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Production of chitin and chitosan from crustacean waste and their use as a food processing aid.

by
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A Thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfillment of the requirements of the degree
of Master of Science

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Exactly what do these large frontal lobes do? They may well have several functions, certainly, and yet do one very specific and important thing. They enable you to think of actions in the future, and wait for a reward then.

J. Bronowski

(The Ascent of Man).

Abstract

Chitin is a polysaccharide found in abundance in the shell of crustaceans. In this study, chitin was first extracted from shrimp waste material previously demineralized, using proteolytic enzymes, i.e., chymotrypsin and papain. The conditions used for the deproteinization were optimized with respect to 3 factors, viz., pH, temperature, and enzyme to waste (E/W) ratio, using response surface methodology (RSM). Of the factors studied, pH had the most important influence in the models obtained. Optimum conditions for deproteinization by chymotrypsin were determined to be around 40°C, pH 8.0, and E/W ratio of 7:1000 (w:w). With papain, a temperature of around 38°C, pH of 8.7 and E/W ratio of 10:1000 (w:w) gave the optimum response. At these conditions, the yield of protein was maximum, and ranged from 22-48% depending on the starting material. Most important, the residual protein levels in the waste after deproteinization with the enzymes were very low, i.e., 1.3 and 2.8% for chymotrypsin and papain-treated samples, respectively.

In a second part of the study, chitosan was prepared from chitin by partial deacetylation and its capacity to preserve whole and headless fresh shrimps was evaluated. The shrimps (*Pandalus borealis)* were dipped in chitosan (1 and 2% w:v), and stored on ice. Chemical indicators such as pH, drip loss, total volatile bases (TVB), nucleotide degradation, and sensory indicators of appearance (including melanosis) and odor, as well as total microbial counts were monitored during 20 days. Chitosan dips significantly reduced the total microbial counts and TVB levels in whole shrimps, which shows the antimicrobial properties of chitosan. In headless shrimps, melanosis and nucleotide degradation were significantly reduced by chitosan.

As a third and final part, the antimicrobial properties of chitosan were evaluated using several microorganisms implicated in food spoilage and/or food poisoning outbreaks, especially those associated with fish and seafood products. Chitosan prepared from crab offal and used in the previous study on shrimp preservation was compared with commercially available chitosan from Sigma Chemical Co. (St-Louis, MO). Concentrations of chitosan tested were 0.005, 0.0075, 0.01, 0.02, 0.05 and 0.1%, at a pH of 5.6. The laboratory prepared chitosan was more efficient than its commercial counterpart in inhibiting the various organisms tested, with the exception of *S. aureus*. Chitosan increased the lag phase of growth, and reduced the cell numbers in the culture media. The efficiency of chitosan in inhibiting microbial proliferation was found to be

comparable to many commonly used chemical preservatives.

Résumé

La chitine est un polysaccharide qui compose une grande partie de la carapace des crustacés. Lors de cette étude, la chitine fut d'abord extraite des déchets de crevette préalablement déminéralisés, à l'aide d'enzymes protéolytiques, i.e., la chymotrypsine et la papaine. Trois des facteurs utilisés lors de la déprotéinisation ont été optimisés, soit le pH, la température d'incubation, et le ratio enzyme/déchets, grâce à la technique des surfaces de réponse. Des facteurs étudiés, le pH a la plus grande influence sur les modèles obtenus. Les conditions optimales pour l'utilisation de la chymotrypsine sont un pH de 8.0, une température de 40°C, et un ratio enzyme/déchets de 7:1000 (p:p). Pour la papaine, un pH de 8.7, à 38°C, avec un ratio de 10 pour 1000 (p:p) donnèrent une réponse maximale. A ces conditions, le taux de protéines solubilisées était maximum et de l'ordre de 22-48, dépendant de la qualité des déchets utilisés. Notamment, les taux de protéines résiduels dans les déchets après déprotéinisation étaient très bas, i.e., 1.3 et 2.8% avec la chymotrypsine et la papaine respectivement.

Dans la deuxième partie de cette étude, la chitosanne fut préparée à partir de la chitine par déacétylation partielle, et ses capacités à préserver des crevettes fraîches entières et partiellement préparées (têtes et pattes enlevées) fut évaluée. Les crevettes (*Pandalus borealis*) étaient plongées dans des solutions de chitosanne (1 et 2%), puis égouttées et conservées sur glace. Des indicateurs chimiques (pH, rétention de liquide, bases volatiles totales, dégradation des nucléotides) et sensoriels (évaluation de l'apparence, incluant la mélanose, et de l'odeur) et le dénombrement total des microorganismes ont été utilisés pour suivre l'évolution de la dégradation des crevettes durant 20 jours. Les solutions de chitosanne ont significativement réduit les dénombrements microbiens et la formation des bases volatiles chez les crevettes entières, ce qui démontre les propriétés antimicrobiennes de la chitosanne. Chez les crevettes partiellement préparées, la mélanose et la dégradation des nucléotides furent significativement réduites par la chitosanne.

En troisième et dernière partie, les propriétés antimicrobiennes de la chitosanne furent évaluées sur plusieurs microorganismes impliqués dans la détérioration des aliments et/ou les empoisonnements alimentaires, spécialement ceux associés aux poissons et fruits de mer. La chitosanne, préparée à partir de déchets de crabe et utilisée pour l'étude précédente sur la conservation des crevettes, a été utilisée en parallèle avec une chitosanne commerciale (Sigma Chemical Co., St-Louis, MO). Les concentrations de chitosanne

testées étaient 0.005, 0.0075, 0.01, 0.02, 0.05, et 0.1% et le pH était ajusté à 5.6. La chitosanne préparée à partir de déchets de crabe démontra plus d'efficacité que celle du commerce dans sa capacité à inhiber les différents microorganismes étudiés, à l'exception de *S. aureus*. La chitosanne augmente la durée de la phase d'attente avant l'apparition de la croissance, et réduit les comptes cellulaires dans les milieux de culture. L'efficacité de la chitosanne comme agent antimicrobien est comparable à celle de plusieurs agents de conservation chimiques utilisés couramment.

Preface

Claim to original research

Parts of this thesis are considered to be original work by the author:

- The use of enzyme to deproteinize crustacean wastes had been investigated before, however, the conditions used for the protein hydrolysis had never been optimized, and the effect of the individual factors influencing the hydrolysis such as temperature, pH, and enzyme to waste ratio were never reported.

- The preservation of fresh shrimps with chitosan dips has never been reported in the literature, neither other forms of chitosan based products for such a purpose.

- Although few reports on the antimicrobial properties of chitosan have been published, originality of the study presented in this work resides in the longer period of observation and the control of pH in the culture media, as well as the use of a liquid media coupled with plating on a solid media for the microbial growth study. Furthermore, *Proteus* spp. and *Pseudomonas* spp. have never been investigated to the knowledge of the author.

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List of Abbreviations

DEAE	Diethylaminoethyl
Glc NAc	N-Acetyl-D-glucosamine
Hx	Hypoxanthine
HPLC	High performance liquid chromatography
Ino	Inosine
IR	Infra-red
LD_{50}	Lowest dosage to obtain 50% mortality rate
M.W.	Molecular weight
NOCC	N-O-carboxymethyl chitosan
TMC	Total microbial counts
TVB	Total volatile bases
UDP-Glc NAc	N-Acetyl-D-glucosamine-uridine 5'-diphosphate
U.V.	Ultra-violet

Foreword

This thesis is presented in the form of original papers suitable for journal publications. The introduction presents the rationale and objectives of the study, followed by chapter 1, a general literature review of the background information on the subject of the thesis, and chapters 2 to 4 which contain the integrality of papers to be published in appropriate journals. The last section is a general conclusion including salient features of the study and perspectives for future works. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions stated in the guidelines concerning thesis preparation, section B, paragraph 2, "Manuscripts and Authorship":

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- Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g., in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis
- In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph. D. oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

The totality of the work reported in this thesis is the responsibility of the author, under the

supervision of Dr. B.K. Simpson of the Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

Part of this work has been accepted for publication, viz.:

N. Gagné and B.K. Simpson (1993). Use of proteolytic enzymes to facilitate the recovery of chitin from shrimp waste. Food Biotechnol. (In press).

Part of this work has been submitted for publication as follows:

N. Gagné and B.K. Simpson (1993). Utilization of chitosan for the preservation of fresh shrimp (*Pandalus borealis*). J. Food Science.

N. Gagné, B.K. Simpson, and L. Adambounou (1993). Antimicrobial activity of chitosan toward selected microorganisms. Food Microbiol.

Introduction

Humans have learnt through ages to exploit nature's resources in many ways and have, from time immemorial, recognized the wealth and potential of the sea. Seafoods have served as energy and nutrient sources for humans for centuries, and have become a delicacy praised by many gastronomists. In addition to its use as energy and nutrient sources, seafoods have recently been shown to be excellent sources of useful biochemicals, such as chitin, a natural biopolymer with unique properties.

Approximately 70% of the landed value of shellfish is rejected as offal. This abundant waste material has either to be discarded at considerable cost or else be converted to value added products. Concerns for environmental health and safety have compelled researchers/seafood processors to enhance value of seafood processing wastes, and this has led to the production of several useful biochemicals and nutrients, such as chitin, pigments, seafood peptones, etc., from this resource material.

Chitin is a relatively "new" polymer in research and food processing applications. Muzzarelli (1977) made an extensive review on chitin, including its sources, preparation, chemistry and applications. Amongst the various publications on chitin, it is evident that the preparation of chitin from crustacean shells (the most common source), using chemical products to deproteinize and demineralize the source material, leads to a product with variable qualities and properties. The chemicals and the conditions used are known to deacetylate and depolymerize chitin to various extents. For the deproteinization step, use of enzymes have been attempted in the past (Takeda and Abe, 1962; Takeda and Katsuura, 1964; Broussignac, 1968; Shimahara and Takiguchi, 1988), however, the conditions were not optimized in order to obtain a complete or almost complete deproteinization.

The use of proteolytic enzymes have some advantages over the use of chemicals, such as preserving the natural state of chitin, avoiding the neutralization of the effluents, and others. Chitinous polymers have been shown to exhibit antimicrobial as well as film forming properties. Thus, the overall project objectives were (i) to optimize the conditions for the enzymatic deproteinization of shrimp wastes with the use of response surface methodology and (ii) to apply chitosan dips to control postharvest quality deterioration of raw shrimp during iced storage. The project hypothesis was that the use of chitinous polymers would control shrimp spoilage by delaying both enzymatic (i.e., browning by polyphenoloxidase) and microbial acitivity. The first part of the study on the optimization of enzymatic procedures to prepare chitin is presented in chapter 3, under the title:

"Use of proteolytic enzymes to facilitate the recovery of chitin from shrimp wastes".

Furthermore, chitosan, the deacetylated form of chitin, has found more applications than chitin due to its relatively higher polyelectrolyte nature. Recently, its ability to form semi-permeable films and its antimicrobial properties have been exploited to preserve fruits and vegetables (El Ghaouth et al, 1991a,b; Lidster, 1987a,b). Fresh shrimp is a highly perishable product for which few means of preservation are available. The most widely used method of preserving fresh shrimp is by iced storage, and even with this approach, prime quality is maintained for up to 5 days only. The use of sulfiting agents extends premium quality of fresh shrimp for up to 14 days on iced storage. However, the use of sulfites in raw shrimps is not permitted in Canada. Because of this, it was decided to evaluate the feasibility of using chitosan dips to preserve fresh shrimps, and this study is presented in the fourth chapter of this thesis, viz.,

" Utilization of chitosan for the preservation of fresh shrimps (*Pandalus borealis*)".

One of the effects of chitosan observed during the preservation study on fresh shrimp was that it had a strong antimicrobial activity against the microorganisms associated with shrimps. In order to confirm this and to ascertain/evaluate the effectiveness of chitosan as an antibacterial agent, several microbial strains related to seafood spoilage were chosen, and the effect of chitosan on their growth was studied. These results are presented in chapter 5, entitled:

"Antimicrobial activity of chitosan toward selected microorganisms".

Chapter 1

Literature review

1.1. Crustacean wastes in the province of Quebec

Several crustacean species are commercially harvested in the province of Quebec, and this industry generates large volumes of waste material which constitutes a disposal problem for the shellfish industry. In 1989, the total amount of crustacean wastes produced in Quebec was 9587 tons. Approximately 3300 tons were put to better economic use, while the main bulk, about 6041 tons, was buried, and the remaining 228 tons were dumped in the sea (Alibhay et al, 1990). However, disposing of waste is becoming very costly due to increasing concerns about environmental pollution as well as health/safety concerns. A better economic use of this abundant waste material is to convert them to value-added forms such as nutrients (e.g., proteins, minerals) and other useful biochemicals (chitin, pigments, flavours, etc.), which are all present in appreciable quantities in crustacean wastes.

1.2. Chitin and chitosan structures

Polymers like cellulose are economically important because of the many applications in which they are used. Like cellulose, chitin is a ß(1-4) linked glycan, but is composed of 2-acetamido-2-deoxy-D-glucose, or N-acetylglucosamine units. Chitin is the second most plentiful natural polymer, after cellulose, with which it bears a structural resemblance (Ruiz-Herrera, 1978).

The N-deacetylated form of chitin, chitosan, is a natural, non-toxic, biodegradable, high molecular weight polycationic polymer. It has been described as "nature's most versatile biomaterial" (Sandford, 1989). Chitosan is composed primarily of glucosamine, or 2-amino-2-deoxy-D-glucose linked together by ß(1-4) glycosidic bonds (Sandford, 1989).

Structural similarities between chitin, chitosan, and cellulose are illustrated in Fig. 1.

The term chitosan is preferred when the nitrogen content is higher than 7% by weight, or

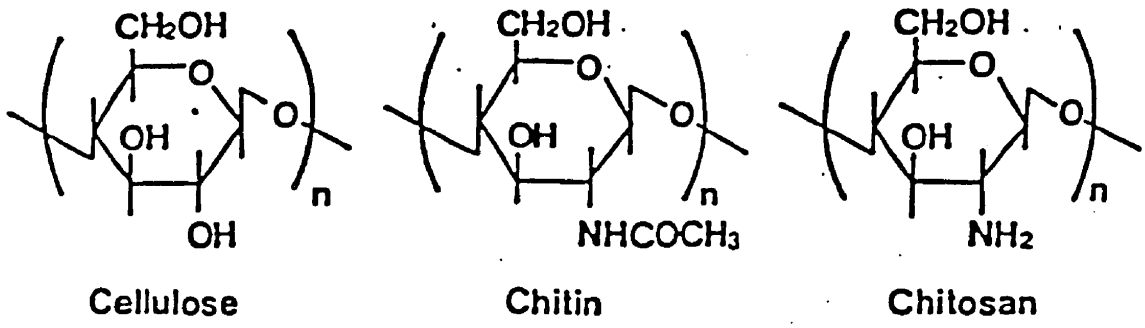

Fig. 1. Chemical structures of cellulose, chitin, and chitosan.

when the degree of deacetylation is over 60%. Chitin and chitosan may also be differentiated on the basis of their solubility in dilute acids, chitosan being more soluble in those solutions unlike chitin (Muzzarelli, 1985; Tsugita, 1990).

1.3. Sources of chitin and chitosan

Chitin is found in abundance in marine invertebrates, insects, fungi, etc., as the conjugated form with proteins. It is frequently present as a cell wall material in plants, and in the cuticule of animals. In addition, chitins in animal tissues are frequently calcified, such as in the case of shellfish. Some fungi contain chitosan, however, it is commercially produced by the deacetylation of chitin (Tsugita, 1990).

The content of crude chitin varies between species, as illustrated in Table 1.

1.4. Production/processing of chitin and chitosan

1.4.1. - Chemical methods

Methods of production of chitin and chitosan vary considerably, however, they all invariably involve deproteinization and demineralization of the crustacean shells. Protein extraction is usually accomplished with a mild alkaline solution, such as 1 or 2% sodium hydroxide, at 60-70^{o} C, for a few hours, and the extracted proteins can be recovered for other uses, such as animal feed.

Removal of calcium carbonate and calcium phosphate is accomplished by dissolution with dilute acids. Hydrochloric acid is used most commonly, but other acids can also be used. Austin et al, (1981) indicated that the use of EDTA for decalcification of the shells minimizes both chain hydrolysis and deacetylation.

Chitosan is produced from chitin by deacetylation with highly concentrated (40-50%) solutions of sodium hydroxide at high temperatures (100-150^{o}C), exclusive of air, for about an hour (Johnson and Peniston, 1982).

Table 1. Chitin content of some organisms.

Organism	Chitin content (% dry waste)	Organism	Chitin content (% dry waste)
Snow crab (*C. opilio*)	18.7 - 32.2%[a]	Crab	13-15%[b]
Shrimp	17.0%[a]	*Aspergillus niger*	42%[c]
Shrimp	14-27%[b]	*Penicillium notatum*	18.5%[c]
Mucor rouxii	44.5%[c]		

[a] From Naczk and Shahidi (1990).

[b] From Ashford et al (1977).

[c] From Kong (1975). Percent of dry cell wall

Highly deacetylated chitosan is usually not obtained under the conditions mentioned above. Effective deacetylation is attained by intermittently washing the intermediate product in two or more changes of water during the alkali treatment. According to Mima et al, (1983), the procedure did not significantly degrade the polymer, and recovered a product of molecular weight of about 5×10^5 with nearly 100% deacetylation.

Domard and Rinaudo (1983) added thiophenol during the deacetylation process. This product traps oxygen thus preventing degradation and exerts a catalytic effect. They achieved better deacetylation with less degradation. These workers found that the best conditions for deacetylation were as follows:

(i) the amount of NaOH necessary was approximately four times that of the total amino-groups in the polymer (concentration range 2% w/v);
(ii) one hour reaction at 100°C; and
(iii) successive treatments were better to prepare fully deacetylated chitosan. Nevertheless it is necessary to reduce the NaOH concentration and the time of contact with the polysaccharide.

The elimination of lipids and pigments is accomplished with solvents such as 95% ethanol, acetone, etc. Peroxide or chlorine bleaches are often utilized as bleaching agents.

1.4.2. - Enzymatic methods

Proteases can be used for the deproteinization of crustacean shells for the production of chitin/chitosan. Shimahara and Takiguchi (1988) used bacterial protease from *Pseudomonas maltophilia* in a culture medium with crustacean shell, and observed that after 24h, the protein content remaining in the shells was only about 1%.

Earlier attempts to use proteolytic enzymes to deproteinize crustacean waste were made by Takeda and Abe (1962), Takeda and Katsuura (1964) and Broussignac (1968). These workers used such enzymes as tuna proteinase at pH 8.6 and 37.5°C, papain at pH 5.5-6.0 at 37.5°C, or a bacterial proteinase at pH 7.0 and 60°C for over 60 hr. After treatment with the enzymes, the amout of protein still associated with chitin was about 5%. Broussignac (1968) recommended the use of papain, pepsin or trypsin for the deproteinization of crustacean shells during chitin preparation.

