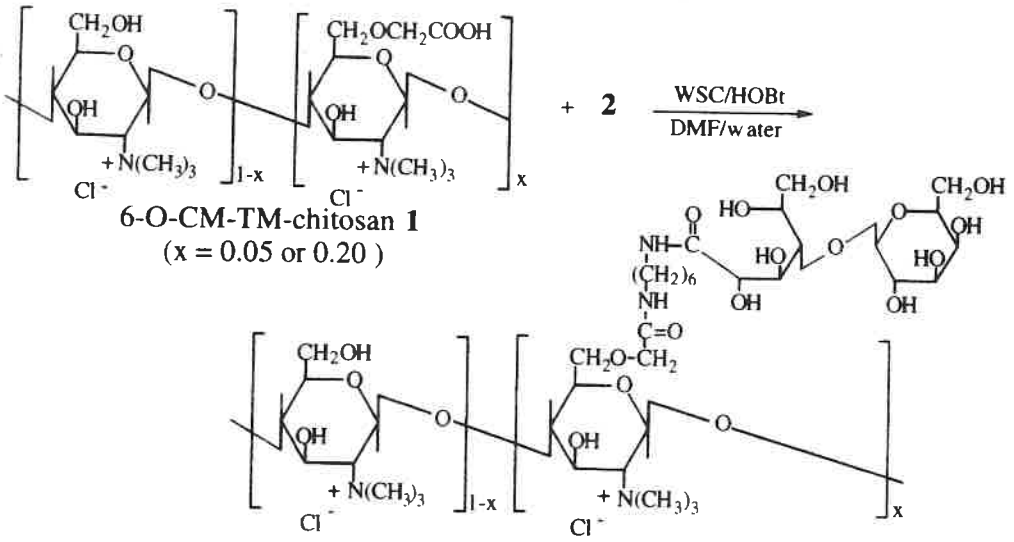
and HOBt (11 mg, 82 μmol) were added at 0°C for 1 h. And then **2** was added and stirred at room temperature over night. After this reaction mixture was evaporated and dissolved in water, it was ultrafiltrated to remove unreacted reagents. The above reaction was repeated to remove residual carboxyl groups. TM-chitosan/galactose conjugate (DGal=20 mol%/sugar unit: **TC-Gal20**) was synthesized by the same method as that of **TC-Gal5**.

The synthesis of TM-chitosan/tetragalactose antenna conjugate (the degree of substitution of tetragalactose antenna residue per sugar unit (DGal4A)=20 mol%/sugar unit: **TC-Gal4A20**) was performed as shown in Scheme 4. Compound **1** (DCM = 20 mol%/sugar unit, 50.4mg, 201μmol/sugar, and 40.1μmol/COOH) were dissolved in DMF/water (1:1 v/v) and WSC (11.5mg, 59.9μmol) and HOBt (6.7mg, 49.6μmol) were added at 0°C for 1 h. Then **3** (90.9mg, 48.9μmol) was added and stirred at room temperature over night. After this reaction mixture was evaporated and dissolved in water, it was ultrafiltrated to remove unreacted reagents. The above reaction was repeated to remove residual carboxyl groups. The objective conjugate was isolated by gel filtration chromatography (HW-40, ϕ2×27cm, eluent : water) and freeze-dried to be afforded as powder, 31.2mg.

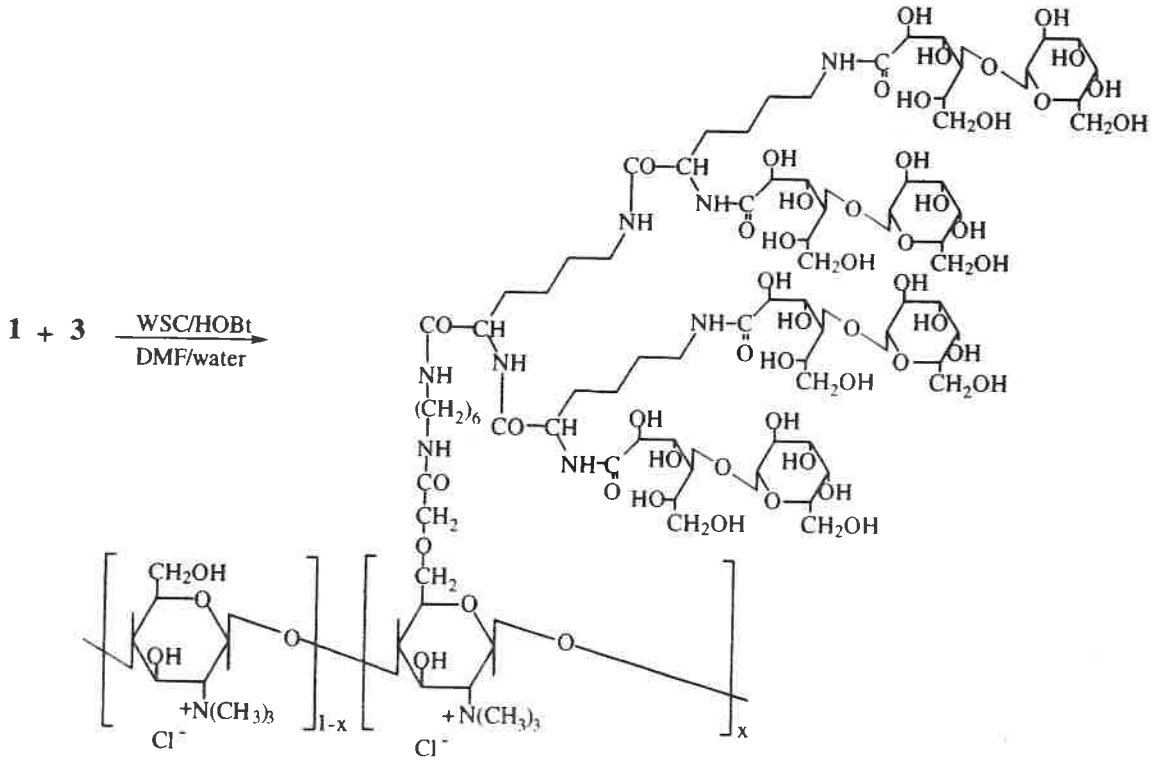
Each polyelectrolyte complex with DNA (pSVluc : 6046 bp) was electrophoresed on 1 % agarose gel at 50 V for 45 min and stained with ethidium bromide to be visualized.

The interaction of polysaccharides with RCA_{120} lectin was evaluated using a biosensor based on surface plasmon resonance (BIAcore system,.Pharmacia Biotech) [13]. The biotin labeled RCA_{120} lectin solution was injected onto the streptavidin pre-immobilized surface of a sensor chip, and various concentrations of polysaccharide were injected to measure sensorgrams. The obtained sensorgrams were used to carry out kinetic analysis of the interaction between RCA_{120} and polysaccharides; the apparent affinity constants of polysaccharides were calculated.

The β-galactosidase activity of the conjugates was measured



Scheme 3. Synthesis of TM-chitosan/galactose conjugate.



Scheme 4. Synthesis of TM-chitosan/tetragalactose antenna conjugate.

against HepG2 human hepatoma cells *in vitro*. The tumor cell suspension containing 1×10^4 cells in DMEM containing 10% FCS was distributed in a 96-well microplate and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. After adding a fresh medium containing 100μM chloroquine and

incubating in a humidified atmosphere containing 5% CO_2 at 4°C for 1 h, the suspension was changed into FCS free DMEM containing 5mM $CaCl_2$ and/or 50mM lactose for the inhibition test and added to each sample. After incubating at 4°C for 1h, it was changed into FCS free DMEM and incubated for 48 h. After adding X-gal solution [14], it was incubated for 48 h and measured using a microplate reader at 420 nm.