Microbial synthesis of chitosan has been identified in various organisms including *Mucor rouxii*, *Phycomyces blakesleeanus*, and others. Cell cultures of these organisms have been used for the production of chitosan, and the yields obtained were improved by addition in the culture medium of a chitin source such as *Aspergillus niger*, thus the mechanism of production was attributed to chitin deacetylation. The yields after 96 hr of incubation were of 26.9% (±1.9) of dry cell wall material, with a chitin source included. Furthermore, the degree of acetylation of chitosan could be reduced to values ranging from 8.9 to 0.6% (Knorr and Klein, 1986). This is significantly lower than values obtained by common practices using sodium hydroxide which results in products with a degree of deacetylation ranging from 22 to 43%.

Chitin deacetylase, the enzyme responsible for the deacetylation of chitin to chitosan, was isolated and purified by Araki and Ito (1974). This enzyme shows a pH optimum at 5.5 and is markedly inhibited by acetate. It showed no effect against chitin in its isolated form, but was active against a soluble glycol chitin substrate.

Similarly, Arcidiacono et al, (1989) used *Mucor rouxii* to produce chitosan and obtained maximum weight after two days of cultures, with a decrease after that period. Molecular weights obtained were over 1 x 10^6 after two days, with 10L batch cultures. The deacetylase activity of their DEAE-Sephadex purified enzyme showed a pH optimum of 4.5, and the activity at this pH varied markedly with the buffer employed. Tris-HCl appeared to be the most suitable for high enzyme activity. In general, unlike the batch systems, molecular weights did not decrease over time in continuous culture systems. In addition, molecular weights were generally higher in the defined culture medium (ammonium sulfate as the nitrogen source, trace of metals and glucose as the carbon source) than in the complex media (nutrient broth, yeast extract, and glucose), although yields of chitosan were significantly lower.

Chitin deacetylase extracted from *Colletotrichum lindemuthianum* exhibited similar properties but had a pH optimum at 8.5, and was not inhibited by sodium acetate. The enzyme is found in the cell extracts and also secreted by this plant pathogen, so it is more easily isolated from the culture filtrate (Kauss and Bauch, 1988).

One important fact about chitin deacetylase is that it is ineffective against preformed (microfibrillar) chitin but it readily attacks nascent chitin chains. In other words, chitosan is made by deacetylation of chitin provided that the deacetylation process occurs in

tandem with chitin synthesis, those requirements being met when both chitin synthetase and chitin deacetylase are present simultaneously. Seemingly, the two enzymes operate in tandem, one polymerizing GlcNAc units from UDP-GlcNAc, the other removing acetate moieties from the nascent chains (Bartnicki-Garcia, 1989).

1.5. Chitin solvents

Chitin, like cellulose, is insoluble in ordinary solvents; it is crystalline, and sometimes oriented in its natural state. Strong acids, fluoroalcohols, chloroalcohols and certain hydrotropic salt solutions are chitin solvents but degrade the chitin or are inconvenient to use. An effective solvent system reported by Austin et al, (1981) comprise dimethylacetamide (DMAc) containing 5% lithium chloride. Mixtures of 2-chloroethanol with hydrochloric, phosphoric, sulfuric or nitric acid have their compromise properties which leads to greater stability of chitin in such solutions (Muzzarelli, 1977).

The dissolution of chitin presents an important problem inherent with operating difficulties. These are mainly due to the existence of both intra and intermolecular hydrogen bonds for this natural polymer in the solid state (Vincendon, 1985).

1.6. Chitosan solvents

The preparation of chitosan is usually designed to ensure a product completely soluble in dilute hydrochloric acid or organic acid but insoluble in neutral or alkaline aqueous solvent. Chitosan is also soluble in dilute nitric acid, marginally soluble in 0.5% H_3PO_4, and insoluble in sulphuric acid at any concentration at room temperature. Chitosan is not soluble in any common organic solvent (e.g., dimethylformamide and dimethyl sulfoxide), but it dissolves well in acidified polyols. The best solvent for chitosan was found to be formic acid, where solutions are obtained in aqueous systems containing 0.2 to 100% of formic acid (Kienzle-Sterzer et al, 1982).

1.7. Chemical properties of chitosan

1.7.1. - Anion-exchanger, acid and neutral pH behavior

Chitosan can be considered a strong base as its primary amino groups (pK_a is 6.3), easily form quaternary nitrogen salts at low pH values. Thus, in acidic solutions chitosan has high anion capacity. At higher pH values, however, it is a weak base because the primary

amino groups are not protonated and therefore do not interact with anions and do not dissociate neutral salts; for instance, they do not retain chloride from neutral solutions of sodium chloride. This is a peculiar feature of chitosan, in so far as it would be classified as a strongly basic anion exchanger with no dissociation capacity for neutral salts. The complexing ability of chitosan is a direct consequence of the base strength of the amino groups and is most effective for those metals which form complexes with ammonia, e.g., copper, zinc and mercury (Muzzarelli, 1978).

1.7.2. - Water-holding property

Chitosan has a water-holding property that is exploited in many fields. For example, sports wear made from chitosan fibers are already on the market (Tsugita, 1990). Muzzarelli (1977) reported that the hygroscopicity of chitin is close to that of mercerized cellulose fibers and considerably greater than that of ramie fibers, but the chitin surface is less active and permeable to water than cellulose fibers. Water uptake of chitin, microcystalline chitin, and chitosan range from 325 to 440% (w/w), and the difference is possibly due to differences in the amount of salt-forming groups, and differences in the protein contents of the materials. The addition of microcrystalline chitin to wheat-flour breads and protein-fortified breads (8% replacement level of the flour by potato-protein concentrate) at different levels of water addition resulted in increased specific loaf volume with increasing microcrystalline chitin addition. Optimum water addition was found to be 65% (flour basis) (Knorr and Betschart, 1981).

1.7.3. - Gelation property

Aldehydes or carboxylic anhydrides react with chitosan in aqueous organic acid solution to give various forms of gels (Hirano, 1989).

Gualtieri et al, (1990) made a gelatin-chitosan embedding medium to perform scanning electron microscopy. The cell suspension of interest was mixed with an equal volume of gelatin (10% aqueous solution, warmed to 37°C), gently stirred, and then mixed with an equal volume of chitosan (2% solution in 0.5% acetic acid). This mixture was spread and then cooled at 4°C to harden.

The ionotropic gelation of chitosan with different anionic counterions as carried out by

Vorlop and Klein (1981) resulted in gels with a mechanical stability similar to those of calcium-alginate gels.

1.7.4. - Adsorption and immobilization of enzymes and other products

The N-arylidene or alkylidene derivatives of chitosan have gelation properties that make them particularly suitable for the immobilization of cells and enzymes (Hirano, 1989).

The beads are easy to make, and porosity can be varied for the desired application. They are stable to phosphate buffers, Na^+ and K^+ ions, and they have high cell/load activities (Sandford, 1989).

Hwang et al, (1985) suggested an approach for controlling the porosity of the capsule membrane used for encapsulation of proteins and cells. They observed that the hydrodynamic volume or intrinsic viscosity plays a significant role in the structure of the capsule membrane, and these properties are dependent on the ionic strength and the pH of the chitosan solution. As the ionic strength increases, the inter- and intra-molecular electrostatic repulsions between chitosan chains are reduced due to the shielding effect of the counter ions. This permits the chains to come closer together and thus lower the intrinsic viscosity or the hydrodynamic volume of the chitosan molecules (Fig. 2).

This effect enhances the inter- and intra-chain hydrogen bonding and causes the chains to form a special network structure that results in the increase of the average pore size in the matrix. Similarly, if the pH of the solution is increased, the intrinsic viscosity will also decrease and the average pore size in the matrix increase.

The fixing of enzymic activity on chitosan matrices by insolubilization of enzymes such as lactase and invertase was achieved by Masri et al, (1978). Several strategies exist for such purpose. In one procedure, chitosan that had been solubilized by neutralization with dilute acids to about pH 4-5 was mixed with an enzyme dissolved in water or a suitable buffer, and then back titrated with alkali, in an amount equivalent to the acid used for solubilizing the chitosan. The resulting precipitated chitosan product retains enzymic activity, however, this method of preparation is less attractive since the products obtained by precipitation are difficult to filter and appear gelatinous. It is difficult to wash unbound or

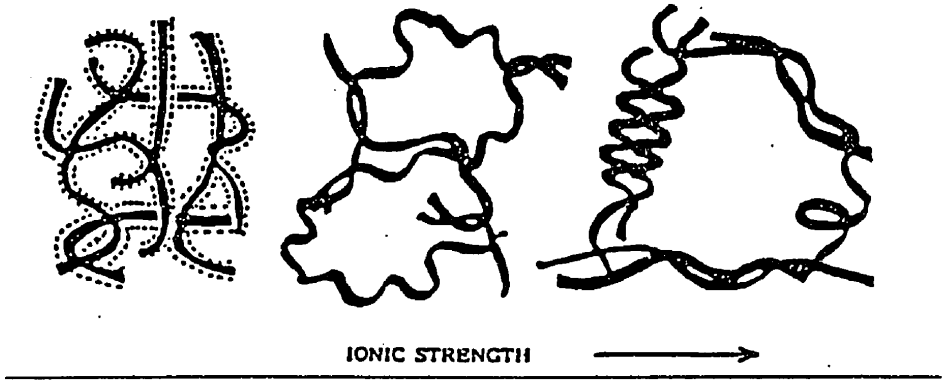

Fig. 2. Effect of ionic strength on the network of chitosan chains.

unencapsulated enzyme from such preparations. Alternatively, the chitosan sulfate is first prepared (from acid-solubilized chitosan and inorganic sulfate or by direct titration of chitosan flakes with sulfuric acid) without the dissolved enzyme and then used to fix the enzyme. In another strategy, the acid solubilized chitosan (pH 4-5) is mixed with the enzyme solution. Then, a difunctional or polyfunctional crosslinking agent such as glyoxal, glutaraldehyde or solubilized dialdehyde starch is added. This results in conversion of the mixture to a gel within minutes. The gel then hardens within hours and can be broken into granular form with retained enzymic activity. The Schiff base polymer produced in this way can be reduced with retention of activity. The advantage of reducing the gel is to enhance its conversion to a granular form and to stabilize the Schiff base formed. The products obtained are very hydrophilic, retaining about 85-90% water after being sucked dry on a filter. The ε-amino groups of lysyl residues of the enzyme probably participate in the Schiff base formation, and thus the enzyme becomes covalently bound to the crosslinked polymer. The enzyme can also be fixed in the polymer, due to physical entrapment or interfacial deposition. However, the high water content of the polymers may not be desirable, as the enzyme may be exposed during preparation to acidic solutions of about pH 4.2 to which they might not be compatible and thus result in inactivation. A variation of the above procedure is to fix the enzyme by crosslinking with dialdehydes or polyaldehydes on chitosan flakes suspended in water rather than on solubilized chitosan.

Muzzarelli (1978) used chitosan as a medium for adsorption chromatography, as a means of immobilization of lyzozyme. Aqueous solutions of lyzozyme at the desired pH were introduced into chitosan columns and kept together for 30 min at 30°C. The columns were then washed with water (60 mL) at the same pH, and water fractions were collected for spectrophotometric analyses. The optimum pH for lysozyme retention was found to be 9.0. Lysozyme was easily and quantitatively eluted from chitosan columns by using 2% propylamine solutions at pH 11.5. Good elution yields can also be obtained with carbonate buffers.

Chitin in a powdered form was used by Takeda (1978) as a thin layer for the separation of some phenols, amino acids and nucleic acid derivatives. Performance of chitin was almost equal or superior to that of crystalline cellulose, silica gel and polyamide layers. The developing time was generally much shorter when operating with the chitin layer than with other layers. On chitin layer, the basic amino acids travelled faster, while the acidic amino acids were slower, than on silica-gel layer.

1.7.5. - Emulsifying properties

The O-acyl derivatives of chitosan are emulsifying agents (Hirano, 1989). Microcrystalline chitin showed good emulsifying properties with an emulsion capacity of 900 ± 47 mL oil/g chitin when sonication was used to produce emulsions (Knorr, 1982).

In a study by Knorr and Betschart (1981) on bread loaf volume and chitin addition, it was demonstrated that a specific loaf volume of about 6 cm^3/g could be obtained even for the protein-fortified samples although the bread formula contained no emulsifiers or shortenings. This indicates surfactant properties of chitin which can overcome the loaf volume depression that occurs when potato proteins (and others) are added to wheat-bread formulations.

1.7.6. - Flocculating agent

Chitosan is a natural flocculant for waste-water treatment. Cationic chitosan reacts with anionic polymers to form polyelectrolyte complexes (Hirano, 1989). Chitosan has been found to be particularly effective in aiding the coagulation of protein from food process wastes. For example, in poultry processing waste, 6 mg of chitosan per liter of effluent at a pH of 6.7 removed 75% of suspended solids, and reduced the chemical oxygen demand by 62% (Muzzarelli, 1977). It has been evaluated in vegetable processing waste, egg breaking operation, meat, shrimp, cheese and fruitcake processing, and found to be as effective as the synthetic polymers tested.

1.7.7. - Films and membrane formation

Biodegradable polymers for food packaging are becoming increasingly desirable from an environmental and functional viewpoint. The film-forming qualities of chitosan were recognized early, and the basic technique for casting them was to dissolve chitosan in a weak organic acid, typically acetic or formic acid at about 2%. This forms a suitably viscous solution, and the film is cast onto a smooth surface. At this point the chitosan is in the form of a complex salt, which has been formed with the dissolving acid, and the anion must be removed if the chitosan is to exhibit any resistance to water. The film may be heated to about 90°C to remove volatile acid components or dried at a lower temperature

and then immersed in a weak caustic solution; the excess acid is neutralized and the soluble products are washed out by rinsing in water. The resultant films are described as flexible, tough, transparent and clear with a tensile strength of about 9000 psi (Averbach, 1978).

The effect of molecular weight (M.W.) of the chitosan used is important, as significant losses in extensibility occur as M.W. increases (Kaplan et al, 1989). The advantageous property of low oxygen permeability was exhibited by all the films prepared from chitosans of different molecular weights. A crosslinked chitosan with 5% epichlorohydrin gave the best results since it improved functional properties such as tensile strength. Since chitinase is found commonly in nature, those films are highly biodegradable (Kaplan et al, 1989).

Yang and Zall (1984) studied the effect of chitosan concentration used to produce films for reverse osmosis applications, as well as the effect of pressure, salt concentration, pH, on the flux rate. They found that flux rate decreases with higher concentrations of chitosan in the casting solution, and that the salt rejection was slightly lower at lower pH (4.0 to 7.0).

Membranes can be prepared by evaporating solvents from solutions of chitosan, chitin or their derivatives, or by N-substituting chitosan membranes. Hirano (1978) studied the properties of various types of chitosan membranes. N-acylchitosan membranes were colorless and transparent or semitransparent, and were stable in cold or boiling water, in ethanol, in 0.5M NaOH, and 0.1M HCl solutions at room temperature for more than one month. N-arylidenechitosan membranes were colorless or brown and transparent or semitransparent, and they were stable in alkaline solutions but were easily solubilized in acidic solutions because of the acid-labile structure of the Schiff base involved. Their thermal decomposition points ranged from 123°C to 246°C. Tensile strengths were 410-440 and 300-360 kg/cm^2 for N-acyl- and N-arylidene-chitosan membranes respectively. The N-acetylation of the chitosan membranes increased the flow rates of water through them, but the partial O-acetylation of N-acetylchitosan membranes slightly decreased the flow rates. Urea, NaCl, $CaCl_2$, and low-molecular-weight compounds (MW < 2900) passed through these membranes, but cytochrome C (MW 13 000) did not. An increase in the thickness of chitosan membranes decreased the permeability of the compounds, as observed with the flow rates of water, but these characteristics were not observed with N-acetylchitosan membranes. The xerogels of these gels were assemblies of many micropores which were surrounded with thin membranous walls as examined by scanning electron

microscopy, and the pore dimensions were 30-50 x 80-300 μm for N-acetylchitosan xerogels, and 40-70 x 50-100 μm for N-methylenechitosan xerogels. These pores have enough space for free movement of these small MW compounds. The micropores are pressed by air-drying, and the membranous walls are in contact with each other to afford the present membranes in which the micropores do not exist. The membranes consist of many layers of the membranous walls, and small MW compounds pass through these layers. These membranes have potential as general membrane filters, a solid support for immobilized enzymes and other applications.

Since chitosan is also edible, it can be applied to fabricated foods, encapsulating agents, or packaging materials. Samuels (1981) observed morphological structures varying from spherulites to rods. These structures influence the mechanical properties of the films. Chitosan can exist in two distinct crystal forms, and the many different patterns observed by X-ray diffraction represent different mixtures of the two forms. The use of higher-molecular-weight chitosan produces a less crystalline, smaller crystallite, rod structured film, while chitosan with lower M.W. forms a more crystalline, larger crystallite spherulitic film. Also, different solvent treatments and wash time can enhance spherulitic production. The degree of deacetylation did not significantly influence observed changes in the X-ray and infrared spectra with treatment, but did affect the morphological structure of the films they obtained.

Aiba (1991) prepared chitosan films by dissolving chitosan in 2% acetic acid, pouring into petri dishes, drying, neutralizing by ethanolic NaOH solution, followed by washing with ethanol-water mixtures. The chitosan and the acetylated chitosan membranes do not possess any small pores which can be observed by a light microscope or an electron microscope; they are homogeneous and have high mechanical strength, especially tensile strength. Moreover, they have high permeability toward water. Due to this fact, a membrane does not need to be processed in order to form a porous structure. A membrane whose thickness makes it self-supporting can be used as a good reverse osmosis membrane, which is advantageous as compared with cellulose acetate membranes. They are also insoluble in alkali The chitosan membrane allows permeation of urea and low molecular weight organic compounds and rejects high molecular weight compounds (Muzzarelli, 1977).

1.8. Characterization of the polymer

1.8.1. - Degree of acetylation

Various methods exist for the determination of the degree of acetylation of chitin and chitosan. Muzzarelli and Rocchetti (1985) proposed a simple method based on the use of the first derivative spectra of N-acetyl-D-glucosamine in 0.01 M acetic acid. The calibration curve of absorbance from 5 to 40 mg/L at 199nm gives a straight line. Correction factors for glucosamine contribution (when the ratio of N-acetylglucosamine to glucosamine is below 0.11) are derived from the reference curve.

Infrared spectroscopy is often used for the determination of the degree of acetylation. The infrared spectra of chitin and chitosan have been published by many authors on both naturally occurring chitins and purified or modified chitins and chitosan. The most significant parts of the spectra are those showing the amide bands at 1665, 1555 and 1313 cm^{-1}, all of which show perpendicular dichroism and which are, respectively, assigned to the C=O stretching, to the N-H deformation in the CONH plane and to CN bond and CH_2 wagging. The chitosan spectrum differs from that of chitin in that the new band at 1590 predominates over the one at 1665 cm^{-1} and the band at 1555 is absent (Muzzarelli, 1977).

When chitosan is highly deacetylated, imprecision arises in the interpretation of IR spectroscopy results. Miya et al, (1980) used the amide I band at 1655 cm^{-1} and found that it gave better results than the amide II band at 1550 cm^{-1} employed by Sannan et al, (1978) for highly deacetylated samples. When the ratio of the absorbance of the 1655 cm^{-1} band to that of the CH stretching band at 2867 cm^{-1} was plotted against the degree of deacetylation measured by the colloid titration method, a smooth curve which is nearly linear over the range 90-100% deacetylation was obtained (Sannan et al, 1978). It was concluded that the estimation of CONH content of chitosan samples gives excellent accuracy, but this method should be limited to the samples deacetylated by >90% per glucosamine residue.

Domszy and Roberts (1985) evaluated the use of the 3450 cm^{-1} absorption band (hydroxyl) as an internal standard in the determination of the extent of N-acetylation of chitosan from the absorbance of the amide I band at 1655 cm^{-1} and found that the technique gave consistent results that were relatively unaffected by whether the sample was air-dried or oven-dried.

The amide II band appears at 1595 cm^{-1} at low degrees of N-acetylation and shifts to 1550 cm^{-1} as the degree of N-acetylation increases. This is presumably due to disruption of hydrogen bonding involving the amide group, with increasing extent of N-acetylation. The presence of hydrogen bonding in simple secondary amides has been found to increase the frequency of the amide II absorption band. Such a shift would have an adverse effect on the accuracy of measurements based on this band. The intensity of the 2878 cm^{-1} absorption band at a given degree of N-acetylation will depend upon the chain length of the N-acyl group. Thus a different calibration curve would be required for each N-acyl group and determination of the extent of N-acetylation in mixed derivatives would be impossible. Furthermore, a plot of the values for the degree of acetylation obtained by chemical methods such as salicylaldehyde uptake and titration of chitosan hydrobromide, against either the ratio A_{1550}/A_{2878} or the ratio A_{1655}/A_{2878} gives sigmoidal curves rather than the rectilinear plot obtained with the ratio A_{1655}/A_{3450}.

Aiba (1986) estimated the degree of N-acetylation of chitosan by ultra-violet (U.V.) spectroscopy, based on the absorbance of acetamide groups at 220nm. This principle was applied in high pressure liquid chromatography with an aqueous gel permeation support and a U.V. detector. It was found that U.V. absorption peak areas in chromatograms were proportional to the concentration of acetamide groups and by this relationship the degree of N-acetylation could be estimated. This method is useful for the simultaneous characterization of chitosan regarding its degree of acetylation and molecular weight.

1.8.2. - Molecular weight

Wu (1988) examined the molecular weight distribution of chitosan products with different times of deacetylation by size exclusion with HPLC, and found the average molecular weight to vary from 1.46×10^6 at 30 min of deacetylation (50% NaOH), to 7.2×10^5 after 5 hr of deacetylation.

The molecular weight of a polymeric coagulating agent has been considered to be one of the most important characteristics affecting functionality of the polymer. A rapid method used to measure the MW distribution of chitosan is size exclusion chromatography in the HPLC mode. Dextran MW standards can be used to obtain a calibration curve. Wu et al, (1976) used pore glass supports having a covalently bonded glycerol coating (Glycophas-G/CPG) to inactivate free silicic acid groups on the surface of the glass particles as

packing materials for HPLC columns. Pore sizes included 40, 100, 250, 550, 1500 and 2500 A. A loading study showed that an injection load of 500 µg, i.e., 100 µl at 5 g/L or 50µl at 10 g/L (w/v) was the optimal load to give reproducible elution volumes, precision in quantitation, and minimum viscosity effects. Analysis time is less than 20 min per sample. A refractive index detector was used to determine elution volumes and molecular weight distributions. A UV detector was used to assist in measuring the separation of different chitosan components.

Generally speaking, the viscosity of a solution of a macromolecular compound is related to the degree of polymerization through the constant K_m, and depends on the nature of the solute and the solvent, the type of bond and the molecular shape as follows:

$$\lim_{c \to 0} (n_{sp}/c) = K_m \times P^n$$

where, c is the concentration, sp means specific, P is the degree of polymerization, and n is between 0 and 2. Currently, the intrinsic viscosity n is related to the average molecular weight M_w in the Staudinger equation:

$$\log | n | = \log K + a \times \log M_w$$

where "a" is unity for a long molecule kinked in a random fashion, and approaches zero for a chain coiled into a ball. Lee (1974) obtained the values K = 8.93 x 10-4 and "a" = 0.71 for chitin.

The molecular weight of chitin was measured by Shimahara and Takiguchi (1988) using viscometry by dissolving chitin powder in a solution of 5% of lithium chloride in N,N-dimethylacetamide, and measuring viscosity of each solution at 30°C with a Ubbeholde-type viscometer. Intrinsic viscosity was determined graphically by plotting the viscosity data against concentration. The molecular weight of chitin (M) is then calculated from the following equation:

$$[n]=8.93 \times 10^{-4} M^{0.71}$$

where [n] is the intrinsic viscosity.

Chitosan in solution exhibits polyelectrolyte effect: in the absence of salt, there is an

abnormal increase in the viscosity of the more dilute solutions because of an enlarged effective volume due to charge repulsion and stretching out of the molecules. When sufficient salt is added to neutralize this charge effect, the viscosity behavior is normal. Muzzarelli (1977) recommended a solvent system comprising 0.2M acetic acid and 0.1M sodium acetate for viscosity measurement of chitosan.

The values of "a" and K_m obtained by Roberts and Domszy (1982) differ from those obtained previously by Lee (1974) but are similar to those reported for related ioinic polysaccharides. The Mark-Houwink equation for chitosan in 0.1M acetic acid + 0.2M sodium chloride can be expressed as:

$$[n] = 1.81 \times 10^{-3} M_v^{0.93}$$

and, in 0.1M acetic acid + 0.02M sodium chloride, as

$$[n] = 3.04 \times 10^{-5} M_v^{1.26}$$

Molecular weights of chitin and chitosan are very different, since a severe degradation of the chain takes place during the production process. This happens during the decalcification step, when the wastes are exposed to acid solutions, or during the deacetylation step which involves harsh treatment with alkali at high concentration and at high temperature (Muzzarelli, 1977).

1.9. Product variability

The variation of chitin properties are, to a large extent, due to the methods of preparation and purification. There are also, to a lesser extent, natural species variations in chitin with regards to degree of polymerization and acetylation, crystallographic structure, solubility, and reactive behavior (Brine and Austin, 1981). The type and amount of protein in the chitin-protein complexes vary widely among various species (Austin et al, 1981).

Chitin and chitosan vary in their properties mainly because of preparation methods and conditions as explained previously. Muzzarelli et al, (1981) analyzed various chitosans from different producers and organisms, and reported acetyl percentages varying from 22 to 35%, and pK of the chitosans ranging from 6.0 to 7.0. However, one chitosan preparation made by their laboratory in 6 different batches showed consistent properties

between the samples.

Salient properties of chitin that are influenced by the method of isolation and purification include the following:

1- as a polyacetal, it is sensitive to and easily hydrolyzed by acids, even more so than cellulose.
2- it is quite stable to dilute alkali.
3- it is sensitive to heat, even in the 40-50°C range and may denature with time.
4- it is a potent metal-sequestering agent; hence water washing should be done with deionized water to the greatest extent possible.
5- hydrates, alcoholates, or ketonates of chitin are formed when chitin in solution is precipitated by the appropriate nonsolvent.
6- **chlorine bleaches** should be avoided as they form N-chloro compounds with chitin; peroxide bleaches are preferred.
7- chitin isolates usually have a few free amino groups, perhaps one for every six glucose units.
8- chitin is a chiral polymer by virtue of both its glucose moieties and its helical structure (Austin, 1988).

1.10. Chitosan derivatives

Alkaline chitin (chitin metal salt or alkoxide) is a key intermediate for its chemical modification (Hirano et al, 1991).

Chitosan can be selectively and extensively sulfated at the primary hydroxyl group by using either a 2:1 mixture of sulfuric and chlorosulfonic acid or a pyridine-SO_3 complex, following protection of the 2-NH_2 and 3-OH groups with copper ions. Introduction of a sulfate group at the C-6 of chitosan, while leaving the amino group unsubstituted could have an important influence on solution and rheological properties of the polysaccharide. In alkali solution, chitosan 6-O-sulfate should behave like a typical polyanion, while at low pH values, a zwitterionic structure is present (Terbojevich et al, 1989).

N-carboxybutyl chitosan is obtained by reaction of chitosan with levulinic acid (4-oxo-pentanoic acid, $CH_3COCH_2CH_2COOH$) and has the advantage of being soluble in water and in water-ethanol mixtures, and gives more viscous solutions than the corresponding chitosans. This derivative retains the film-forming ability of chitosan. By slow evaporation

of aqueous and water-alcohol solutions, membranes were obtained (Muzzarelli et al, 1989).

N-carboxymethyl chitosan is another water-soluble derivative which is much more effective than the parent chitosan in binding Cu(II) and Pb(II) (Muzzarelli, 1989). N-O-carboxymethyl chitosan (NOCC) possesses several useful functional groups, i.e., primary and secondary amines, carboxyl, and hydroxyl groups. Its salts with the alkali metals, magnesium, and ammonium ions, are water soluble. NOCC reacts with and precipitates heavy metal and large organic cations. It is also soluble in solutions whose pH is above 6 or below 2 (Davies et al, 1989).

N-acylation of chitosan was performed by Moore and Roberts (1978) and monitored by IR. Carboxylic-acid anhydrides in methanol were used as the reaction media. Acetic, propionic, butyric, hexanoic and benzoic acids gave their respective derivatives. The induction period for the reaction increased with the size of the acid-anhydride molecule and decreased with an increase in the temperature. The induction period has been attributed to the time required for the methanol to swell the film before diffusion of the anhydride into the chitosan takes place.

1.11. Antimicrobial properties of chitosan

Polycations exhibit antimicrobial properties. On the surface of microorganisms the polycations, in contrast to divalent cations such as Mg^{2+} or Ca^{2+}, produce a destabilizing effect on the outer cell wall structure (Popper and Knorr, 1990). Microscopic examination made by Sudarshan et al, (1992) showed that chitosan caused cell agglutination at pH 5.8.

Chitosans are used as natural antibacterial agents (Tsugita, 1990). For example, the growth of *Escherichia coli* was inhibited in the presence of >0.02% chitosan in its culture medium, and the growth of several plant pathogens was also inhibited in the presence of chitosan. Its N-alkyl derivatives also exhibit antimicrobial properties.

Chang et al, (1989) used low molecular weight chitosan (M.W. 35 000) at a level of 75 ppm to completely inhibit the growth of *E. coli*. Gram positive bacteria such as

Table 2. Chitosan levels and inhibition of microorganisms.

ORGANISM	CHITOSAN LEVEL	ORGANISM	CHITOSAN LEVEL
Escherichia coli	100 ppm	*Vibrio* sp.	100 ppm
Staphylococcus sp.	50 ppm	*Salmonella* sp.	2000 ppm
Bacillus sp.	50 ppm	*Saccharomyces* sp.	100 ppm
Pseudomonas sp.	100 ppm		

(Chitosan M.W. 35 000).

(From Chang et al, 1989).

Staphylococcus sp. and *Bacillus* sp. were also inhibited (Table 2). However, the pH of the culture medium was not indicated, and this is of great importance. The shelf life of Mulkimchi (pickle type Kimchi) containing 0.2% chitosan was 10 days longer than control stored at 5°C.

The growth of *Vibrio parahaemolyticus* was enhanced on media containing from 0.5 to 1% chitin, with lower levels having no effects. *V. parahaemolyticus* seemed to be preferentially adsorbed on chitinous material of certain shellfish and copepods (Anand et al, 1981).

N-carboxymethyl chitosan, a water-soluble derivative, has an antibacterial action due to its sequestering ability for transition metal ions, especially Cu, which are thus unavailable to the bacteria. Twenty-six strains of *Staphylococcus aureus*, *Streptococcus* spp. and *E. coli* were all inhibited by low concentrations of N-carboxymethyl chitosan (317 to 2250 μg/mL of culture media) (Muzzarelli, 1989).

Muzzarelli et al, (1990) studied the effect of N-carboxybutyl chitosan on various pathogens. Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* were completely inhibited at concentration of 0.8% of N-carboxybutyl chitosan in the culture medium. Gram-negative bacteria, e.g., *E. coli, Enterobacter* spp., *Proteus* spp., *Salmonella* spp., *Pseudomonas aeruginosa*, etc., were completely or almost completely inhibited at a concentration of 0.9% of N-carboxybutyl chitosan in the agar medium.

Given the polycationic nature of N-carboxybutyl chitosan, it is likely that it can interact and form polyelectrolyte complexes with acidic polymers produced at the bacterial cell surface (e.g., lipopolysaccharides, teichoic, and teichuronic acids, or capsular polysaccharides) (Muzzarelli et al, 1990). It is noteworthy that chitosan can agglutinate a number of bacteria, and moreover, polyelectrolyte and macromolecular complexes of chitosan with glycosaminoglycans have been reported (Muzzarelli, 1985).

Chitosan lactate and chitosan hydroglutamate were used as antibacterial agents against *E. coli*, *S. aureus*, and *Saccharomyces cerevisiae* (Papineau et al, 1991). A concentration of 0.2 mG/mL of chitosan lactate in distilled water appeared most effective against *E. coli*. Within 1 hr, the population was reduced by approximately 4 log cycles for *S. aureus*. The

reduction was about 6 log cycles within 1 hr. For yeast, inactivation was complete within approximately 17 min of exposure to 1 mG/mL of chitosan lactate.

Chitosan and chitin are present in the cell walls of phytopathogens, and at the same time, they have been shown to inhibit phytopathogens growth. Hirano and Nagao (1989) observed the antimicrobial effect of chitosan (0.1% at pH 5.6) against several fungi, e.g., *Fusarium, Alternaria, Rhizopus,* etc. Similarly, Allan and Hadwiger (1979) demonstrated the antifungal property of chitosan, and noted that chitosan was less effective against organisms having a cell wall composed of chitin/chitosan or chitin-B-glucan.

For example, Wang (1992) showed the importance of pH on the antimicrobial activity of chitosan. *S. aureus, E. coli, Yersinia enterocolitica, Listeria monocytogenes* and *S. typhimurium* were all inhibited by concentrations of 0.5 to 1% chitosan at pH 5.5, but only *S. aureus* was inhibited by chitosan at pH 6.5, and all the other strains grew in 2.5% chitosan (the highest level studied).

1.12. Chitosan toxicity

Knorr (1986) mentioned that although approval of chitin or chitosan as food ingredient or processing aid by the US Food and Drug Administration (FDA) has not yet been sought, FDA approval for chitosan as a feed additive has been granted in 1983 and use of chitosan for potable water purification has been approved by the US Environmental Protection Agency (EPA) up to a maximum recommended level of 10 mg/L.

Bough and Landes (1978) showed in rat feeding trials that chitosan was completely harmless below 5% concentration. The animals were all healthy and vigorous and displayed no overt symptoms of physiological distress. Arai et al, (1968) also reported that chitosan caused no harm when fed up to 18 g per kg of body-weight per day to mice.

Shrimp wastes contain high levels of protein (around 44%) with high levels of lysine and methionine (6.2 and 2.5 g per 100 g protein) (Oke et al, 1978). The protein is easily digestible and has a good protein-efficiency ratio (PER) of 2.1 when fed to rats at a 10% level. Shrimp waste is also high in chitin (12.5%) and ash (27.3%), which are made up mainly of calcium and sodium chloride, and this might affect its value as animal feed. The composition is comparable to the compositions of fishmeal and of bonemeal. In addition, it contains an appreciable amount of carotenoids which makes it valuable as chicken feed

and as a fish (salmonids) feed. The amount of chitin is closely related to the crude-fiber content. It is very doubtful that chitin is utilized by monogastric animals, though it may possibly be used by ruminants, and this lowers the nutritive value of shrimp waste. A problem that might be posed in the use of shrimp waste, beside the high mineral content, is the variable composition of the meal, because the final composition of the meal depends on the proportions of the various parts that are mixed together. In addition, the method of processing will also have an effect on the composition, as well the time lapse before processing is important, as a loss in nutritive value of 10% was observed in the protein when the heads were allowed to spoil for 24 hr. The rapid degradation is brought about by the enzymes concentrated in the head. This autolytic and microbiological degradation can be reduced by a treatment with acid for 6-24 hr, followed by neutralization, which destroys the enzymes (Oke et al, 1978).

1.13. Chitosan uses in the food industry

1.13.1. - Clarifying aid in the fruit juices industry

The use of chitosan for the clarification of fruit juices is approved by the FDA at levels lower than 0.1% (Sandford, 1989).

Sapers (1992) demonstrated prevention of browning in McIntosh apple juice by the addition of 200 ppm chitosan, followed by filtration with diatomaceous earth filter aid. Chitosan at 1000 ppm was required to prevent browning in juice from ripe Bartlett and Bosc pears. Removal of particulate matter was probably the main effect of chitosan for preventing enzymatic browning.

In the fruit juice industry, chitosan can be an advantageous replacement for conventional clarifying aids including gelatin, bentonite, silica sol, tannins, polyvinylpyrrolidone, or combinations of these compounds. Turbidity of apple juice was reduced to zero with 0.8 kg/m^3 of water-soluble chitosan (Soto-Peralta et al, 1989).

Apple juice turbidity and microbial counts at ambient temperature were significantly reduced by chitosan (Popper and Knorr, 1990).

1.13.2. - Cholesterol lowering effect

Chitosan has the ability of lowering cholesterol levels in the blood. When added as a dietary fiber at a 4% level in the diet of rats, the total cholesterol level in blood decreased significantly after only 2 weeks of feeding (Tsugita, 1990). Furthermore, chitosan acts as a hypolipidemic agent. The hypocholesterolemic and hypolipidemic activity of chitosan is probably due to the inhibition of micelle formation. At pH 6.0-6.5, chitosan begins to precipitate (depending on its origin and quality), and as the chains of the polysaccharide aggregate, they can entrap whole micelles (Muzzarelli, 1985). These features suggest the possibility of using chitosan for new types of health food. In Japan, dietary cookies and noodles containing chitosan are on sale (Hirano, 1989).