Results and discussion

The synthesis of TM-chitosan/galactose conjugates was carried out through reaction steps shown in Schemes 1 and 3. By repetition of the reaction of conjugation, the objective TM-chitosan/galactose conjugate having no residual carboxyl group was obtained. We could synthesize TM-chitosan/galactose conjugates having two values of DGal, **TC-Gal5**, and **TC-Gal20** through the coupling reaction of **2** with 6-O-CM-TM-chitosan **1**. For efficient binding to galactose receptors on the surface of hepatocyte, a tetra-antennary ligand having four β-D-galactosyl residues, *N*-[tetragalactose antenna]-1,6-hexanediamine, was designed. The synthesis of TM-chitosan/tetragalactose antenna conjugate was carried out through the reaction steps shown in Schemes 2 and 4. By the usual method, the *N*-[antenna]-*N*-Z-1,6-hexanediamine•4HCl having hetero terminal groups was made from 1,6-hexanediamine. *N*-[tetragalactose antenna]-1,6-hexanediamine having hetero terminal groups, antennary tetragalactose residue, and amine, was isolated by ion-exchange chromatography. By repetition of the reaction of conjugation, the objective TM-chitosan/tetragalactose antenna conjugate having no residual carboxyl group was obtained.

The results of electrophoresis suggested that the neutral polyelectrolyte complex of cationic polysaccarides with plasmid DNA (pSVluc) was formed when the ratio of cationic group to base pair was 2 : 1 in each polycation. Thus, this ratio was utilized in subsequent all experiments.

In order to evaluate the affinity of obtained conjugates to hepatocyte, the interaction between polysaccharides and RCA_{120} lectin was investigated as a simple model experiment. The mesurement of interaction between polysaccharides and RCA_{120} lectin was carried out by the use of a biosensor based on surface plasmon resonance (BIAcore system, Pharmacia Biotech). RCA_{120} lectin is well known to recognize β-D-galactose and β-D-*N*-acetylgalactosamine residues. The results of the interaction of polysaccharides with RCA_{120} lectin are shown in Fig. 1. The apparent affinity constant of TM-chitosan was the same level as that of DEAE-dextran. **TC-Gal5** and **TC-Gal20** showed a higher

apparent affinity constant than TM-chitosan. **TC-Gal4A20** showed the highest apparent affinity constant in these used polysaccarides. These results suggested that the high apparent affinity constant per galactose residue of **TC-Gal4A20** was caused by the "cluster effect" referred to by Lee *et al.* as branches. It could be expected that novel polysaccharides having a high affinity to hapatocyte were able to be synthesized.

In order to investigate the specific delivery of DNA by **TC-Gal4A20** conjugate, the expression of β-galactosidase activity by the polycation-DNA complex in HepG2 cells was carried out. Furthermore, in order to make sure that the specific delivery of DNA by **TC-Gal4A20** conjugate was caused by receptor-mediated endocytosis, the inhibition test was performed. The results of effect of the inhibitor on the expression of β-galactosidase activity by polycation-DNA complex in HepG2 cells are shown in Fig. 2. In the absence of the inhibitor, the β-galactosidase activity of TM-chitosan was the same level as that of DEAE-dextran. **TC-Gal5** and **TC-Gal20** conjugates showed higher β-galactosidase activity than TM-chitosan and DEAE-dextran. **TC-Gal4A20** conjugate showed the highest β-galactosidase activity in these used polysaccharides. When the amount of galactose residues in the conjugates was increased, the β-galactosidase activity of the conjugates increased.

In the presence of the inhibitor, the β-galactosidase activities of DEAE-dextran and TM-chitosan were only slightly affected. On the other hand, those of **TC-Gal5**, **TC-Gal20** and **TC-Gal4A20** conjugates were significantly decreased by the addition of lactose. These results suggested that the increase of β-galactosidase activity of **TC-Gal5**, **TC-Gal20** and **TC-Gal4A20** conjugates were caused by the specific internalization *via* the galactose receptor on the cellular surface of HepG2 cells and the

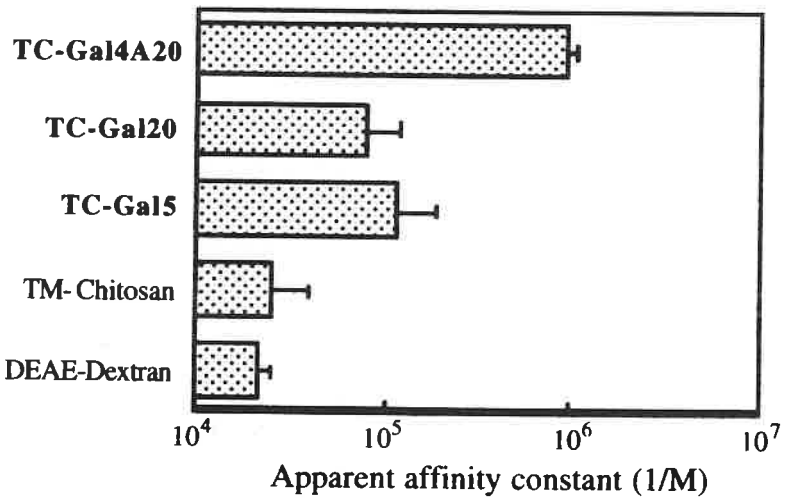


Fig. 1. Apparent affinity constant of polysaccharides against agglutinin RCA_{120} [10].

galactose residue's "cluster effect".

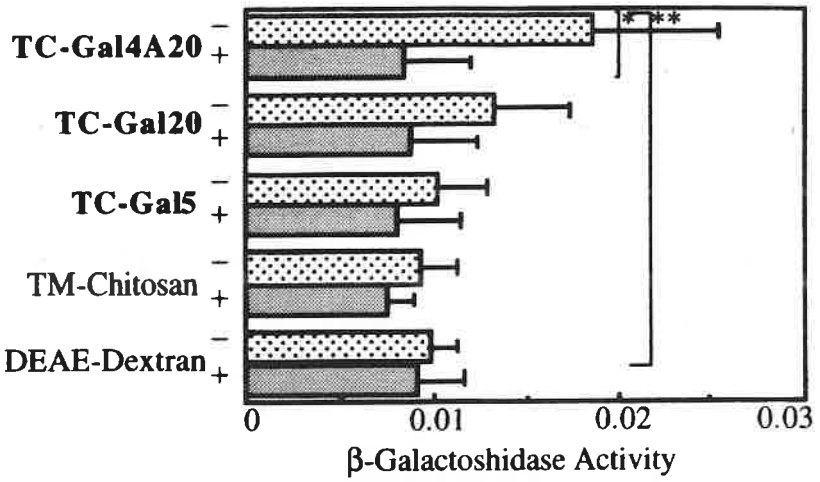


Fig. 2. Effects of inhibitor on expression of β-galactosidase by polycation-DNA complexes in HepG2 cells [10].
DNA dose = 0.5μg/well. Ratio of polycation / DNA = 2 cationic group / 1 bp. - : in the absence of inhibitor(lactose 50μM), + : in the presence of inhibitor(lactose 50μM).
* : $p < 0.05$, ** : $p < 0.01$.

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NEW AREAS OF MICROCRYSTALLINE CHITOSAN APPLICATIONS

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Abstract

Development of microcrystalline chitosan has been resulted in the perfection of its quality and the preparation of new useful forms for special applications. A special interest for utilization of this form of chitosan is connected with agriculture. The new applications of microcrystalline chitosan as the plant biostimulating agent are presented and discussed.

Keywords: Microcrystalline chitosan, agriculture, biostimulation, germination, plant

Introduction

A development of microcrystalline chitosan has been resulted in the perfection of its quality and the preparation of new useful forms for special applications. Microcrystalline chitosan (MCCh) as the product of physico-chemical modification of initial chitosan is characterized by specific properties such as:

- controlled bioactivity
- controlled biodegradability
- direct film-forming from aqueous dispersion
- high adhesivity and miscability
- non-toxicity

The new applications of microcrystalline chitosan have been still generated utilizing above properties. A special interest for utilization of MCCh is related to its application in agriculture.
The aim of this research is to present some abilities for utilization of microcrystalline chitosan in agriculture, mainly as the plant biostimulating agent.

Materials and methods

Microcrystalline chitosan obtained according to the original method (1) in these studies was used. Different grades of initial chitosan were also utilized.

A testing of lettuce seeds and plants of Grand Rapid ritsa was carried out in cooperation with the Famifarm Ltd (Joroinen, Finland).

All tests were carried out in the greenhouses on a production line using the same condition parameters as the standard. A production period was 37 days.

Microcrystalline chitosan with specified properties was used for seeds treatment and modification of soil (2).

The strawberries plants having 3 leaves were grouped for 15 plants in each repeat. Then these plants were treated by microcrystalline chitosan dispersion and initial chitosan solution according to 2 methods:

- young plants were saturated with chitosan dispersion/solution before seadling
- young plants were seadled into soil containing suitable amount of chitosan forms.

All strawberry plants were growing at the greenhouse at temperature of 20-25 °C and lighting for 16 h. The plant roots were washed and dried after 2 weeks of experiment.

The cotton seeds were germinated in a lab conditions using glass dishes containing suitable solution or dispersion of chitosan forms.

Results and discussion

The agriculture seems to be one of the most important area for chitosan application. The plant protection and growth biostimulation by conventional chitosan were described previously (3-4). The research for application of microcrystalline chitosan in selected fields of agriculture has resulted with several extra-ordinary results that a part of them are presented in this paper.

The studies with application of gel-like dispersion of microcrystalline chitosan for biostimulation of germination of lettuce seeds and plant growth were carried out on the production line of Famifarm Oy (Joroinen, Finland) using the standard optimum conditions. The results of germination effectivity and total weight of plants after harvesting, in comparison to control, are presented in Tables 1-2.

Table 1. Influence of lettuce seeds treatment on their germination and plant growth

MCCh concentration used for seed treatment, wt%	Germination effectivity, % to control	Total lettuce weight after harvesting, % to control
0.1	128.6	109.3
0.5	138.7	107.2

Table 2. Influence of lettuce seeds and soil treatment on their germination and plant growth

Concentration of MCCh, wt%		Germination effectivity, % to control	Total lettuce weight after harvesting, % to control
used for seed treatment	in soil		
0	0.3	117.9	114.0
0.1	0.3	138.7	105.4

The results presented in Tables 1-2 shown an increasing effectivity for seed germination according to a type of treatment using microcrystalline chitosan. At the same time an application of 0.3 wt% of MCCh for soil modification as well as 0.5 wt% of MCCh for seed treatment has resulted in increase of lettuce total weight for 14 %, in comparison to control.

Microcrystalline chitosan is also effective biopolymeric material for biostimulation of growth for different plants. A special interest is related to a use of MCCh for biostimulation of plant roots growth, especially applied for seedlings before planting.

The results of microcrystalline chitosan and initial chitosan application for strawberry plants dipping are presented in Table 3.

Table 3. Effect of strawberry plants dipping in different forms of chitosan on their rootage

Chitosan type	Chitosan concentration in solution/dispersion, wt %	Root weight, % in comparison to control
MCCh	0.10	207
Initial *	0.01	160

*) in acetic acid solution with pH ~ 5.5; a higher concentration was phytotoxic

Microcrystalline chitosan dispersion used for dipping of young strawberry plants before planting was affected on the effective root growth of 207 %, in comparison to control. At the same time an initial chitosan in acetic acid solution with pH = 5.5 was able to be used in a very low concentration due to its phytotoxic phenomena (Tab. 3).

The influence of chitosan forms used for modification of soil utilized for young strawberry plants on root weight after 2 weeks from planting is presented in Table 4.

Table 4. Effect of soil modification by different forms of chitosan on the strawberry plant rootage ability

Chitosan type	Chitosan concentration in soil, wt %	Root weight, % in comparison to control
MCCh	0.10	203
Initial *	0.01	145

*) in acetic acid solution with pH ~ 5.5; a higher concentration was phytotoxic

Modification of soil by microcrystalline chitosan was activated the growth of strawberry roots (Table 4.). A positive effect of chitosan on the strawberry plants was also observed in a case of green leaves.

A similar effect is also observed during application of chitosan forms on the cotton plant growth (Table 5.).

Table 5. Influence of cotton seeds treatment by different forms of chitosan on the cotton growth

Type of chitosan form	Chitosan form concentration used for seeds treatment, wt%	Length of shoots, % of control
MCCh	0.1	145.5
	0.5	81.6
Initial chitosan*	0.01	87.7
	0.1	103.4

*) used in acetic acid solution with pH ~ 5.5

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EFFECT OF CHITOSAN ON TISSUE MACERATION AND PRODUCTION OF MACERATING ENZYMES BY *ERWINIA CAROTOVORA* IN POTATO

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Abstract

The effect of chitosan on the maceration of potato tissue by macerating enzymes secreted by *Erwinia carotovora*, a casual agent of soft rot of potato was investigated. Chitosan treatment of potato slices decreased the bacterial count significantly compared to the control slices. The activity of polygalacturonase (PG), pectate lyase (PL) and pectin-methylesterase (PME) were higher with concomitant increase in maceration and cell death. On the other hand, in chitosan-treated tissues which were challenged with the pathogen, significant decreases in the enzymatic activities and tissue maceration were observed, and the effect was more pronounced with increasing concentrations of chitosan. Furthermore, the elevated pH of the inoculated control tissue contributed to the activation of PME which is responsible for the initiation of maceration process, while chitosan treatments decreased the pH of the tissue contributing to protection of the tissue.

Key words : Chitosan, potato, soft rot, *Erwinia carotovora*, macerating enzymes, polygalacturonase, pectate lyase, pectin methyl esterase.

Material and Methods

Erwinia carotovora subsp. *carotovora*, an isolate from potato was used. Shrimp-shell chitosan was purchased from Nova-Chem (Halifax, Canada). Chitosan stock solution (10 $mg.ml^{-1}$) was prepared, pH adjusted to 5.6 and made up to 2, 4, 6 and 8 mg/ml. Potato slices were dipped in chitosan solution for 5 min, excess solution was drained and placed in Petri dishes on moist filter paper to maintain high relative humidity. Each slice was inoculated with 20 ml of bacterial suspension of 10^8 $CFU.ml^{-1}$. The Petri plates were sealed with parafilm and incubated at 27^oC for 24 h.The bacterial population were determined on chitosan treated potato slices, and the tissue maceration was determined by the

method described by Bateman (2) and Quantick *et al.* (7). The texture of the potato slices was measured with Texture Analyzer and the pH of the tissue homogenate was measured using a pH meter.

The methods of Ayers *et al.* (1) was followed to determine polygalacturonase (PG) and pectate lyase (PL) activities. Pectin-methylesterase (PME) activity was determined by incubating a 0.5 ml reaction mixture containing 0.25% (w/v) pectin A, 50 mM Pipes buffer (pH 7.5) and 0.1 ml crude extract at 25°C for 6 h. The reaction was terminated by adding 0.5 ml H_2SO_4 and the amount of methanol liberated was determined by the method of Wood and Siddiqui (9).