1.13.3. - Protein recovery

Whey is a liquid waste from cheese manufacture containing mainly lactose and soluble proteins. As a waste material, it is highly polluting, but recovery of proteinaceous solids is a way of enhancing the value of this liquid waste. Whey proteins have been utilized in beverages, ground meats, and other food. Various methods exist to recover the proteinaceous solids, and chitosan was successfully applied for this purpose (Bough and Landes, 1976). A ratio of chitosan to suspended solids of 2.15% (or 30 mg per liter) was used to flocculate the suspended solids. The lowest turbidity was achieved at pH 6.0. The results of rat feeding studies showed no significant differences between this protein residue containing 2.15% chitosan and the values for casein or for whey solids containing no polymer.

The discharge streams from crustacean processing plants represent a valuable source of organic compounds, notably flavor-related amino acids. No and Meyers (1989) used crawfish chitosan at a concentration of 150 mg/L and a pH of 6.0 to obtain a reduction of 83% in turbidity, and a reduction in the concentration of suspended solids of 97%. Analysis of the recovered solids showed high levels of glutamic acid and aspartic acid, with somewhat lower amounts of leucine, arginine and alanine. These 5 amino acids accounted for 62% of those present in the coagulated solids of crawfish wastewater.

Holland and Shahbaz (1985) reported the use of chitosan in mussel protein recovery. They obtained a percentage of recovery of 80% at pH 6.4, at chitosan dosages of 32 mg/L.

In the same manner, chitosan can be used as an effective **ligand-exchange** column material for recovery of amino acids from processing wastewater of seafood for example. Ligand-exchange chromatography is based on the principle of fixation of a transition metal on a solid support wherein this solid sorbent can be used for exchange of bound ligands of the metal. With this system, ligands such as amino acids and amines can be removed from their medium by formation of complexes with the metal attached to the support, and consequently water or liquid coordinates of the metal will be displaced. Copper chitosan and amino copper chitosan was prepared and used by No and Meyers (1989) to recover amino acids. At pH 8 to 10, asp, thr, ser, pro, glu, gly, ala, val, met, ile, leu, tyr, phy, lys, his, and arg were recovered at high percentages. Specific flavor and flavor-enhancing compounds are recovered by this process.

Chitosan concentration of 30 mg/L of waste effluent at pH 6.4 can reduce suspended solids by 88% and the chemical oxygen demand (COD) by 49% in poultry processing waste effluent. The crude protein content of coagulated solids was 68% of the dry matter (Muzzarelli, 1977).

1.13.4.. - Water-alcohol separation

Chitosan membranes can be prepared in the form of hollow fiber, with controlled permeation flow rate (weight of solution passing through the membrane of 1 m^2/h). This membrane can be applied to reaction systems to which high temperatures cannot be applied. The separation is selective to water only, even with concentration of ethanol over 80% (Tsugita, 1990).

1.13.5. - Chitosan use as a coating (fruits and vegetables)

Edible coatings controlling preservative migration from surface to food bulk could control surface microbial growth which is often the main cause of spoilage for many food products (Vojdani and Torres, 1989).

N-O-carboxymethyl chitosan (NOCC) is prepared from the reaction of chitosan with monochloroacetic acid under alkaline conditions (Davies et al, 1989). NOCC is soluble in solutions whose pH is above 6 or below 2. Strong films of NOCC can be formed readily from aqueous solutions. These films have selective permeability to gases such as oxygen and carbon dioxide and are capable of separating mixtures of gases such as ethylene,

ethane, and acetylene. Toxicology studies have shown that 50 000 ppm of NOCC in the diet of rats for 14 days has a "no-observable-effect" on female rats and a "no-observable adverse-effect" on male rats. It appears from studies on rabbit that the main route of elimination of the polymer when injected in the blood stream is via the urine. There is also significant amounts of activity in the liver which probably reflects uptake by the Kupffer cells. There is no evidence of significant uptake by any organ than those mentioned. Apples coated with NOCC films formed by dipping or spraying with NOCC retained their freshness for more than six months and their titratable acids for approximately 250 days when placed in cold storage. The films give the apples some luster but are transparent and non-waxy to the touch. They are readily removed by washing with water. NOCC films are also effective for the preservation of other fruits such as pears, peaches, and plums. Pears treated with NOCC had a lower incidence of flesh breakdown and decay than did pears stored in controlled atmospheres of 1% or 2% oxygen and were just as firm after 120 days. The Canadian Department of Health and Welfare has approved the use of NOCC on fruit that is to be washed and peeled prior to further processing. Because NOCC retains free amine groups, it should have many of the properties of chitosan and have applications in wound healing and cholesterol lowering effect for example.

El Ghaouth et al, (1991a) reported the effect of chitosan coating on the storability and quality of fresh strawberries. Solutions of 1.0 and 1.5% (w/v) applied as a thin and transparent coating to the fruits were effective in preventing decay in 78 and 81% of the inoculated fruits respectively after 29 days at 13°C, compared to 18% remaining in the control.

Chitosan coating also has a beneficial effect on flesh firmness, titratable acidity and retarded synthesis of anthocyanin in strawberries stored at 4°C. After 31 days, the fruits were firmer and higher in titratable acidity than the control of fungicide-treated berries. Chitosan coating delayed rate of ripening, as indicated by the lower anthocyanin contents. While available coating materials have been found to extend storage-life of produce by acting as a diffusion barrier, chitosan coating afford the added advantage of antifungal activity which is beneficial for highly perishable produce such as strawberries (El Ghaouth et al, 1991a). In addition, chitosan coating is likely to modify the internal atmosphere without causing anaerobic respiration, since chitosan films are more selectively permeable to O_2 than CO_2 (Bai et al, 1988)

Another study by El Ghaouth et al, (1991b) on cucumber and bell pepper fruits showed

that chitosan coatings markedly reduced the weight loss at 13 and 20°C (RH 85%), with the 1.5% chitosan dipping solution being more efficient than the 1% solution. Respiration rate, loss of color, wilting and fungal infection were also reduced by the chitosan coating.

Chitosan-based coating were also tested on green peppers, tomatoes, carrots, rutabagas and English cucumbers. Among those products, only tomatoes and green peppers responded favorably to the coating. No reduction in weight loss, retardation of senescence or prevention of decay was observed for carrots, rutabagas or English cucumbers coated with chitosan-based coatings. The coating may even be detrimental to these commodities by enhancing the potential for decay. The application of a 2% (w/v) chitosan-based coating to green peppers reduced the incidence of decay, delayed ripening, increased internal CO_2 and reduced internal O_2. Chitosan-based coatings were not effective barriers to moisture loss through the stem scar of tomatoes. However, the coating reduced the rate of ripening over a 12-day storage period (Lidster, 1987a).

The effect of this chitosan-based coating on O_2 uptake and CO_2 evolution was studied on apples and pears. A 2% solution used for dipping the fruits was most effective for maintaining firmness, preventing decay, and retaining higher titratable acidity in the fruits, compared with modified atmospheres (Lidster, 1987b).

1.14. Fresh shrimp preservation

1.14.1. - Current methods

The prevention of deterioration in the quality of the shrimp involves two distinct problems, namely, maintaining low numbers of detrimental microorganisms and the control of oxidations, chiefly of phenols, into melanins (Fieger, and Novak, 1961). This latter reaction, called melanosis, is produced by melanin pigments which form on the internal shell surfaces or , in advanced stages, on the underlying shrimp meat. These pigments are produced by an oxidative reaction of tyrosinase on tyrosine. The reaction is accelerated by copper and other metallic ions In American good manufacturing practice (GMP), the treatment of fresh shell-on tails of shrimp involves a 1 min dip in 1.25% solution of metabisulfite, which retards melanosis. After a 15 sec rinse, such shrimp contain in the edible part about 80 µg SO_2 per gram of wet weight (Finne et al, 1986).

1.14.2. - Perspectives

Food irradiation is a process in which food products are exposed to ionizing radiation to improve the shelf life or wholesomeness of that product. Ionizing radiation may damage DNA at the cellular level, thus interfering with normal biochemical processes. Genetic damage caused by exposure to radiation can kill or damage molds, bacteria, and other microorganisms that cause food spoilage or disease. Non-spore-forming pathogenic bacteria that have been identified in seafood products include *Salmonella, Shigella, Vibrionacae,* and *Staphylococcus. Vibrios* can be eliminated with a relatively low dose of irradiation; however, a higher radiation dose has been found necessary to eliminate or inactivate *Salmonella* (Grodner and Andrews, 1991). Gram-positive bacteria such as *Staphylococcus aureus, Micrococci, Bacilli,* and *Clostridium* are among the more irradiation-resistant bacteria.

Modified atmosphere packaging (MAP) where CO_2 is predominant is also being tested for preserving shrimp. Layrisse and Matches (1984) studied the effect of 50 and 100% CO_2 at 0 to 2°C on the preservation of fresh shrimp (*Pandalus platyceros*). Only 100% CO_2 was effective in extending the lag phase of bacterial growth, but the greatest drip was also obtained with this atmosphere. The presence of CO_2 caused a shift in the bacterial population, which became predominantly composed of Gram-positive organisms, e.g., lactobacillus-like species.

Vacuum packaging of seafoods has been controversial since the absence of oxygen and low temperature growth of *C. botulinum* Type E may present a health hazard if not appropriately heat processed after storage, and if the product has been handled at "temperature abuse" conditions (Lampila, 1991). MAP in conjunction with other preservatives, such as antioxidant dips, may be more promising.

1.15. Spoilage/preservation monitoring of shrimp

Several biochemical indicators of shrimp freshness are currently used. Among these, the total volatile base nitrogen measurement, extractable nitrogen, nucleotide profile, pH, water-holding capacity, total microbial counts were used by Chen et al, (1990) to monitor freshness in shrimp.

Hypoxanthine (Hx) is one of the most useful indicators of loss of freshness, since its

increase is due to enzymatic degradation as well as to bacterial activity. The content of Hx increases linearly with time of storage to about 5μM/g wet weight and subsequently declines or remains at that level. The content of Hx correlates well with sensory assessment, particularly with flavor. A limit of 2 μM/g for shrimp is proposed (Fatima et al, 1981).

Indole has also been measured by several authors. Chang et al, (1983) demonstrated that while indole levels indicate decomposition, decomposed shrimp may not necessarily contain indole. Indole is of value in stressing the history of shrimp if high temperature abuse is suspected.

1.16. Microbiology of shrimp spoilage

Shrimps contain far greater amounts of free amino acids than fish, which largely facilitates bacterial growth and explains their ready spoilage. Most shrimp are handled raw. Soon after the shrimp are landed, they generally die. Microbial spoilage starts immediately through marine bacteria on the surface or through microorganisms which happen to contaminate the shrimp on deck, during handling and washing (Fieger and Novak, 1961).

The main group of bacteria present on freshly caught shrimps are *Achromobacter, Bacillus, Micrococcus,* and *Pseudomonas.*

1.17. Response Surface Methodology and its advantages in food research

Response-surface methodology (RSM) is a system for optimizing variables in order to obtain an optimum result or response. RSM tests several variables at a time, uses special experimental designs to cut costs, and measures several effects by objective tests. The second-order equations obtained which define quantitative relationships between variables and responses cover the entire experimental range tested, and include interactions if present. The models can then be used to calculate any, or all combinations of variables and their effects within the test range (Henika, 1982).

In fact, the equations obtained by this method can be graphically represented (the response surfaces) and be used in three ways: (1) to describe how the test variables affect the response; (2) to determine the interrelationships among the test variables; and (3) to describe the combined effect of all test variables on the response (Giovanni, 1983). Also,

RSM can be used to determine the values of the independent variables which are optimum as far as the response is concerned. In this case, the purpose is to determine a region of the total space of the factors in which certain operating specifications are met (Myers, 1971).

The effective use of RSM depends upon several assumptions, e.g., that the factors and the levels of influence which are critical are known; that the factors vary continuously throughout the experimental range tested; that there exists a mathematical function which relates the factors to the measured response; and that the response which is defined by this function is a smooth surface (Giovanni, 1983).

Chapter 2.

Materials and methods.

2.1. Materials

All reagents used for the study were of high purity. Reagents for spectroscopy and HPLC were of appriopriate grade. Enzymes were obtained from Sigma Chemical Company and kept frozen as recommended. Culture media for the microbial study were supplied by Difco Laboratories, Detroit, MICH.

Fresh shrimps and shrimp wastes were obtained from "Les fruits de mer de l'Est du Quebec", Matane, PQ. Fresh shrimps were shipped on ice the same day as they landed and treated for the preservation study as they were received (the following day). The wastes were kept frozen until use. Crab wastes were supplied by "Fishery Products International, St-John's, NF, and were kept frozen until use.

Bacillus cereus, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhymurium*, and *Proteus vulgaris* were provided by the department of microbiology of McGill University. *B. coagulans*, *B. brevis*, *P. morganii (Morganella morganii)*, *Pseudomonas fluorescens* and *Pseudomonas putida* were obtained from the Bureau of Microbial Hazard (Health protection branch, Ministry of Health and Welfare Canada, Ottawa).

2.2. Methods

2.2.1. Chitin and chitosan preparation

Chitin and chitosan were prepared chemically following the steps illustrated in Fig. 8, section 4.3.1. For the enzymatic production of chitin, demineralization of shrimp waste was done using 1.75N acetic acid at room temperature (~25°C) for 12 hr. The deproteinization of the demineralized waste was done with either chymotrypsin or papain at various temperature, pH, and enzyme to waste ratios, as explained in sections 3.4.2 and 3.4.3.

2.2.2. Shrimp preparation

Fresh shrimps were prepared for the preservation study as noted in section 4.3.2.The

dipping solutions were prepared fresh and kept at 4°C prior to use.

2.2.3. Protein measurement

Proteins were measured after deproteinization with the method of Lowry, as modified by Hartree (Hartree, 1972), as explained in section 3.4.4.

2.2.4. Chemical indicators of shrimp freshness

Total microbial counts, pH, drip loss, total volatile bases, and nucleotides were measured to monitor the progression of shrimp deterioration during the preservation study, as explained in sections 4.3.3 to 4.3.6 inclusively.

2.2.5. Sensory evaluation of shrimp freshness

Shrimp freshness was evaluated during the preservation study for appearance, melanosis, and odor. Five different scales were used and details are given in section 4.3.7.

2.2.6. Degree of acetylation

The degree of acetylation of chitosan was measured spectrophotometrically according to the method of Aiba (1986) as explained in section 5.3.3.

2.2.7. Microbial growth measurement

Microbial growth was measured following the method described in section 5.3.5.

2.2.8. Statistical analysis

All statistical analyses were done with SAS software (SAS Institute Inc., 1989).

Chapter 3

Use of proteolytic enzymes to facilitate the recovery of chitin from shrimp wastes.

3.1. Summary

Two proteolytic enzymes, chymotrypsin and papain, were used to hydrolyze proteins associated with demineralized shrimp waste to recover chitin. The conditions used were optimized using response surface methodology (RSM). The factors studied were temperature (3 levels), pH (3 levels) and enzyme to waste ratio (E/W ratio)(5 levels). Of these, pH was the most important factor in the models obtained. Optimum conditions for deproteinization by chymotrypsin were determined to be around 40°C, pH 8.0 and E/W ratio of 7:1000 (w:w). With papain, a temperature of around 38°C, pH of 8.7 and E/W ratio of 10:1000 (w:w) gave the optimum response. At these conditions, the yield of protein was maximum, and ranged from 22-48% depending on the starting material. The residual protein levels in the waste after deproteinization with the enzymes were very low, i.e., 1.3 and 2.8% for chymotrypsin and papain-treated samples, respectively.

3.2. Introduction

Chitin is a high molecular weight polysaccharide composed primarily of N-acetyl-glucosamine units. Chitin and its deacetylated form, chitosan, have found an increasing number of uses in various fields in recent years, from the flocculation of solid particles in waste water to transparent films (Skjak-Braek et al, 1989; Knorr, 1984).

The main source of chitin for commercial use is crustacean wastes, however, it occurs also in fungi, insects, etc. Traditional methods for the preparation of chitin involve demineralization and deproteinization of the waste material with strong acids and bases (e.g., HCl and NaOH)(Muzzarelli, 1977). However, use of these chemicals causes a partial deacetylation of chitin and hydrolysis of the polymer, resulting in a lower molecular weight final product with variable and inconsistent physicochemical properties (Brine and Austin, 1981). These chemical treatments also create a disposal problem, e.g., detoxification and neutralization of effluent waste water. Furthermore, the value of the deproteinization liquid is reduced by the presence of sodium hydroxide.

A few studies on use of proteolytic enzymes for the deproteinization of crustacean wastes have been carrried out. Broussignac (1968) demonstrated that use of papain, trypsin, or pepsin produced chitin with as little deacetylation as possible. Other enzymes used for the deproteinization step include tuna proteinase (pH 8.6 and 37.5 C), papain (at pH 5.5-6.0 and 37.5°C), or a bacterial proteinase (at pH 7.0 and 60°C) for over 60 hr. The residual protein associated with chitin after the enzyme treatments was about 5% (Takeda and Abe, 1962; Takeda and Katsuura, 1964). These studies indicated that the use of proteolytic enzyme was promising, however, reaction conditions were not optimized to obtain a more complete deproteinization.

Response surface methodology (RSM) is a powerful tool for optimization of a process influenced by several factors and their interactions. A combination of the levels of the factors which leads to an optimum response can be established through this approach. The second-order model or the quadratic regression model computed by this technique also helps to predict the response at a given combination of factor levels (Khuri and Cornell, 1987).

Thus, the objective of this study was to optimize reaction conditions for the deproteinization of shrimp waste using selected proteolytic enzymes by the RSM approach.

3.3. Materials

Shrimp wastes were received frozen from "Les fruits de mer de l'Est du Québec" (Matane, Québec), and were stored at -20°C until use. Chymotrypsin, type II (EC 3.4.21.1) and crude type papain (EC 3.4.22.2) were obtained from Sigma Chemical Company (St-Louis, MO, USA).

3.4. Methods

3.4.1. Demineralization

The demineralization of the waste was done using 1.75N acetic acid at room temperature (~25°C) for 12 hr. The ratio of waste to solvent was 1:15 (w:v). The demineralized material was recovered by filtration and thoroughly rinsed with deionized water, and dried at 65°C in a forced air oven.

3.4.2. Deproteinization

The demineralized materials were then deproteinized with various proteolytic enzymes. The buffers used for the chymotrypsin and papain digestions were 0.05M tris-base, pH 6.5 to 9.1 (containing 0.1M $CaCl_2$) and 0.05M L-cysteine, pH 6.8 to 8.8 (containing 2mM Na_2 EDTA), respectively. The ratio of demineralized waste to buffer was 1:20 (w:v), and 4 g of waste were used. The deproteinization was done in Erlenmeyer stoppered flasks incubated for 72 hr at various temperatures in an incubator-shaker (Orbit incubator-shaker, Lab-line Instruments Inc., ILL., USA, model no. 3529-3CC) with constant agitation of 140 rpm.