Results and discussion

The bacterial counts from potato discs treated with chitosan showed significant decrease ($P<0.05$) compared to the inoculated control (Fig. 1). Initial significant decrease ($P<0.05$) in viable counts of bacteria was noticed from all chitosan treatments. However, at 2 and 4 $mg.ml^{-1}$ treatments, the bacteria survived throughout the observation period of 24 h but at a significantly lower rate compared to control. At 6 $mg.ml^{-1}$ chitosan treatment, there was a significant decrease in CFU's from 12 h, and no viable counts were recorded at 24 h. At 8 $mg.ml^{-1}$ chitosan treatment in addition to instantaneous reduction in CFU's, no viable counts were observed after 12 h.

There was significant difference ($P<0.05$) in tissue maceration between the control and the chitosan treatments (Fig. 2). No tissue maceration was observed in the intact control compared to treatments since they were not inoculated. In the inoculated control, the maceration was about 80% and it decreased with increases in chitosan concentration (Fig. 2). As the maceration of the tissue decreased with chitosan concentration, the tissue viability increased. Both tissue maceration and cell viability was related to the bacterial count on the potato tissue. The texture of slices after 24 h of inoculation showed significant difference ($P<0.05$) between control and chitosan treatments (Fig. 3). The texture of the tissue was not affected at 6 and 8 $mg.ml^{-1}$ chitosan treatments and was comparable to the intact control. However the slices treated with 8 $mg.ml^{-1}$ chitosan appeared harder than the intact control. This is presumably due to the chitosan film formation on the tissue.

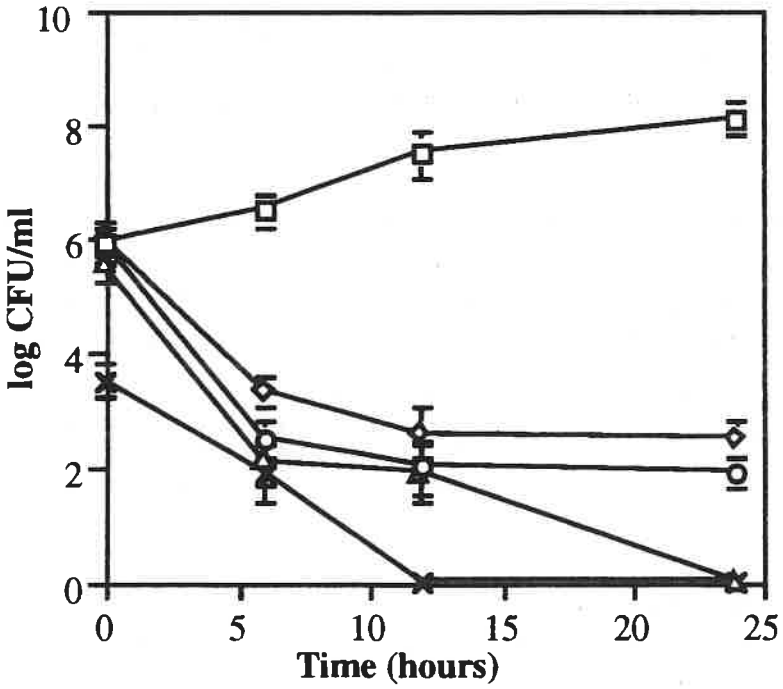

Fig.1. Effect of chitosan treatment on *E.carotovora* population in inoculated potato tissue : control (□), tissue treated with 2 mg (◇), 4 mg (○), 6 mg (△) and 8 mg.ml^{-1} (X).

The pH of the homogenate of the tissue slices changed with chitosan treatments (Fig. 3). The pH of the intact control tissue was 6.4, but it increased to 7.4 in the inoculated control. The pH of the chitosan treated tissue varied between 6.4 and 7.4 in proportion to tissue maceration, suggesting that membrane damage and loss of pH homeostasis occurred in the macerated tissue. However, at chitosan concentrations of 6 and 8 mg.ml^{-1}, the pH of the tissue was comparable to that of the intact control.

Polygalacturonase (PG) and pectatelyase (PL) activities were detected in the crude tissue extracts made after 24 h of inoculation, and the activities decreased with increases in chitosan concentrations (Fig. 4). The decrement in the activities of both PG and PL with chitosan were proportional to the tissue maceration process. Significant activity of pectin methylesterase (PME) (Fig. 4) was detectable in crude tissue extracts from inoculated controls, which decreased with increases in chitosan concentrations. Maceration intensity and tissue viability correlated with bacterial load as well as pH (Figs 1, 2 and 3). The activities of pectic enzymes also correlated with pH as well as viable CFU's of *E. carotovora* on potato tissue treated with different concentrations of chitosan (Figs 1, 3 and 4).

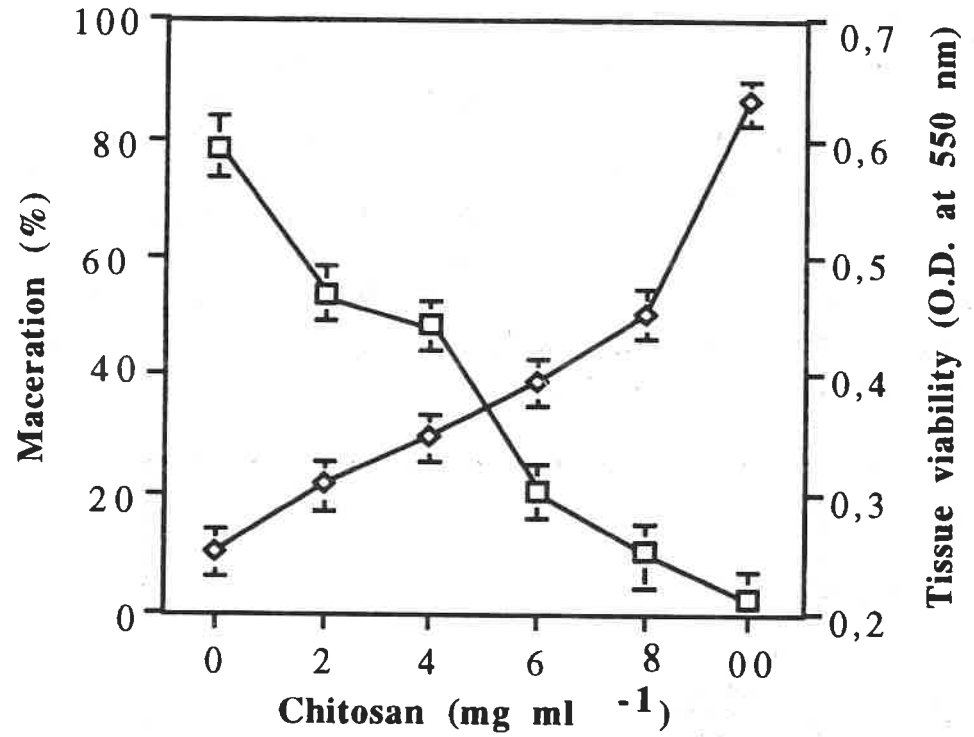

Fig. 2. Effect of chitosan treatments on tissue maceration (□)and cell viability (◇) of potato tissue inoculated with *E. Carotovora* (00 intact control).