3.4.3. Experimental Design

Three factors were studied for each enzyme, viz., temperature of incubation, buffer pH and enzyme to waste ratio (E/W). The levels of each factor for each enzyme are summarized in Table 3.

A full factorial design was applied in both cases, and the 45 possible combinations (3 x 3 x 5) were run, with duplicates for the extremes and middle ratios only (i.e., 2, 6, and 10 mg/ 1000 mg for chymotrypsin and 2, 10 and 18 mg/1000mg for papain), giving a total of 72 runs.

3.4.4. Protein Measurement

The deproteinized material was filtered through a Whatman No.4 filter paper and the filtrate analyzed for protein content using the Lowry method as modified by Hartree (Hartree, 1972).

The protein in the residue recovered after filtration was measured by digesting the dry residue with 2N NaOH (1:25 w:v) at 70°C for 1 hr, and the protein content of the resulting liquid was measured by the Hartree method (Hartree, 1972).

TABLE 3

Levels of Combination Factors used in a Factorial Design for Optimization Experiment.

VARIABLE	LEVELS				
a) **for chymotrypsin hydrolysis**					
Temperature (X1)	25		32		40
pH (X2)	6.5		7.8		9.1
Enz:Waste ratio (X3) (mg/mg)	2:1000	4:1000	6:1000	8:1000	10:1000
b) **for papain hydrolysis**					
Temperature (X1)	25		36		47
pH (X2)	6.8		7.8		8.8
Enz:Waste ratio (X3) (mg/mg)	2:1000	6:1000	10:1000	14:1000	18:1000

3.4.5. Statistical Analysis

The results were analyzed for optimization using the RSM procedure of SAS (SAS version 6.06, 1989) and graphs were produced with the G3D procedure and GCONTOUR procedure of SAS.

3.5. Results and discussion

The results obtained were analyzed by RSM to determine the region in which the enzymatic deproteinization was as complete as possible, without using an excess of enzyme. Generally, at sub-optimum levels of enzyme, the response obtained is proportional to the amount of enzyme used. At enzyme levels closer to the optimum level, the response is no longer proportional to the amount of enzyme added, as this is approaching the saturation limit of the enzyme with the substrate. Using more enzyme beyond the optimum level achieves a very low increase in the response for a high addition of enzyme. At this stage saturation of the enzyme with substrate occurs, and these effects were easier to visualize with the response surface graphs obtained (Figs. 3 and 4).

The complete equation for chymotrypsin and papain hydrolysis of the shrimp waste are respectively:

$$Y = -245.26 + 0.50(X1) + 60.24(X2) + 0.83(X3) - 0.0011(X1)^2 + 0.044(X2*X1) - 3.84(X2)^2 + 0.013(X3*X1) - 0.013(X3*X2) - 0.055(X3)^2$$

and

$$Y = 110.57 + 0.048(X1) - 33.075(X2) + 1.166(X3) + 0.001(X1)^2 + 0.008(X2*X1) + 2.574(X2)^2 + 0.0006(X3*X1) - 0.174(X3*X2) + 0.018(X3)^2.$$

The analysis of variance (Tables 4 and 5) showed no lack of fit for both models. The coefficient of variation and the percentage of variability explained by the model are somewhat high, especially for papain (Table 5). These values were probably affected by the lack of homogeneity of the waste from batch to batch. The sample size used for each run was relatively small, thus heterogeneity could have had some effect on the results. The wastes were used in the uncomminuted form as previous studies with shrimp waste in our laboratory had shown that sample size had no effect on chitin yield (Tetteh, 1991).

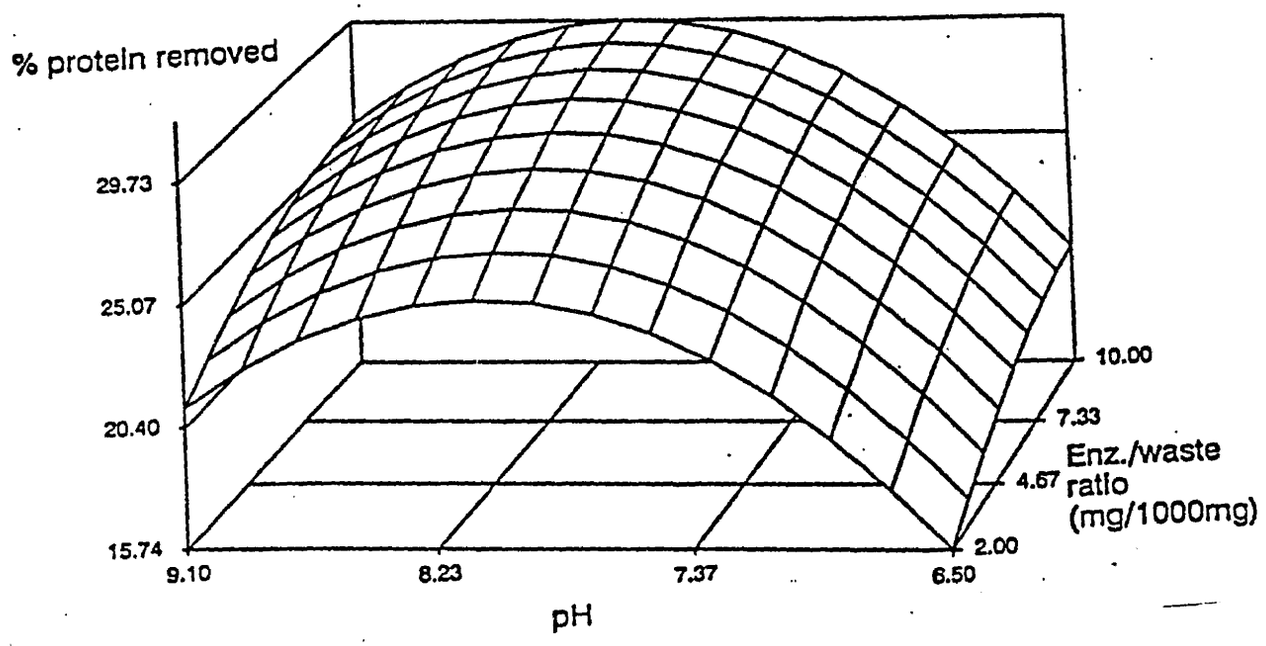

Fig. 3. Response Surface Graph of Protein Removal by Chymotrypsin at 39.7°C.

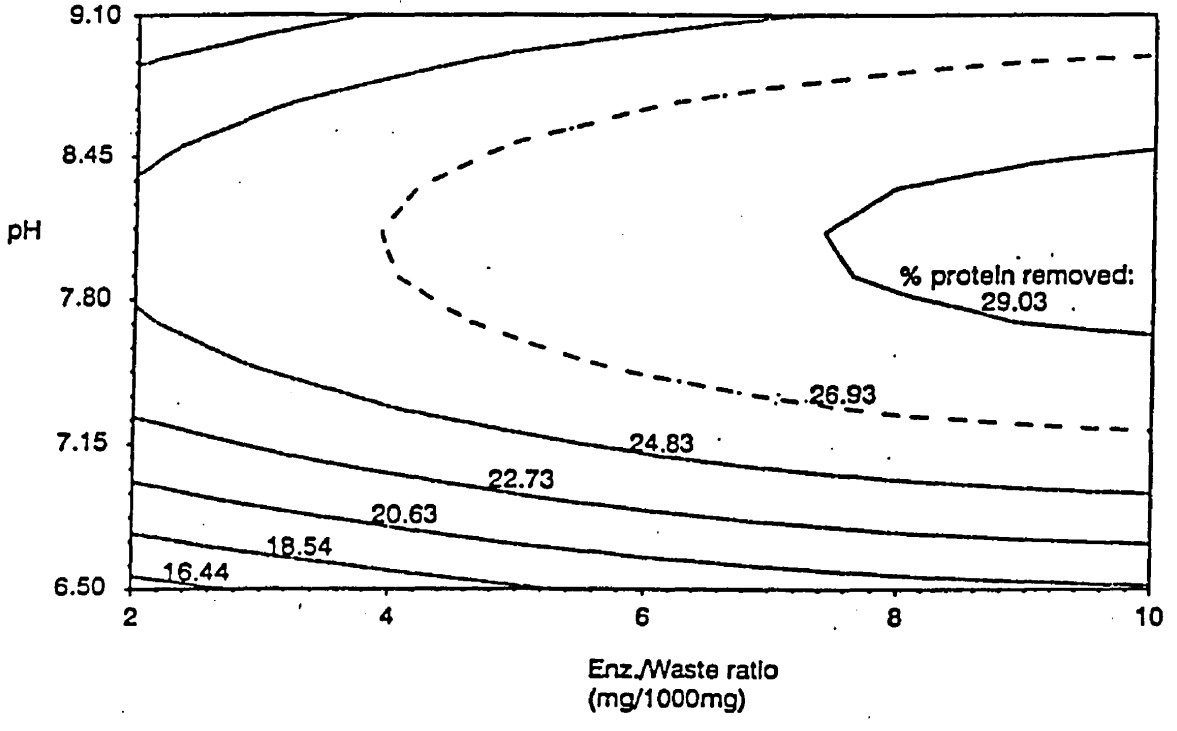

Fig. 4. Contour Plot of Protein Removal by Chymotrypsin at 39.7°C.

TABLE 4. Analysis of Variance of the Regression Parameters for the RSM Model for the Chymotrypsin Hydrolysis.

SOURCE	D.F.	S.S.	F-Ratio
Model	9	2645.476	49.809***
Linear	3	1957.758	110.6***
Quadratic	3	678.087	38.301***
Crossproduct	3	9.632	0.544
Residual			
Lack of fit	35	216.118	1.106
Pure error	30	167.471	
% variability explained (R^2)		0.8734	
Coefficient of variation		12.9651	

* Significant at the 5% level; ** Significant at the 1% level (< 0.01);
*** Significant at the 0.1% level (<0.001)

TABLE 5. Analysis of Variance of the Regression Parameters for the Response Surface Model for the Papain Hydrolysis.

SOURCE	D.F.	S.S.	F-Ratio
Model	9	3360.076	22.209***
Linear	3	3061.699	60.710***
Quadratic	3	209.466	4.153**
Crossproduct	3	88.910	1.763
Residual			
Lack of fit	35	378.816	0.549
Pure error	72	1419.909	
% variability explained (R^2)		0.6513	
Coefficient of variation		24.5252	

TABLE 6. Analysis of Variance of the Factors studied for the Chymotrypsin and Papain Regression Model.

FACTOR	D.F.	S.S.	F RATIO
a) **chymotrypsin regression model**			
Temperature (X1)	4	1896.72	80.35***
pH (X2)	4	1015.60	43.02***
E/W ratio (X3)	4	170.27	7.21***
b) **papain regression model**			
Temperature (X1)	4	354.22	5.27***
pH (X2)	4	2740.58	40.76***
E/W ratio (X3)	4	266.54	3.96**

A test on each factor (temperature, pH and E/W ratio) showed that they were all significant in the model (Table 6). For both enzymes, the influence of pH was more important statistically, as shown by the greater coefficient in the regression model.

The estimated maximum response for deproteinization by chymotrypsin is at a temperature near 39.7°C (Figs. 3 and 4), a pH of 8.0 and an E/W ratio of 7:1000 (w:w). On the contour plot (Fig. 4), it is clear that along the optimum pH of 8.0, the distance between the response level line increases closer to the optimum area. Thus, to achieve an increase of 2% in the yield of protein, for example, it is necessary to add from 4 to 8 mg of chymotrypsin per 1000 mg of waste, which may be undesirable in terms of cost/benefit implications with respect to the enzymes, if large scale production is considered.

For deproteinization by papain (Figs. 5 and 6), the maximum response was at a temperature of around 38°C, a pH of 8.7, and an E/W ratio of 10:1000 (w:w). From the three dimensional plot (Fig.5), it is obvious that pH had the most marked effect, while E/W ratio had a negligible effect at higher pH. The optimization may have been improved by choosing a higher range of pH, but papain requirements for cysteine do not allow higher alkaline pH ranges.

The responses obtained for chymotrypsin and papain hydrolyses were, respectively, 29 and 23% protein removed under the reaction conditions used. However the percentage of protein removed depends greatly on the starting material, so these values should be compared with caution. For example, the protein content of various batches of shrimp waste was found to range from 25 to 43%, depending on the starting material (i.e., how much exoskeleton material and/or meat was associated with the offal).

A more accurate picture of the completion of deproteinization is given by measuring the residual protein level on the chitin obtained at these optimum reaction conditions. Thus, for chymotrypsin, a run at 40°C, pH 8.0, and E/W ratio of 7:1000 (w:w) gave a yield of 46.4% protein. The percentage of protein remaining on the residue was 1.3%, showing the efficiency of the deproteinization at these conditions. The predicted yield of 29.1% protein calculated with the model was obtained at the time with another batch of waste, thus possibly explain i.e. the large difference. For papain, at 40°C, pH 8.6, and E/W ratio of

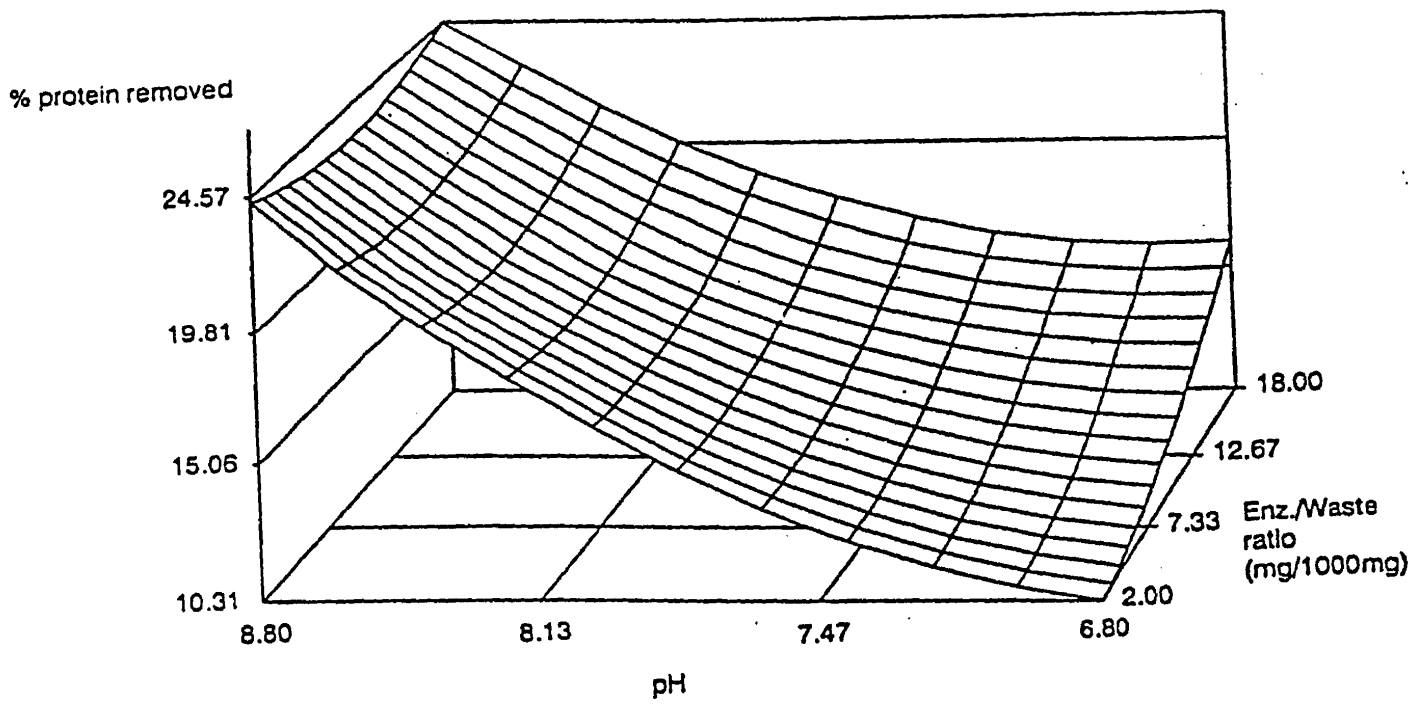

Fig. 5. Response Surface Graph of Protein Removal by Papain at 38.3°C.

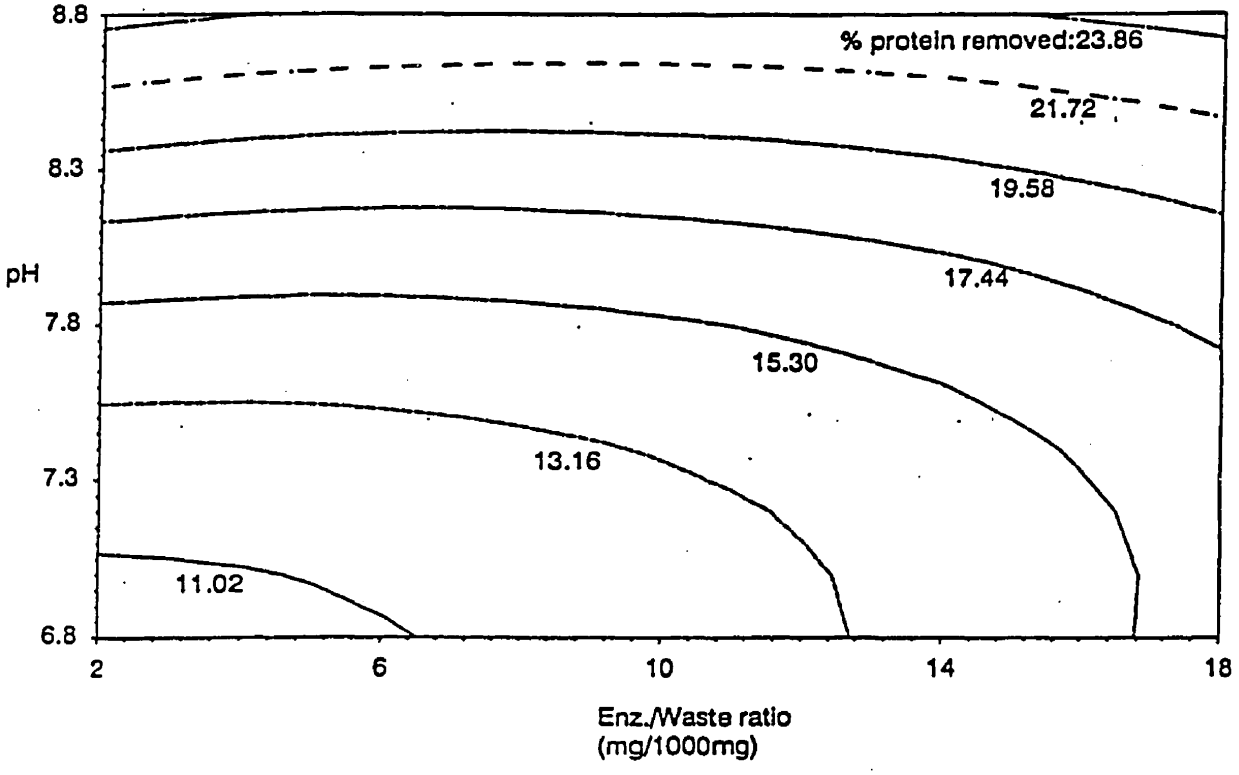

Fig. 6. Contour Plot of Protein Removal by Papain at 38.3°C.