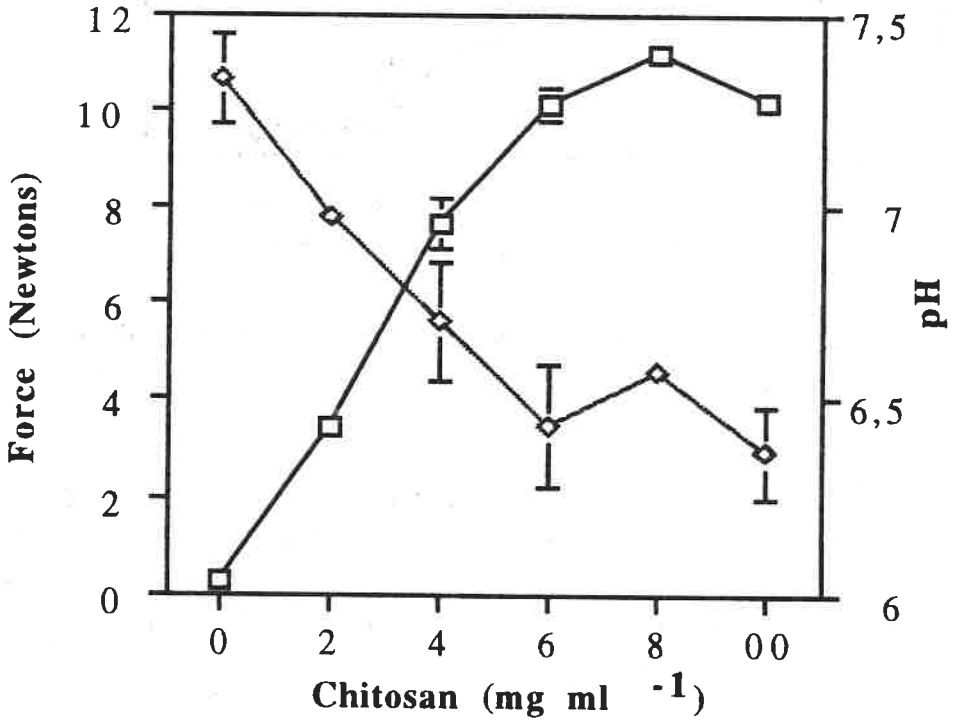

Fig. 3. Effect of chitosan treatments on texture (□) and pH (◇) of the potato tissue inoculated with *E.carotovora* (00 intact control).

The maximum bacterial maceration observed in the inoculated control was related to higher activity of pectic enzymes which are the principal macerating enzymes. A similar observation was recorded by Weber and Wegener (8) wherein levels of PG and PL were found to be closely related to the level of maceration. Treatment of potato tissue with increased concentrations of chitosan prevented the progress of tissue maceration, because the activity of pectolytic enzymes was reduced and multiplication and intercellular spread of the pathogen through the tissue was impeded.

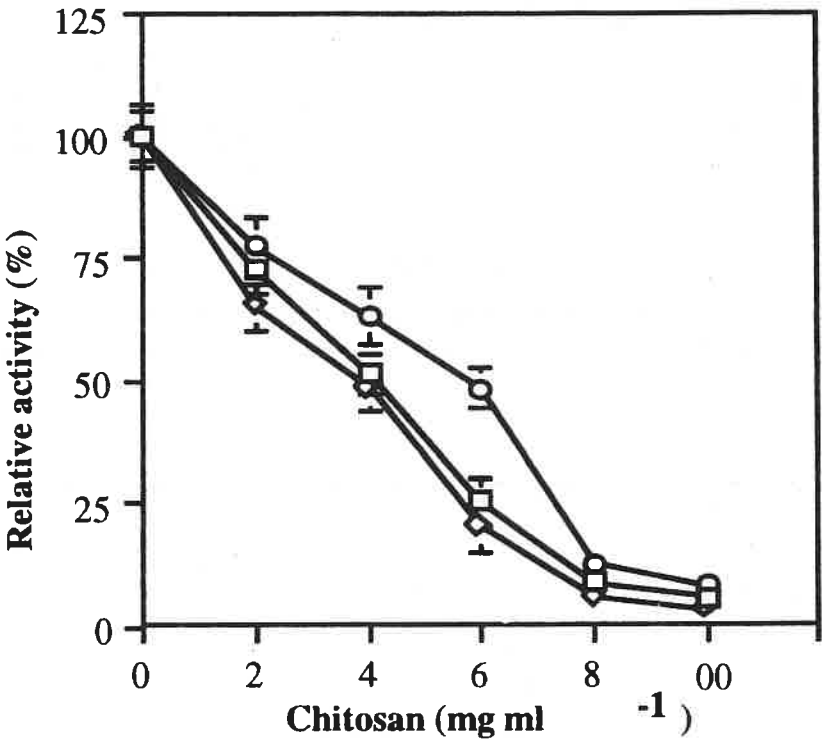

Fig.4. Effect of chitosan treatments on PG (◇) PL(□) and PME (O)activities in potato tissue inoculated with *E. Carotovora* (00 intact control).

Enzymes other than polygalacturonases are involved in the degradation of potato cell wall and this may be a cooperative response. The activity of PME was correlated to tissue maceration (Figs 2 and 6) and the activity of PL which preferentially attacks non-esterified rhamnogalacturonan chains was enhanced, since PME demethylates pectin (3). The result indicate that the activities of enzymes responsible for tissue maceration were controlled by chitosan treatment offering tissue protection against the pathogenic bacteria. One important observation is that the pH did not increase in the intact tissue, and in the tissue treated with chitosan and challenged with the pathogen. Pagel and Heitefuss

(6) have also observed that at lower pH the total pectic enzyme activities were reduced.

Chitosan may play multiple roles in controlling tissue maceration : direct inhibition of bacterial growth, inactivation of pectic enzymes, induction of phenolic compounds in the host and control of tissue pH. Cationic chitosan can bind to the secreted polygalacturonases which are negatively charged (pI : 5.1-6.0) (12). Inhibition of pectic enzymes by chitosan, can give the host time to activate defense reactions such as stimulation of phenylalanine ammonia lyase and hydroxymethylglutaryl CoA-reductase (mevolanic acid pathway) (6). Quantick *et al.* (7) demonstrated that macerating enzymes are inactivated by the host phenolic compounds.

Conclusion

Inoculation of potato tissue with a compatible pathogen causes tissue damage and defense reactions were supressed. With chitosan treatments, the tissue is protected through its antimicrobial activity, interfering with cooperative action of macerating enzymes and possibly by inducing defense reactions in the host (4,5). Although this study was carried out with differentiated tissue, the role of chitosan in inhibiting the soft rot in whole potato tuber and the effect of environmental factors such as storage temperature and relative humidity, need to be investigated.

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APPLICATION OF CHITIN DERIVATIVES AND THEIR COMPOSITES TO BIODEGRADABLE PAPER COATINGS

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Abstract

The paper presents a discussion of the properties of paper coated with chitin derivatives: chitosan, dibutyrylchitin, their films and composites. The casting technique was applied to obtain the coatings.

Almost all mechanical properties like, breaking strength, burst resistance or tearing strength of paper coated with chitosan and dibutyrylchitin are improved compared to those of non-coated paper and chitosan films. Breaking length due to growing mass of the paper samples, decreases, while it reveals rising values for the films. Elongation at break is smaller in cases of paper samples in contrast with the films, but is improved for paper coated especially on both sides. Oil absorbency decreases with growing initial concentrations of chitosan in solution which confirms perfect hermetic properties of the obtained samples.

Two factors like whiteness and double folds number do not improve after coating. Some of the given properties like tearing strength or double folds number were investigated along and across the paper fibers.

Neither air permeability nor surface picking is observed in case of all coated samples. Microphotographs of crossected samples allowed for their morphological analysis and comparison with commercially available PE-coated paper wrapping.

Keywords: biodegradable coating, chitin, chitosan, dibutyrylchitin, paper properties

Introduction

Non-degradable plastic packages and their composites with paper [1] are produced in great amounts all over the world and comprise a large proportion of the waste deposits from many branches of life and industry [2]. They are known to be totally resistant to enzymatic or microbial deterioration.

The application of both, artificial polymer composites with degradable additive (like poly(vinyl)-acetate with dibutyrylchitin or PE with starch [3]) and biopolymers (such as poly(esther)-urethanes [4], chitosan [5] and dibutyrylchitin) in packaging market becomes an issue of significant interest and importance. The biopolymers can create biodegradable and non-toxic films by themselves or be composed with starch or cellulose [6].

Materials and methods

Applied to this study chitosan was made in the Sea Fishery's Institute in Gdynia by thermochemical deacetylation of α-chitin obtained from Antarctic krill's shells. The same substrate α-chitin, additionally purified from residual calcium carbonate by treatment its powder with 2N hydrochloric acid, was used to prepare dibutyrylchitin [7].

All chitosan films were prepared from a 1% acetic acid solution with chitosan concentrations of 2%, 3%, 4% by weight and of its deacetylation degree DD=86,73%.

Dibutyrylchitin films - from 100 % methanol solution with dibutyrylchitin concentration of 5% by weight. The solutions were mechanically stirred and stored overnight at room temperature to let the substrate dissolve. The films were obtained by the casting an exact amount of chitosan and dibutyrylchitin solutions onto glass slides and evaporating the solvent.

Paper of grammage 130 g/m^2 (Canson Co., France) was coated with chitosan and dibutyrylchitin solutions with a paintbrush, pulled over to avoid paper deformations and left for 24 h to dry at room temp. Then, chitosan films and chitosan-coated paper were treated with 1N NaOH to regenerate chitosan [8], thoroughly washed with distillated water and dried at room temp. Both the chitosan and dibutyrylchitin samples were additionally dried in vacuo at 40°C for 6 hours.

All samples containing dibutyrylchitin were covered with Petri glasses after casting to prevent too rapid evaporation of the solvent.

The samples of paper coated with chitosan and dibutyrylchitin were tested in term of their typical paper properties [9] to compare with non-coated paper which was treated with an appropriate solvent and dried in the same conditions. All samples were conditioned before the following tests:

1. Breaking strength P [N] and elongation at break λ [%] analysis were carried out in Shopper's apparatus with time of breaking 20±5 s and with the length of samples 10cm.

2. Breaking length S [m] was estimated on the basis of previous tests and the following equation:

$$S = (\Sigma P * l) / (\Sigma m * 9.81),$$ where

ΣP - the sum of values of breaking strength for 10 samples of the same kind

l - length of the sample

Σm - the sum of mass of 10 samples cut at the grip.

3. Oil absorbency A_o [1/mm] was tested in IGT apparatus at pressure of 60 Pa; constant velocity of 0.2 m/s and with application of butyl phthalate dyed with Sudan IV, in the amount of 0.006 cm^3 equal to 1 droplet from the syringe containing a needle PE 14;

$$A_o = 1000 / l,$$ where

l - length of oil stain

4. Air permeability P_A [ml/s]- in Shopper's apparatus with the time of experiment t=60 s for non-coated and maximal t=20 min for coated paper.

5. Tearing strength E [mN] was tested along and across paper fibers in an Elmendorf apparatus and the tearing strength factor was counted from the following equation:

$$E = 16 * z / k * n,$$ where

z - arithmetic mean of all values for one direction

k - pendulum coefficient

n - number of samples teared in one test.

6. Double folds number DFN [-] was estimated in Shopper's apparatus for two directions - across and along the paper fibers and in the middle of each sample; with the bending angle of 180°; the number of folds per minute equal to 110-120 and a constant load.

7. Picking velocity P_v [cm/s] - in IGT apparatus (AC 2 type) with applications of high viscosity pick test oil (ξ=1550 P, REPROTEST B.V.); increasing velocities 0 - 3 m/s; pressure of 40 Pa and a printing aluminum roll with a rubber layer.

8. Whiteness W [%] was investigated on both sides of the paper samples in ELREPHO apparatus (OPTON Co.) in relation to a standard sample of whiteness of 86%.

9. Burst resistance B [MPa] - in Mullen's apparatus.

10. Morphology analysis was carried out with a Multiscan computer programme in Polarized Interference Microscope (magnification 5x) on the crossections of the coated and non-coated paper.

11. Thickness of all samples was checked by micrometer.

Some of the given tests were applied to investigate the properties of pure chitosan films.

The ability of coating was also investigated in the case of heterogeneous PVAc/DBCH composite. Dibutyrylchitin in the form of 5 wt % acetone solution (acetone: C_3H_6O - 58.08 g/moll; $1dm^3$=0.79kg) was composed at room temperature with 8 wt % solution of commercial poly(vinyl)-acetate in proportion of: 0% - 60%, 80%, and 100% by weight. The samples were stirred with ultrasounds for 30 min. to overcome the poor miscibility of both substances and spread with laboratory rod onto paper and glass slides. The composites were left to dry at room temperature and then additionally dried in the drying machine for 120 min. at 50°C in vacuo and left in it for 24 hours.

Results and discussion

Most of the investigated paper properties turned out to be better for paper coated with chitosan (fig.1,2,3,4,7) and dibutyrylchitin (DBCH) (fig.1,2,3,4,5,7) than for non-coated one or for chitosan films (fig.1 and 2). Nevertheless, DBCH-coated paper does not have as good strength characteristics as chitosan ones do. Both, breaking strength (fig.1) and burst resistance (fig.2) are increased in the cases of chitosan-coated paper, especially when coated on both sides. The values for chitosan films are much lower, however, a slight growth of them can be observed for films prepared from solutions of higher chitosan concentrations. Breaking strength (fig.1) and burst resistance (fig.2) of DBCH-coated paper are comparable to those described above.

Tearing strength (fig.3) increases rapidly for the chitosan-coated samples and is higher for paper coated on both sides. For 5% DBCH one side coating it is only a little higher than for non-coated paper. Chitosan films, even those of 1% or 2% are much stronger.

Chitosan films reveal higher values of elongation at break (fig.4) than the paper samples. However, coatings especially on both sides of the paper cause significant growth of this factor comparing with non-coated one. DBCH-coated paper samples were found to be the strongest in this term.