10.7:1000 (w:w), the yield was 48.8%, with 2.8% protein remaining in the residue. Compared to a predicted yield of 22.1%, the effect of the starting material on the yield of protein is evident.

3.6. Conclusion

The use of proteolytic enzymes is a simple and inexpensive alternative to chemical methods currently employed in the preparation of chitin. It could be especially useful for the preservation of the natural state of this polymer. Also appealing is the fact that the deprotenization liquid is "ready-to-use" for protein recovery for purposes such as animal feed/supplement, and with a much lower level of minerals (ash). Both enzymes studied were effective in achieving optimum deproteinization of the waste material.

Chapter 4

Utilization of chitosan for the preservation of fresh shrimp (*Pandalus borealis*).

4.1. Summary

The capacity of chitosan dips (1 and 2% w/v) to preserve the premium quality of whole and headless fresh shrimps (*Pandalus borealis*) during iced storage was studied by monitoring chemical indicators such as pH, drip loss, total volatile bases, nucleotide degradation, and sensory indicators of appearance (including melanosis) and odor as well as total microbial counts. Chitosan dips significantly reduced the total volatile base levels at the end of the 20 days of observation, as well as the total microbial counts, in whole shrimps, attesting to the antimicrobial properties of chitosan. In headless shrimp, melanosis and nucleotide degradation were significantly reduced. However, the nucleotide degradation pattern was not influenced by the chitosan treatment for whole shrimp.

4.2. Introduction

Chitosan is the deacetylated form of chitin (fig. 7), a high molecular weight polysaccharide found in abundance in crustacean wastes. It is non-toxic, with a LD_{50} value of 18g/kg of body weight in mice (Arai et al, 1968). It is also odorless and tasteless, biodegradable, and shows antimicrobial properties against a large spectrum of microorganisms (Wang, 1992; Hirano and Nagao, 1989). In recent years, a great deal of applications has been found for this polymer, including its use for the preservation of fruits and vegetables, based on the ability of the polymer to form a thin layer / film around the product which then acts as a gas barrier, or a semi-permeable coating, as in modified atmosphere packaging (Lidster, 1987a, b; El Ghaouth et al, 1991a, b).

Fish and seafoods have gained popularity amongst Canadian consumers and their counterparts elsewhere in recent years (MAPAQ, 1991). However, these products are highly perishable in the fresh state, thus, in order to keep up with the increasing demand for premium quality fresh products (MAPAQ, 1992), more effective methods of preservation are needed to extend the shelf life of these products. Thus, the present study was aimed at evaluating the efficacy of chitosan dips in controlling post-harvest quality deterioration of these products and thereby extend their premium quality during chilled

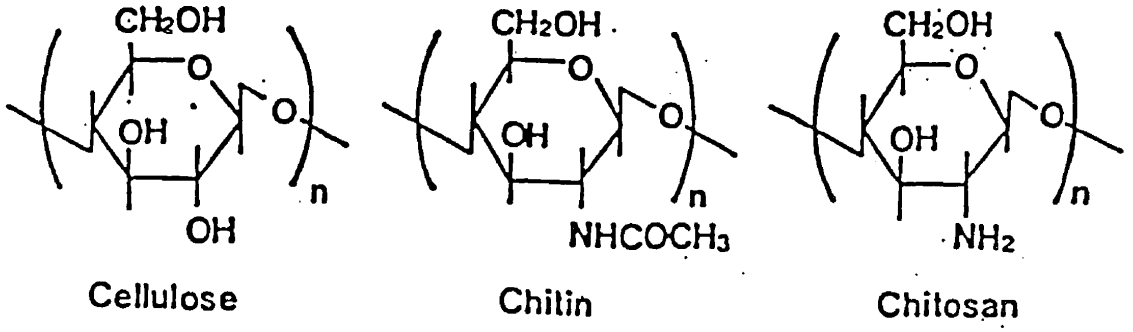

Fig. 7. Chemical structures of cellulose, chitin and chitosan.

storage.

4.3. Materials and Methods

4.3.1. Chitosan preparation

Chitin was obtained from crab wastes suppplied by the Fishery Products International, St-John's, NF, and deacetylated to obtain chitosan using a modification of the method of Mima et al, (1983) as summarized in Fig. 8.

4.3.2. Shrimp preparation

The fresh shrimp *Pandalus borealis* samples were shipped on ice from Les fruits de mer de l'est du Québec, Matane, PQ, and treated as soon as they were received in the laboratory from the processing plant. The treatment process involved washing with tap water and/or deheading (with the legs removed) prior to dipping in the chitosan solution, followed by draining, packing in sterile plastic bags and then storage at 4°C on ice.

The various dipping solutions tested comprised 2 mM NaH_2PO_4, 0.1% tween 80, and either 0, 1, or 2% chitosan. The pH of the dipping solutions was adjusted to 5.6 for all treatments. Sampling for chemical and sensory analysis was done every 4 days, over a 20 day period of storage.

4.3.3. Total microbial counts (TMC) and pH measurement

Samples of shrimp meat (5g) were homogenized in 50 mL of sterile saline (0.9% NaCl) using a Polytron (Brinkmann, model PT3000) at a low speed setting of 4000 rpm to avoid foaming. An inoculate of 0.1 mL was plated on Plate Count Agar (PCA) supplemented with 0.5% NaCl, after appropriate serial dilutions. The plates were duplicated and incubated at 20°C, and the number of colony forming units (CFU) was determined after sufficient growth. The homogenate used for TMC was also used to measure the pH at ~20°C.

4.3.4. Drip loss measurement

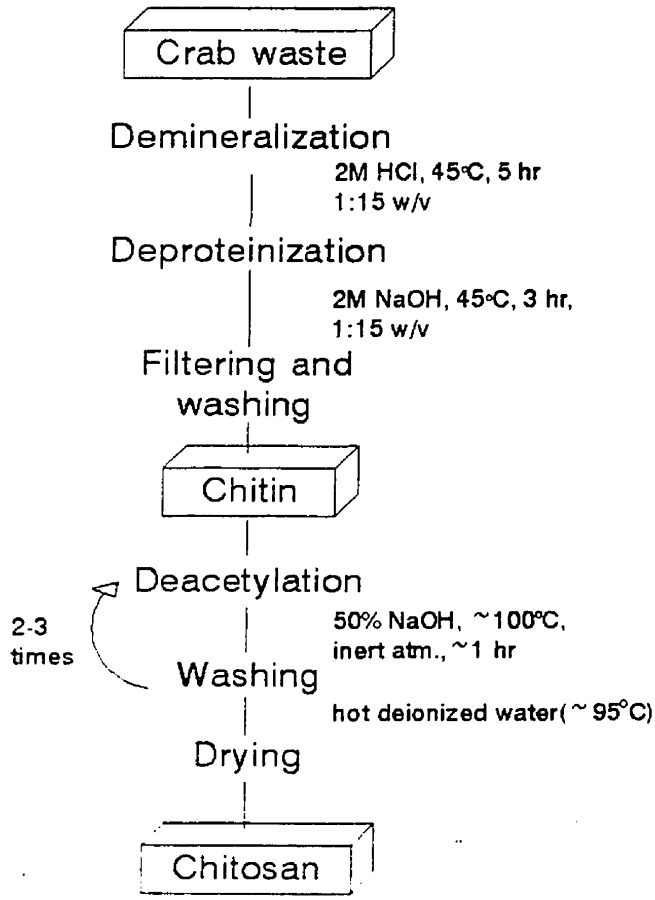

Fig. 8. Chitin and chitosan preparation steps.

Drip loss measurement was used as an indicator of the water holding capacity of the meat and was measured by centrifuging shrimp meat samples (1-3 g) wrapped with a filter paper at 31000 g and 4°C, for 20 min. The difference in the weight after centrifuging and drying is an indicator of the drip loss, and was expressed as the percentage of moisture retained by the meat after centrifugation over the total moisture of the meat.

4.3.5. Total volatile bases (TVB)

Volatile amine, nitrogen, and other non-protein nitrogenous compounds were measured using the microdiffusion method of Conway (1957) as modified by Cobb et al, (1973). This involved addition of trichloroacetic acid (TCA, 7% w/v) to a shrimp meat sample (1:2 w/v) and homogenizing with a Polytron at 10 000 rpm for 2 min. The homogenate was centrifuged at 31 000 g, at 4°C, for 30 min. One milliliter of Na_3PO_4 saturated in KOH was added to 1 mL of the supernatant in the outer well of a microdiffusion plate and the plate was immediately covered. The trapping agent was 4% boric acid with an indicator (Kjelsorb, Fisher Scientific). After complete release of the TVB (90 min), titration of the boric acid solution was done with 0.02M HCl. The results were expressed as mg N/ 100 g of meat.

4.3.6. Nucleotide analysis

Nucleotides were measured on the TCA extract used for TVB analysis after neutralization to a pH of 5.8 with 1M KOH and appropriate dilution. An HPLC system (Beckman, system Gold) equipped with a programmable solvent module (model 126), a diode array detector (model 166), a 20µL sample loop injector, and a RP-C18 150mm ultrasphere column was used to separate and quantitate the nucleotides. The running buffer comprised 4 mM KH_2PO_4 (pH 5.8) and 1% methanol (HPLC grade), at a flow rate of 1 mL/min., and dectection was done at 254 nm (Phenomenex, 1991). The results were expressed as µM/ g of meat.

4.3.7. Sensory evaluation

The shrimps were boiled for 5 min in water and allowed to cool before sensory evaluation. For melanosis evaluation, a 5 level scale was used, as follows:

5- no darkening discoloration;
4- a few black spots on the head, feet, and/or tail;
3- black spots on head, feet and tail;
2- many black spots on head, feet and tail; and
1- severe darkening discoloration on whole body.

For evaluating the appearance, 4 to 6 panelists were asked to classify the shrimps on a scale of 9 (like extremely) to 1 (dislike extremely), with a ranking of 5 representing satisfactory samples.

To evaluate odor / smell, 3 different scales were used, (1) hedonic scale (similar as appearance evaluation); (2) a 4 grades scale (fresh, good, poor, spoiled); and (3) a 3 level scale of classes, 1 referring to an acceptable product, 2 for products showing early signs of spoilage, and 3 for definitely spoiled shrimps. Two shrimps per treatment (total of 6 shrimps) were evaluated each time.

4.3.8. Statistical analysis

A complete randomized block design of the 3 treatments was used to perform two-way analysis of variance, with SAS software (SAS Institute Inc., 1989), the day of sampling being the blocking factor. Each 20 days experiment was repeated 3 times, for each type of shrimp.

4.4. Results and Discussion

The initial pH of the shrimps was determined as 7.2-7.6 and this increased gradually with storage time to 7.9-8.0, irrespective of the treatment and the type of shrimp used. Chung and Lain (1979) reported a pH of 7.8 to be a critical margin for acceptability. This value was obtained at day 14-16 for whole shrimp and at day 12-14 for headless shrimps. As will be shown later, this critical value does not relate well with chemical and sensory evaluation for whole shrimps and headless shrimps.

TMC were significantly reduced in whole shrimp samples by the chitosan treatment ($p<0.05$) (Fig. 9) but not significantly in headless shrimp samples. By the end of the 20 day storage period, the difference between the control and the 2% chitosan treated samples

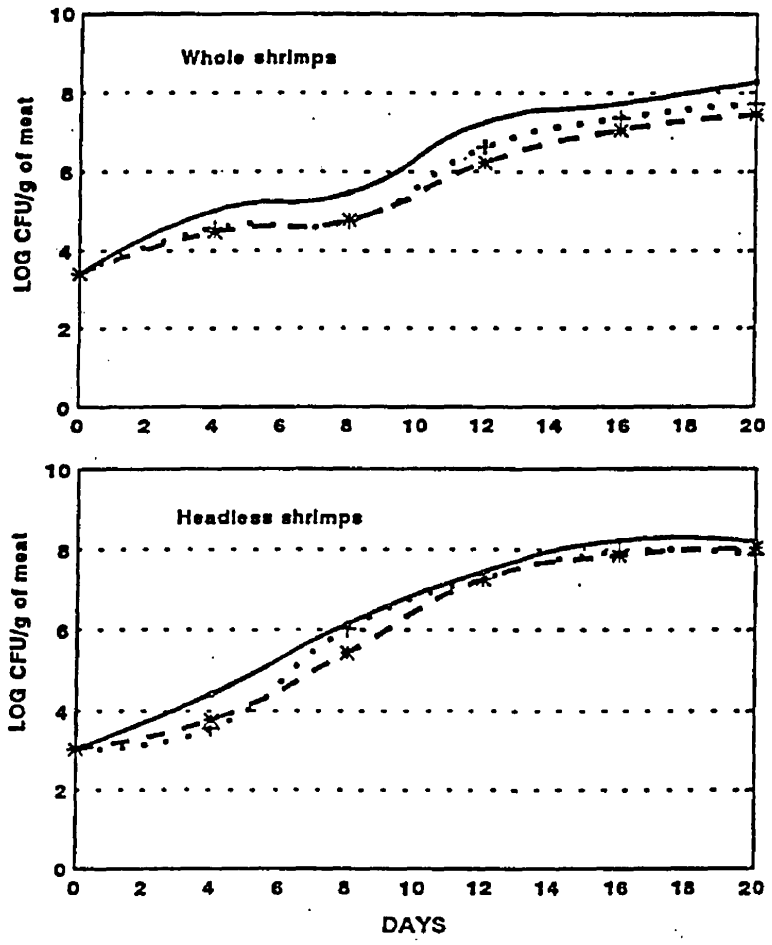

Fig. 9 TMC of whole and headless shrimps. Control ——; 1% chitosan ••••; 2% chitosan - - - -

was about 1 log cycle for whole shrimps. A log of 6 is used as a reference for food safety (Fieger and Novak, 1961) and for whole shrimps treated with 2% chitosan, this level was attained after 12 days, compared to 10 days for control samples. For headless shrimps, the trend was similar, however, microbial proliferation was more rapid. This is surprising and may be attributed to two factors: the shrimps were harvested in summer and early fall, which is a period more favorable for microbial contamination, since shrimps are kept on board for longer periods before landing and deheading, and also, the manipulations involved for deheading and removing the legs may have increased contamination through handling. In any case, the differences in the TMC levels for treated versus control samples indicate that chitosan exhibit antimicrobial activity toward microorganisms associated with fresh shrimp.

The data on drip loss presented in fig. 10, indicate that the water holding capacity of shrimp meat decreased with time throughout storage. In whole shrimps (fig. 10) there was a clear reduction in the water holding capacity of the shrimp meat. For headless shrimps, the water holding capacity remained constant over the 20 days of observation, presumably indicating that the meat maintained a good quality. For both types of shrimps, the chitosan treatments did not appear to affect the results.

Chitosan treatment significantly reduced ($p<0.05$) TVB formation in both whole and headless shrimp samples (fig. 11). The acceptable level of TVB for shrimps is given as $\leq$ 30mg N/100g of meat (Sikorski et al, 1990). This level was attained after 5 days in the control and the 1% chitosan treated samples, but not until 8 days for the 2% chitosan treated samples for whole shrimps (fig. 11). In the case of headless shrimps, a TVB value of 30mg N/100g of meat was attained after ~ 9 days for control and 1% samples, and after 10 days for 2% chitosan treated shrimps. TVB evolution was much slower in the headless shrimps after this period than for whole shrimps, and considering that TVB production is mainly due to specific microbial activity, it may be inferred that the microorganisms involved in the spoilage of the two forms of the shrimp samples were probably different, since we would have expected the TVB evolution of headless shrimps to be correlated with the microbial counts.

ATP degradation in seafood after death follows the following pathway: ATP → ADP → AMP → IMP → Ino → Hx (Sikorski et al, 1990).

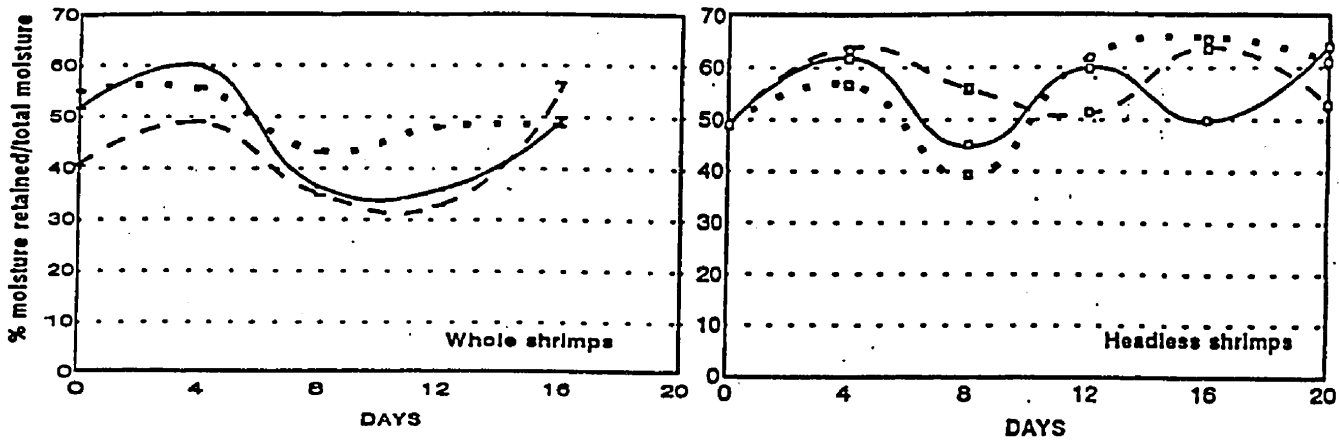

Fig. 10 Drip loss results for whole and headless shrimp. Control ——; 1% chitosan • • • • ;2% chitosan - - - -

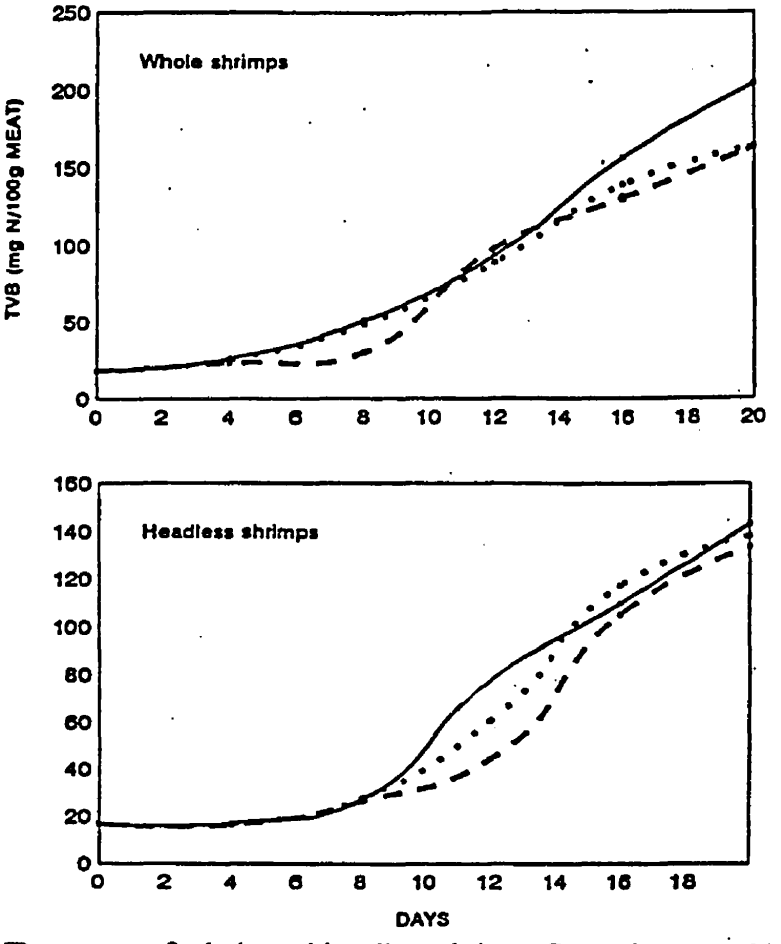

Fig. 11 TVB content of whole and headless shrimp. Control ——; 1% chitosan • • • • ;2% chitosan - - - -

The first steps of degradation occur rapidly, and the degradation of ATP to IMP is almost complete in the first two days after catch (Sikorski et al, 1990). Hx is suggested to impart an undesirable flavor while Ino and IMP contribute to the characteristic smell of fresh shrimps. The formation of Hx is used as an index of quality deterioration since it is very slowly decomposed to xanthine and uric acid. In a similar manner, Ino is slowly decomposed during degradation and can be used as an indicator of freshness.

Hx levels increased gradually (fig. 12) in a similar fashion for both types of shrimp, irrespective of the treatment, while inosine decreased to non-detectable levels after an initial build-up due to ATP, ADP and AMP depletion. The level of Hx proposed for shrimp rejection is 2 μM/g of meat, and this level was attained after 6 to 8 days for whole shrimps, and after 10 days for headless shrimps. In headless shrimp samples, nucleotide degradation in the control samples was faster during the first 10 days, which is shown by the more rapid and higher levels of inosine (significant at 0.001 level) due to the degradation of ATP, ADP, AMP and IMP.

Sensory evaluation showed that melanosis appears fast, especially in the head region (fig. 13). Whole shrimps received a score of 3 (between a few and many black spots on head, feet and tail) at ~ day 13, and chitosan treatment did not reduce melanosis. However, for headless shrimps, the 2% chitosan treated samples underwent significantly lesser degree of melanosis by the end of the 20 days of preservation ($p<0.05$), and were on average a little over the score of 4 at that time. The difference between the two types of shrimps may be related to the absence of heads and legs, which are both susceptible to faster and intense melanosis.

Melanosis affects the evaluation of general appearance, and the appearance evaluations shown in fig. 14 correlate with what was observed for melanosis. For headless shrimps, control samples were given a score of 5 (limit of acceptability) by the end of the 20 days of observation, while the 2% chitosan treated shrimps were ranked to be more acceptable, although the difference is not statistically significant. The data for whole shrimps reflect the rapid onset of melanosis and scores of 5 were assigned before 9 days of preservation.

Odor scores of whole shrimps (fig. 15) indicated that early signs of spoilage were detected after 8 days of storage in whole shrimps, and this correlated with both the TVB and the Hx rejection levels observed. For headless shrimps, odor scores were generally higher

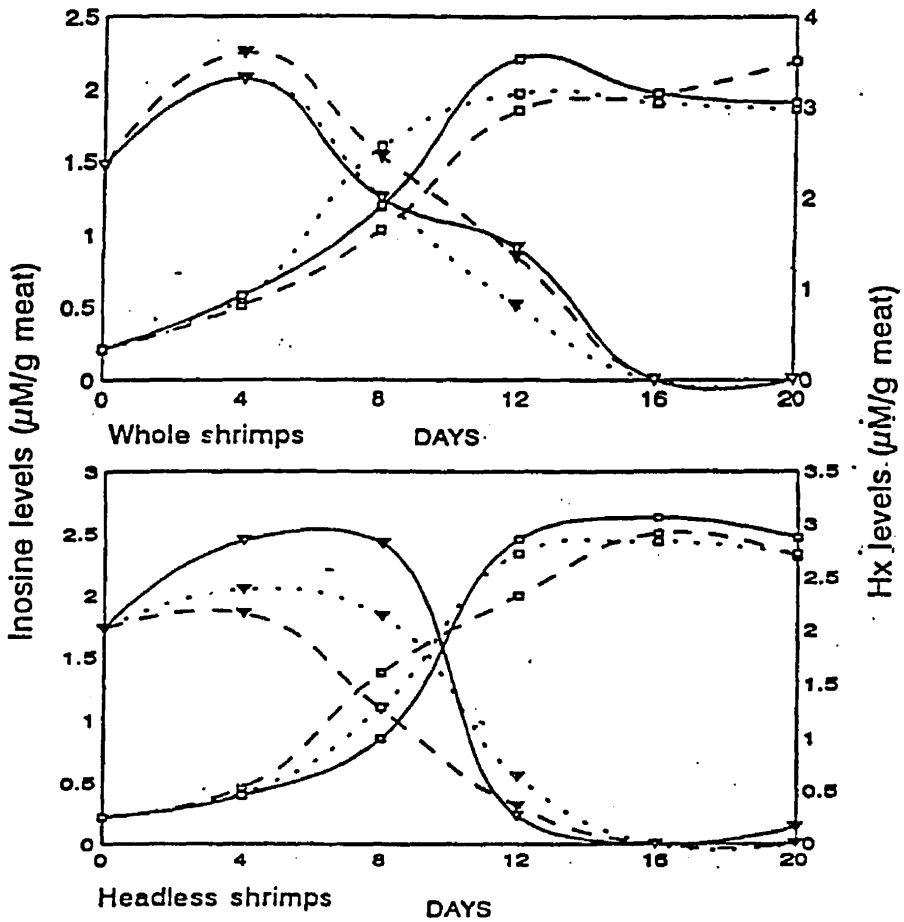

Fig. 12 Hx and Inosine evolution in whole and headless shrimp. Hx ■, Inosine ∇, control ——; 1% chitosan • • • • ; 2% chitosan - - - -

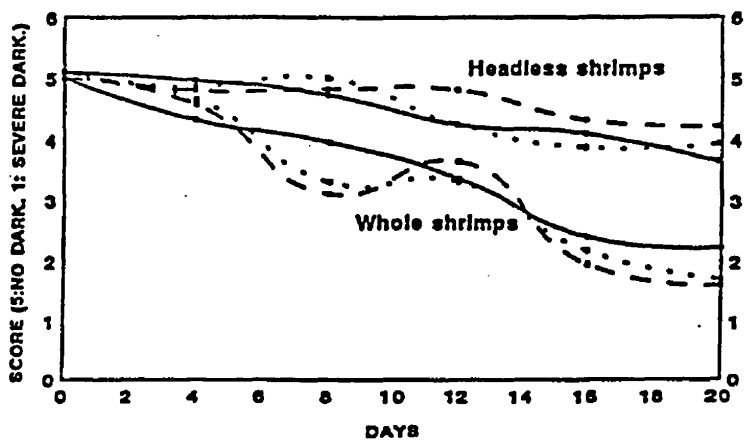

Fig 13. Melanosis evaluation of whole and headless shrimp. Control ——; 1% chitosan • • • • ; 2% chitosan - - - -

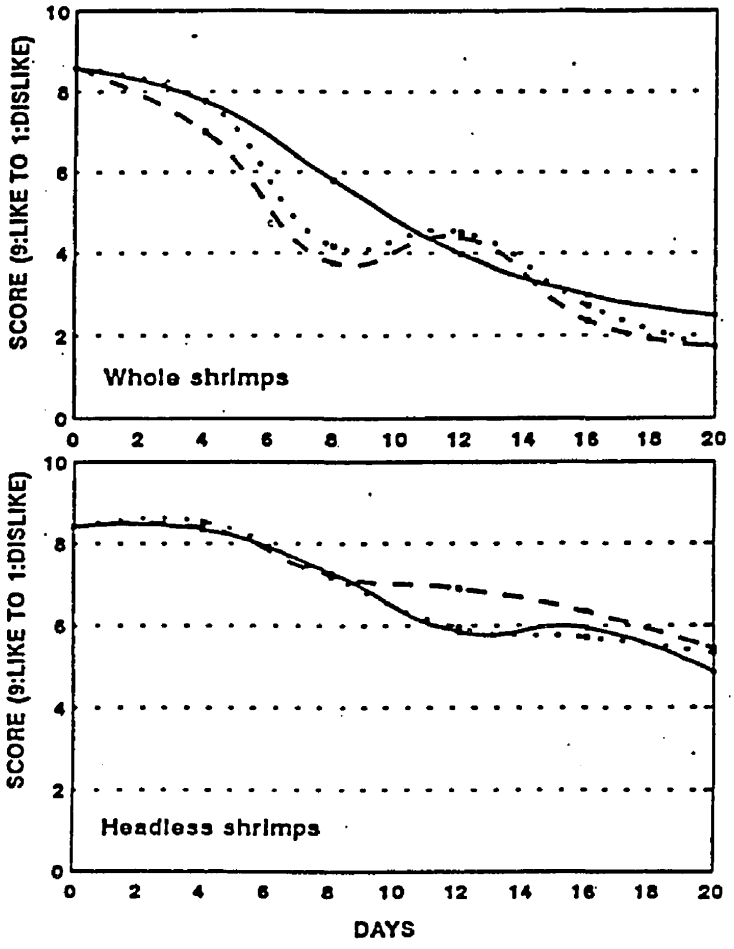

Fig. 14 Appearance evaluation (hedonic) of whole and headless shrimp. Control ——; 1% chitosan • • • • ; 2% chitosan - - - -

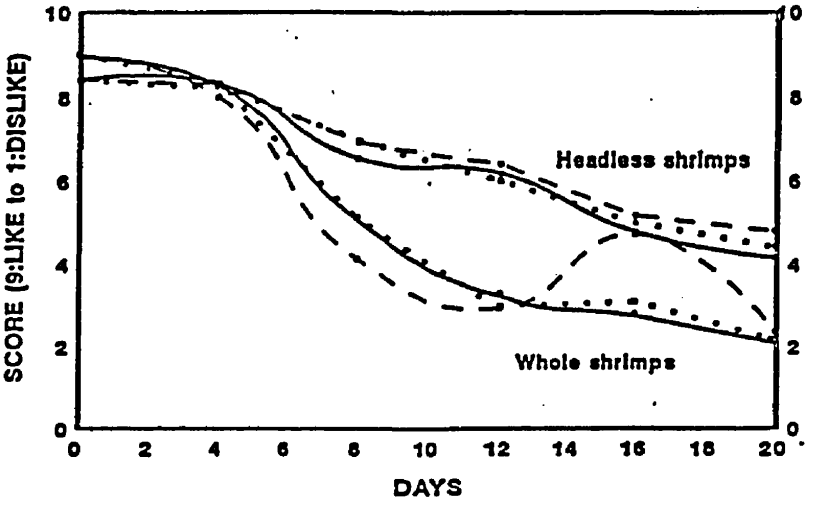

Fig. 15 Odor scores (hedonic) of whole and headless shrimp. Control ——; 1% chitosan • • • ; 2% chitosan - - - -

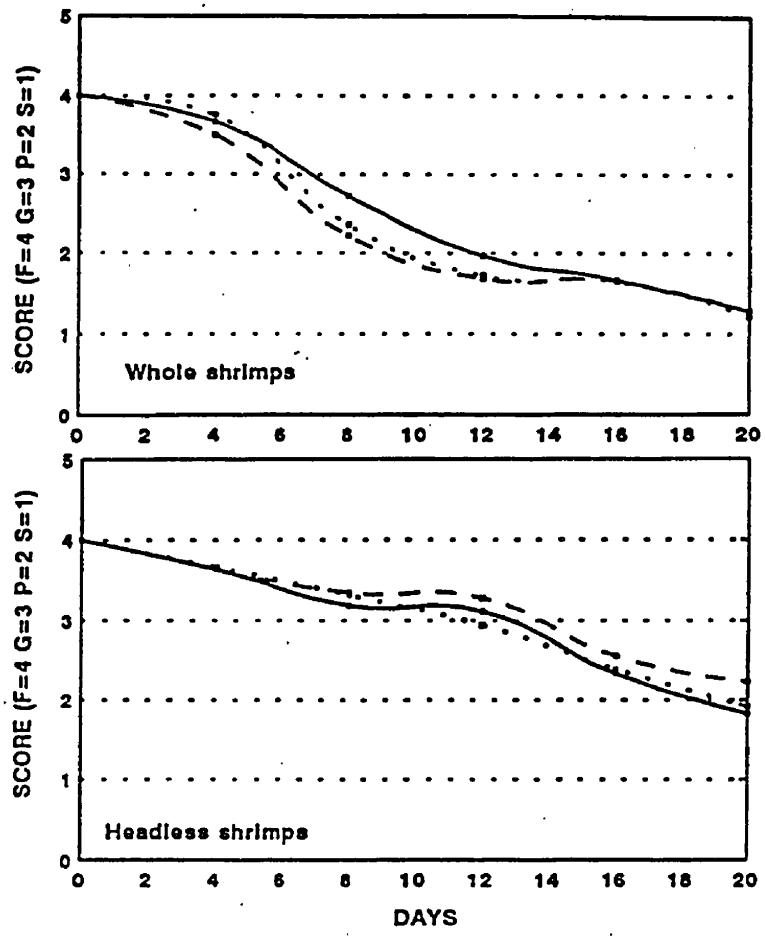

Fig. 16 Odor evaluation as fresh, good, poor or spoiled. Control ——; 1% chitosan ••••; 2% chitosan - - - -

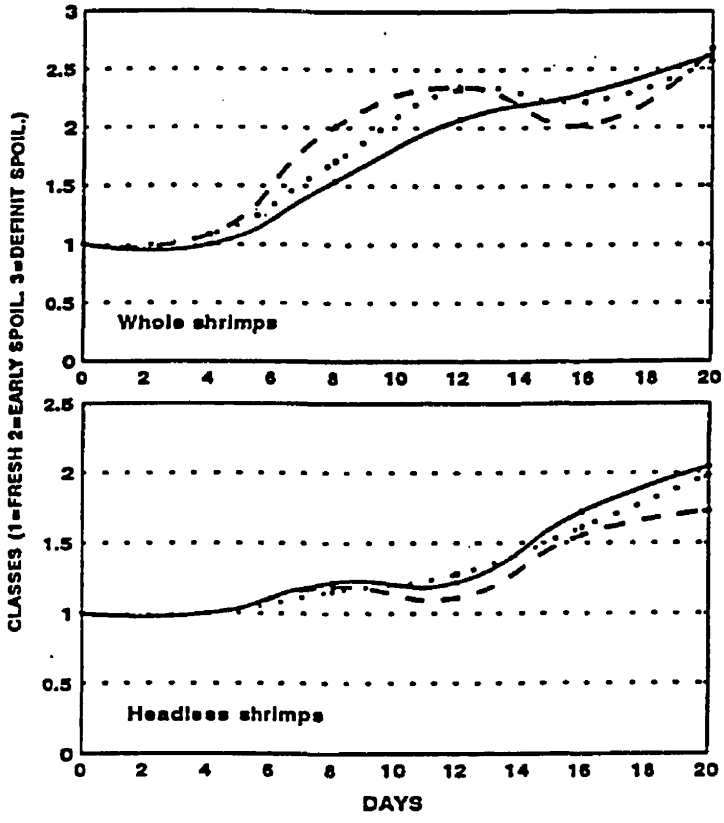

Fig. 17 Classification according to freshness of whole and headless shrimp. Control ——; 1% chitosan • • • • ; 2% chitosan - - - -

(better appreciation) and the 2% chitosan treated shrimps were still considered "satisfactory" (middle score of 5) at the end of the 20 days of observation, while 1% and control samples were slightly disliked. Judging by the theoretical limits of rejection for TVB and Hx, these were reached by day 10, when headless shrimps were still acceptable to the panelists. The difference observed for the various treatments were not statistically significant for both types of shrimp.

When panelists were asked to classify the shrimp samples as fresh, good, poor or spoiled (fig. 16), whole shrimps were ranked between good and poor (2.5 on the scale) at ~8-10 days, which again correlated the rejection limits shown by chemical analysis discussed previously. Headless shrimps reached that state at ~ day 14-16. The 2% chitosan treated shrimp samples were generally judged best of the three treatments, but the differences were not statistically significant.

Similarly, when asked to classify the shrimp in 3 simple classes (as acceptable, early spoiled, or definitely spoiled), whole shrimp showed early signs of spoilage as early as at day 8, while it took 19 and 20 days for control and 1% chitosan treated headless shrimps to reach that state (fig. 17). Headless shrimps dipped in 2% chitosan were still considered class 1 (going toward class 2) at the end of the 20 days of observation. However, the differences between chitosan and control samples were not statistically significant for both types of shrimp samples.

4.5. Conclusion

From these results, it is evident that the chitosan exerted mainly an antimicrobial effect during preservation of shrimp. This is reflected in the evolution of volatile bases which is slower in the presence of chitosan, since they are mainly evolved from the outer portion of the meat, due to the action of specific microorganisms. The indicators which reflect deterioration due to biochemical or enzymatic activity, such as nucleotide degradation, were not affected by the chitosan treatment for whole shrimps, but chitosan had a positive effect in reducing the enzymatic degradation and the melanosis of headless shrimps. The reduction of melanosis was probably more due to exclusion of O_2 by the chitosan film, than to inhibition of the polyphenol oxidase enzymes responsible for the discoloration. Sensory evaluations were more favorable for headless shrimps, probably because the head region is the first part affected by melanosis, and contains most of the microorganisms and

softer tissues more susceptible to microbial degradation.