* - values for paper coated with 5 % DBCH on one side included in the following figures:

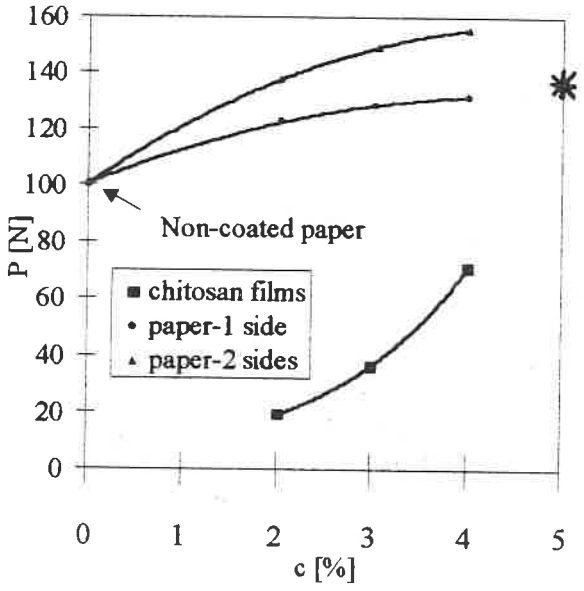

Fig.1 *Breaking strength P [N] of chitosan films and paper coated with chitosan solution on one and both sides versus initial chitosan concentration c [%] in solution.*

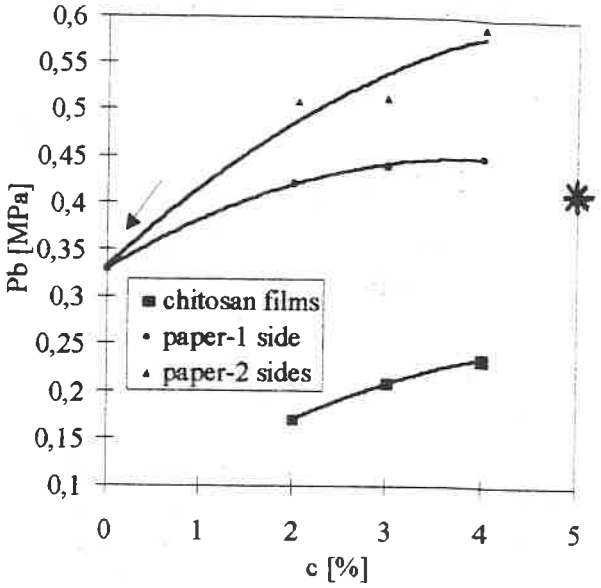

Fig.2 *Burst resistance Pb [MPa] of chitosan films and paper coated with chitosan on one and both sides versus initial chitosan concentration c [%] in solution.*

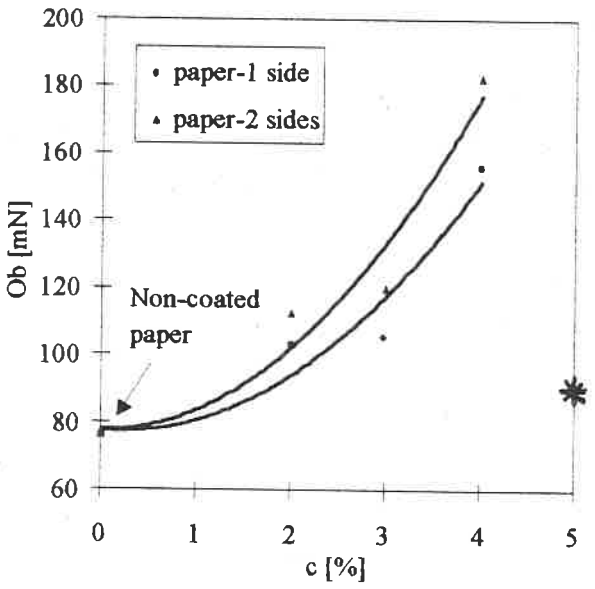

Fig.3 *Tearing strength Ob [mN] of paper coated with chitosan on one and both sides versus initial chitosan concentration c [%] in solution.*

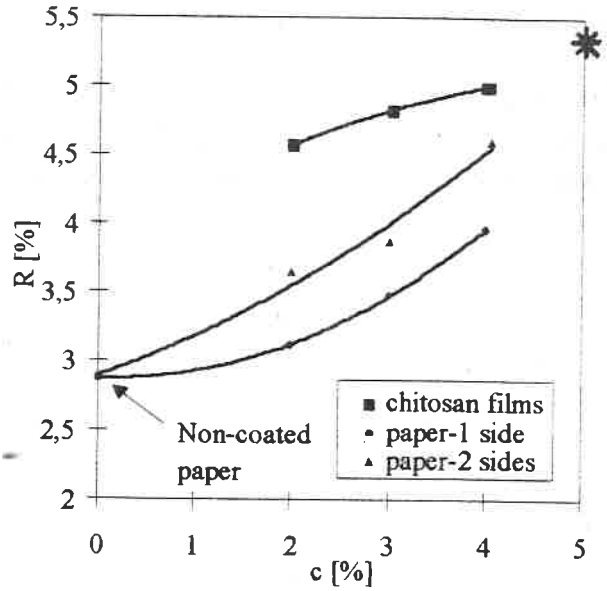

Fig.4 *Elongation at break R [%] of chitosan films and paper coated with chitosan on one and both sides versus initial chitosan concentration c [%] in solution.*

Breaking length is a parameter which describes the length of sample that causes its breaking. Because paper gains additional mass load due to polymer coating, the coated sample is heavier than non-coated one and a smaller length of the sample is required to break it. The higher chitosan concentrations, the bigger the mass of the samples and the breaking length (fig.5) decreases with chitosan concentration. It shows, however, growing

values for more elastic chitosan films. DBCH-coated paper seems to be either very light or very strong and elastic because the value of this factor is very high in comparison to chitosan and even non-coated paper.

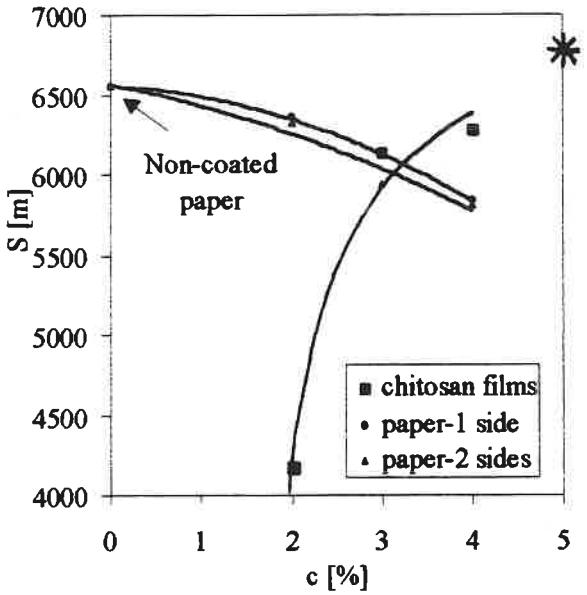

Fig.5 *Breaking length S [m] of chitosan films and paper coated with chitosan on one and both sides versus initial chitosan concentration c [%] in solution.*

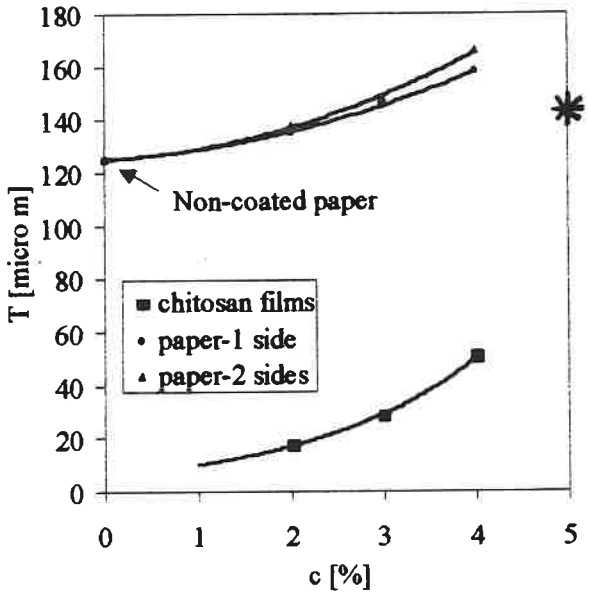

Fig.6 *Average thickness T [micro m] of chitosan films and paper coated with chitosan versus initial chitosan concentration c [%] in solution*

The higher chitosan concentration in solution, the thicker the layers, the thicker (fig.6), more yellowish (fig.8) and rigid the chitosan-coated paper. It was observed that the DBCH layer influences paper the same way, but is much thinner than the chitosan one (fig.6)

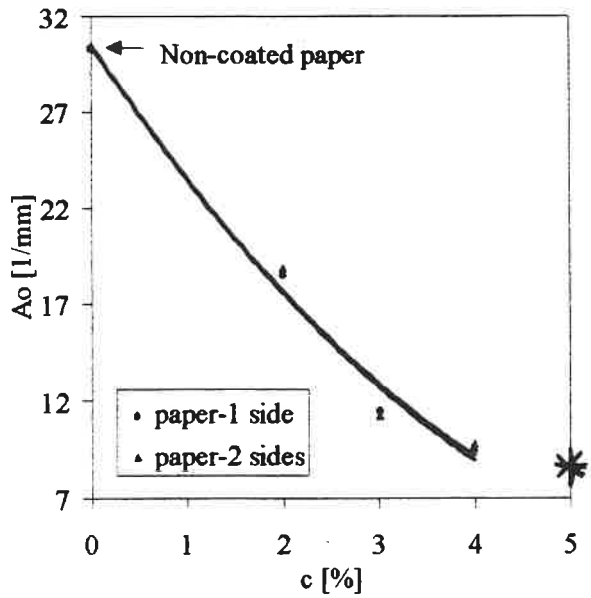

Fig.7 *Oil absorbency Ao [1/mm] of chitosan coated side of paper versus initial chitosan concentration c [%] in solution.*

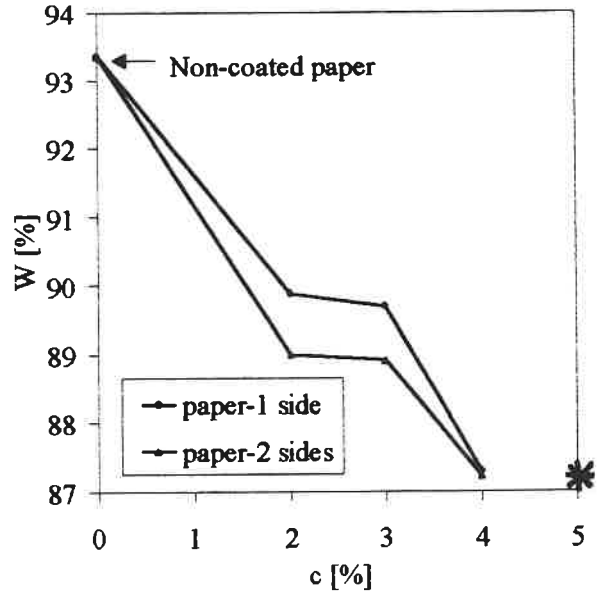

Fig.8 *Whiteness W [%] of chitosan coated side of paper versus initial chitosan concentration c [%] in solution.*

Coating with both chitosan and DBCH has improved, to a large extent, hermetic properties of the paper. The values of oil absorbency factor (fig.7) for all coated samples are much better than for non-coated ones.

The thickness has also affected the double folds number (fig.9) which diminished for the thicker samples. As fig.9 shows paper has different, but better strength properties along than across paper fibers - curves (a) and (b). It was also investigated that paper coated with 2% chitosan is stronger than the one coated with 5 % DBCH solution. Air permeability A_o and picking velocity P_v for non-coated paper were adequately: A_o = 2,1 ml/s and P_v =96 cm/s. No air permeability and no surface picking was observed in the cases of all coated with chitosan and dibutyrylchitin samples - perfect adhesion and hermetic properties.

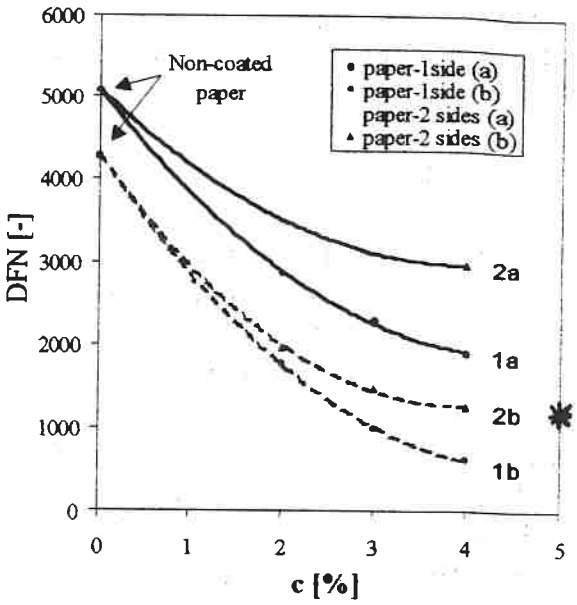

Fig.9 *Double Folds Number DFN [-] of paper coated with chitosan on one and both sides, along (a) and across (b) paper fibers versus initial chitosan concentration.*

Crossection of the samples allowed for their morphological analysis (fig.10-11). The chitosan and DBCH layers are thinner than PE ones (fig.11). It is probably because of partial absorption of chitosan and DBCH solutions by paper and filling its pores with the biopolymer. This happens due to different coating processes. However, the chitosan and DBCH layers are thick enough to be a barrier for water and gases.

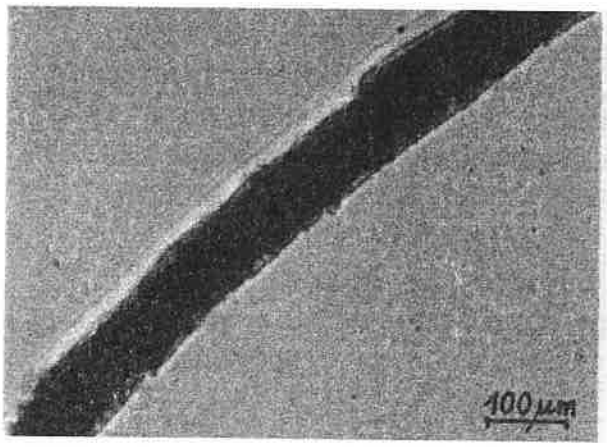

Fig.10 *Microphotograph of crossected paper coated with chitosan on both sides, initial chitosan concentration in solution - 4%.*

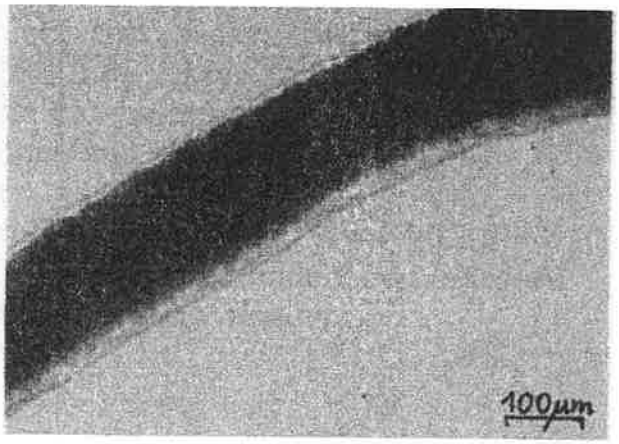

Fig.11 *Microphotograph of crossected, commercially available PE-coated paper wrapping.*

Conclusions

The given investigations show significant growth of the majority of the typical properties of paper coated with biodegradable chitosan [10] or dibutyrylchitin solutions.

The casting method of obtaining the coatings allows for a good adsorption of the biopolymer at the base paper. Part of the solution fills the pores of paper and part of it stays on top. This way paper becomes double strengthen and hermetic. Additional advantage of the application of chitosan and DBCH to paper coating is that the layers are biodegradable, non-toxic and easy to obtain.

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