Chapter 5

Antimicrobial activity of chitosan toward selected microorganisms.

5.1. Summary

The antimicrobial properties of chitosan were evaluated using several microorganisms implicated in food spoilage and/or food poisoning outbreaks, especially those associated with fish and seafood products. Chitosan prepared from crab offal was compared with commercially available chitosan from Sigma (St-Louis, MO). Concentrations of chitosan tested were 0.005, 0.0075, 0.01, 0.02, 0.05 and 0.1%, at a pH of 5.6. The laboratory prepared chitosan was more efficient than its commercial counterpart in inactivating the various microorganisms tested, with the exception of *S. aureus*. Chitosan increased the lag phase of growth, and reduced the cell numbers in the culture media. The efficiency of chitosan in inhibiting microbial proliferation was found to be comparable to many commonly used chemical preservatives.

5.2. Introduction

The preservation of food by physical and chemical methods is an important area to the food industry. Food preservatives have to meet several regulatory standards, e.g., they must be (i) efficient over a broad range of spoilage organism; (ii) tasteless and odorless, and (iii) non toxic, safe and inexpensive. When physical means of preservation are not available or desirable, chemical preservative(s) must be used, and the choice of one or more preservative is primarily based on the chemical composition of the food, its pH, etc. No preservative is universal, and the list of GRAS (generally recognized as safe) preservatives is short (VanDemark and Batzing, 1987). There is also a growing demand from the public for natural preservatives, and for these reasons, alternative sources of safe, effective, and acceptable preservatives need to be developed.

Chitosan is the deacetylated form of the natural polymer chitin, a polysaccharide composed of N-acetyl D-glucosamine and D-glucosamine units, and extracted mainly from crustacean waste by demineralization and deproteinization. Over 60% deacetylation of chitin yields chitosan (Tsugita, 1990). Recently, research on chitosan and its properties have shown its potential for use as a food preservative. Chang et al, (1989) achieved a 10 day shelf life extension of mulkimchi (pickle type kimchi, i.e., chinese cabbage) at 5^{o}C by

incorporating 0.2% chitosan compared to control samples.

Chitosan has also been tested as a dipping agent for "coating" fruits and vegetables (Lidster, 1987a,b). El Ghaouth et al (1991a) used chitosan dips to preserve strawberries and achieved a 60% reduction of infected berries after 30 days of storage at 13°C, compared to the control samples. In our laboratory, we tested the effect of chitosan solutions (1 or 2%) on whole fresh shrimps (*Pandalus borealis*) and found a reduction of 1 log in total microbial counts, after 20 days of iced storage (unpublished results). Based on the foregoing, the present study was aimed at investigating the effect of chitosan on selected microorganisms implicated in food spoilage and food poisoning, particularly fish and seafoods.

5.3. Material and methods

5.3.1. Organisms

Bacillus cereus, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhymurium*, and *Proteus vulgaris* were provided by the Department of Microbiology at McGill University. *B. coagulans*, *B. brevis*, *P. morganii (Morganella morganii)*, *Pseudomonas fluorescens* and *Pseudomonas putida* were obtained from the Bureau of Microbial Hazard (Health protection branch, Ministry of Health and Welfare Canada, Ottawa).

5.3.2. Chitosan

Chitosan was obtained from crab residue (ground crab waste from which most carotenoproteins had been extracted as per the method of Simpson and Haard (1985)). The crab residue was further deproteinized with 2M NaOH (1:15 w/v, 45°C) for 3 hr. After filtration and washing with deionized water, the residue was demineralized with 2M hydrochloric acid (1:15 w/v, 45°C) for 5 hr, and the crude chitin obtained was filtered, washed and dried under a fume hood. Chitosan was prepared from the crude chitin following the method of Mima et al, (1983) by boiling the chitin with 50% NaOH (1:15 w/v) under nitrogen for 1 hr. The residue was washed with hot deionized water and the boiling and rinsing steps were repeated once or twice to deacetylate chitin until a product soluble in dilute acetic acid solution was obtained. The soluble product thus obtained was the chitosan. Commercial chitosan (Sigma Chemical Co.) was used without further purification.

5.3.3. Degree of acetylation

The degree of acetylation of chitosan was measured according to the method of Aiba (1986), at 220 nm, using an average molar absorption coefficient of 175 for the N-acetyl glucosamine residue. Chitosan solution of 0.1% (w:v) in 0.1M acetic acid were used, and corrections were made for the absorbancy of the acetic acid solution.

5.3.3. Culture Media

The culture media were formulated in order to minimize co-precipitation of chitosan with reactants. After trials, it was found that positively charged amino acids such as arginine precipitate with chitosan. Thus, the culture media formula comprised (per litre) 5 g NaCl, 3 g yeast extract*, 2 g glucose, 5 g casamino acids*, and 10 g Bacto-peptone*, in order to minimize precipitation/cloudiness and to provide sufficient nutritional support for microorganisms.

The various culture media were prepared by mixing equal parts of chitosan solution (in acetic acid) with culture medium solution. Both solutions were adjusted at a pH of 5.6 before mixing. The final concentrations of chitosan used were 0, 0.005, 0.0075, 0.01, 0.02, 0.05, and 0.1% (w/v).

5.3.5. Growth Measurement

Fresh cultures of microorganisms in tryptic soy broth (cells/mL) were inoculated (0.1mL/50mL broth) in the culture media maintained at 37°C, and growth was monitored by turbidimetry. The absorbancies at 540 and 650 nm were measured with a diode array spectrophotometer (DU-7500, Beckman Instruments, USA) at various time intervals over a period of 150 hr. The average absorbancy was corrected by subtracting the absorbancy of the culture medium before inoculation. Samples of the cultures were inoculated at times on plate count agar (PCA) to confirm absorbance readings.

Experiments were done in duplicate (or triplicate) with the laboratory prepared chitosan and once (or duplicated) with Sigma chitosan. When more than one specie of a genus was used, only one specie was replicated.

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5.4. Results and discussion

Bacteria's architecture and resistance to antimicrobial agents vary considerably with strain, age, and nutrition, this difference being most pronounced between Gram-positive and Gram-negative organisms (Popper and Knorr, 1990).

Bacillus species are widespread in foods, and can be found on freshly harvested shrimp (Miget, 1991). They are occasionally involved in food poisoning due to the production of enterotoxins (Freeman, 1985). Among the Gram-positive organisms tested, *B. cereus* showed a great susceptibility to chitosan (Table 7). At 0.02% chitosan or less, no growth was observed in all replicates, for both types of chitosan. The lag phase of growth was increased in the presence of chitosan (Fig. 18). Differences among replicates (Table 7) show the importance of the age of the strain, since experiments were conducted for up to 6 months. Chitosan was bactericidal for most of the cases where no growth was observed, as plate counts showed no colony growth. *B. brevis* and *B. coagulans* were even more sensitive to chitosan, with growth observed only for *B. coagulans* in 0.005% chitosan culture medium.

Chang et al, (1989) observed that chitosan at a concentration as low as 0.005% was capable of inhibiting *B. cereus* growth. These authors, however, used a low molecular weight chitosan (M.W. 35 000), which could explain the differences between the studies, as discussed later. As well, the pH at which their experiment was conducted was not specified.

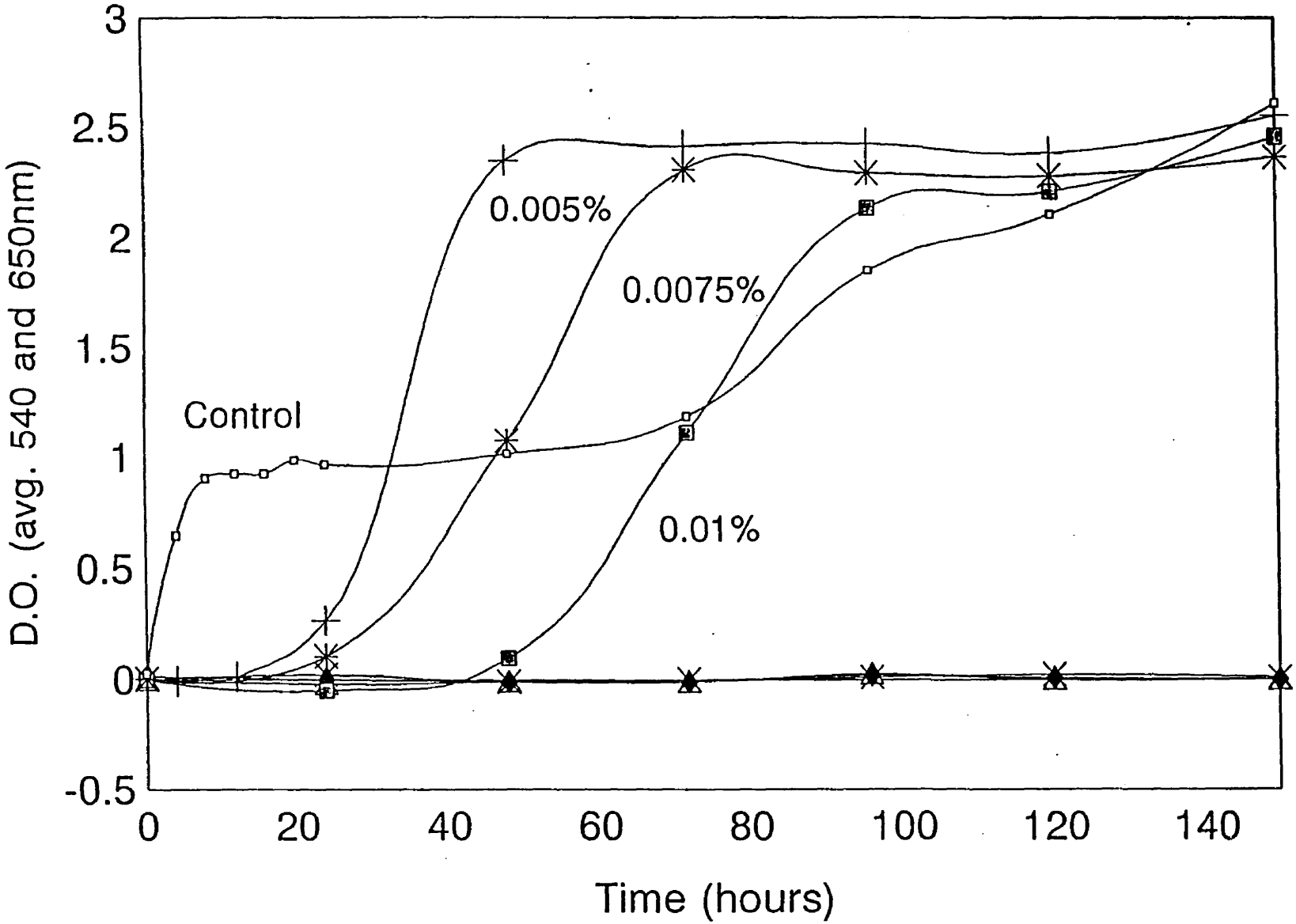
Staphylococcal food poisoning is a major public health concern. When environmental conditions are favorable, *S. aureus* will grow and produce thermostable harmful enterotoxins (Kraft, 1992). *S. aureus* was also found to be sensitive to low chitosan concentrations, and no growth was observed at 0.05% chitosan or less (Table 7). Sigma chitosan appeared to be more effective against *S. aureus* than the laboratory fabricated chitosan. The lag phase was also increased in the presence of both type of chitosan (Fig. 19). Wang (1992) found that 0.5% chitosan was inhibitory at pH's of 5.5 and 6.5, although the bactericidal effect was slower at the latter pH. It is not clear whether the latter researcher investigated the effects of lower concentrations. Chang et al, (1989) found a concentration of 0.005% to be inhibitory, however, they stopped their experiment

TABLE 7. Chitosan's inhibitory levels at pH 5.6

SPECIE	REPLICATE	INOCULUM (cells)	% CHITOSAN[a]
B. cereus	1	2.4×10^7	1×10^{-2}
	2	5.7×10^7	2×10^{-2}
	3	9.8×10^2	5×10^{-3}
idem, Sigma[b]	1	1.3×10^8	1×10^{-2}
B. coagulans	1	3.1×10^6	7.5×10^{-3}
B. brevis	1	1.3×10^7	5×10^{-3}
S. aureus	1	9.4×10^5	5×10^{-2}
	2	6.0×10^7	2×10^{-2}
idem, Sigma[b]	1	1.8×10^8	7.5×10^{-3}
	2	4.7×10^7	7.5×10^{-3}
E. coli	1	7.3×10^7	7.5×10^{-3}
	2	4.0×10^7	7.5×10^{-3}
idem, Sigma[b]	1	1.1×10^8	2×10^{-2}
S. typhimurium	1	1.1×10^7	5×10^{-3}
	2	7.0×10^7	1×10^{-2}
idem, Sigma[b]	1	9.7×10^7	2×10^{-2}[c]
P. fluorescens	1	1.0×10^6	2×10^{-2}, 1×10^{-1}[c]
	2	1.9×10^8	1×10^{-1}
	3	7.3×10^7	1×10^{-2}
idem, Sigma[b]	1	1.4×10^8	2×10^{-2}
	2	1.0×10^8	1×10^{-2}
P. putida	1	8.8×10^6	7.5×10^{-3}
Proteus vulgaris	1	8.6×10^7	5×10^{-3}
	2	6.2×10^7	1×10^{-2}
idem, Sigma[b]	1	1.4×10^8	5×10^{-2}
	2	6.2×10^7	1×10^{-2}
Proteus morganii	1	1.7×10^9	1×10^{-2}

[a] Chitosan concentration where no increase in absorbancy was observed over a 150 hr period of incubation.
[b] With Sigma chitosan.
[c] See discussion for explanations on this result.

Fig. 18 Inhibition of *B. cereus* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◇ 0.05%; △ 0.1%).


3
2.5
2
1.5
1
0.5
0
-0.5
D.O. (avg. 540 and 650nm)
0.005%
0.0075%
Control
0.01%
0
20
40
60
80
100
120
140
Time (hours)

after 60 hr which is shorter than the lag phase observed at 0.0075% and 0.01% chitosan in the present study (Fig. 19). Hitherto, Chang et al, (1989) used a low molecular weight chitosan in their study.

Some strains of *E. coli* are pathogenic (i.e., enteropathogenic or enterotoxigenic), to humans and animals, and they may produce foodborne disease (Kraft, 1992). *E. coli* was more susceptible to the laboratory fabricated chitosan than its Sigma counterpart, as shown in Table 7. Plate counts at 47 hr showed no colony growth or less than 3×10^2 CFU/mL for 0.0075% chitosan culture media and higher concentrations (Fig. 20). However, at 150 hr, plate counts of 0.0075% and 0.1% culture media were up to 1×10^6 and 4.4×10^4 cells/mL, respectively, in one of the replicates. This may indicate a lag phase prolonged over the 150 hr period of observation.

Wang (1992) found that *E. coli* was inhibited by 0.5% chitosan (the lowest concentration used) after 2 days of culture, at pH 5.5, but observed growth at concentrations up to 2.5% (their highest concentration used) at pH 6.5. Chang et al, (1989) studied the effect of the molecular weight on chitosan inhibition of *E. coli*, and found that 0.2% chitosan (M.W. 2 800 000) was necessary to inhibit *E. coli* growth while 0.01% was sufficient using chitosan of average molecular weight of 35 000.

S. typhimurium serotypes produce an endotoxin which can cause foodborne diseases (Kraft, 1992). *S. typhimurium* was inhibited by lower concentrations of laboratory fabricated chitosan, i.e., 0.01% or less, compared to Sigma chitosan (Table 7). With Sigma chitosan, growth was observed in all the media except the one with 0.02% chitosan, but plate counts of 0.02%, 0.05% and 0.1% were negative at the end of the 150 hr period of observation, showing that death had occurred after an initial growth phase at these concentrations. With the laboratory fabricated chitosan, there was early growth development on plated cultures in all culture media, but plate counts at 150 hr were all negative for 0.01% or more chitosan (Fig. 21), which again may indicate that chitosan had a delayed bactericidal effect on *S. typhimurium*.

Chang et al, (1989) needed 0.2% chitosan to inhibit *S. typhimurium*, with their low molecular weight chitosan. This is not consistent with our findings, and in contradiction with the trend observed with their low molecular weight product.

Pseudomonads are a group of bacteria producing extracellular proteinases and are frequently involved in the spoilage of meat, poultry and fish products (Kraft, 1992).

Growth of Pseudomonads was observed at all concentrations except at 0.1% chitosan in the two first replicates for *P. fluorescens* (Fig. 22). After 6 months, the same strain was used again and this time, no growth occurred for chitosan concentrations higher than 0.0075%. Experiments with Sigma chitosan were performed also with the aging strain and as well, growth was not observed when chitosan concentration was over 0.0075%. *P. putida* was also inhibited with 0.0075% or more chitosan.

Proteus spp. is another group of organisms occasionally involved in spoilage of various food products, including fish and seafoods (Miget, 1991). *P. vulgaris* and *P. morganii* (Fig. 23) exhibited high susceptibility to the laboratory fabricated chitosan and at levels $\geq$0.0075% chitosan, no growth was observed. With Sigma chitosan, *P. vulgaris* grew at concentrations up to 0.02% chitosan, but growth was delayed in time and reduced in quantity.

As a comparison, inhibitory levels of commonly used chemical preservatives are shown in Table 8. For many of these chemicals, the spectrum of action is limited to yeast and molds, and to the pH of the medium. It is obvious from Table 8 that chitosan was effective at comparative levels of the chemical preservatives.

Polycations such as chitosan are known to exhibit antimicrobial properties (Popper and Knorr, 1990). On the surface of microorganisms, the polycations produce a destabilizing effect on the outer cell wall structure. This indicates a strong interaction of chitosan with the lipopolysaccharide layer of Gram-negative bacteria as well as with the peptidoglycan of Gram-positive species (Popper and Knorr, 1990). It is noteworthy that chitosan can agglutinate a number of bacteria, as well as mammalian cells (Muzzarelli 1977). It was noted earlier that Chang et al, (1989) found a greater inhibitory effect with a lower molecular weight chitosan. However, Stossel and Leuba (1984), showed the reverse situation with *Fusarium oxysporum*. The degree of acetylation is probably important for the antimicrobial activity of chitosan, as it determines the global charge of the polycation. It has been shown that chitosan has no antibacterial activity at pH 7.0, where it is no longer significantly charged (Sudarshan et al, 1992). The degree of acetylation of the laboratory prepared chitosan was 7.5% compared to 15.5% for Sigma chitosan. Since there are more reactive groups on the former product, this could explain the higher

Fig. 19 Inhibition of *S. aureus* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◊ 0.05%; △ 0.1%).

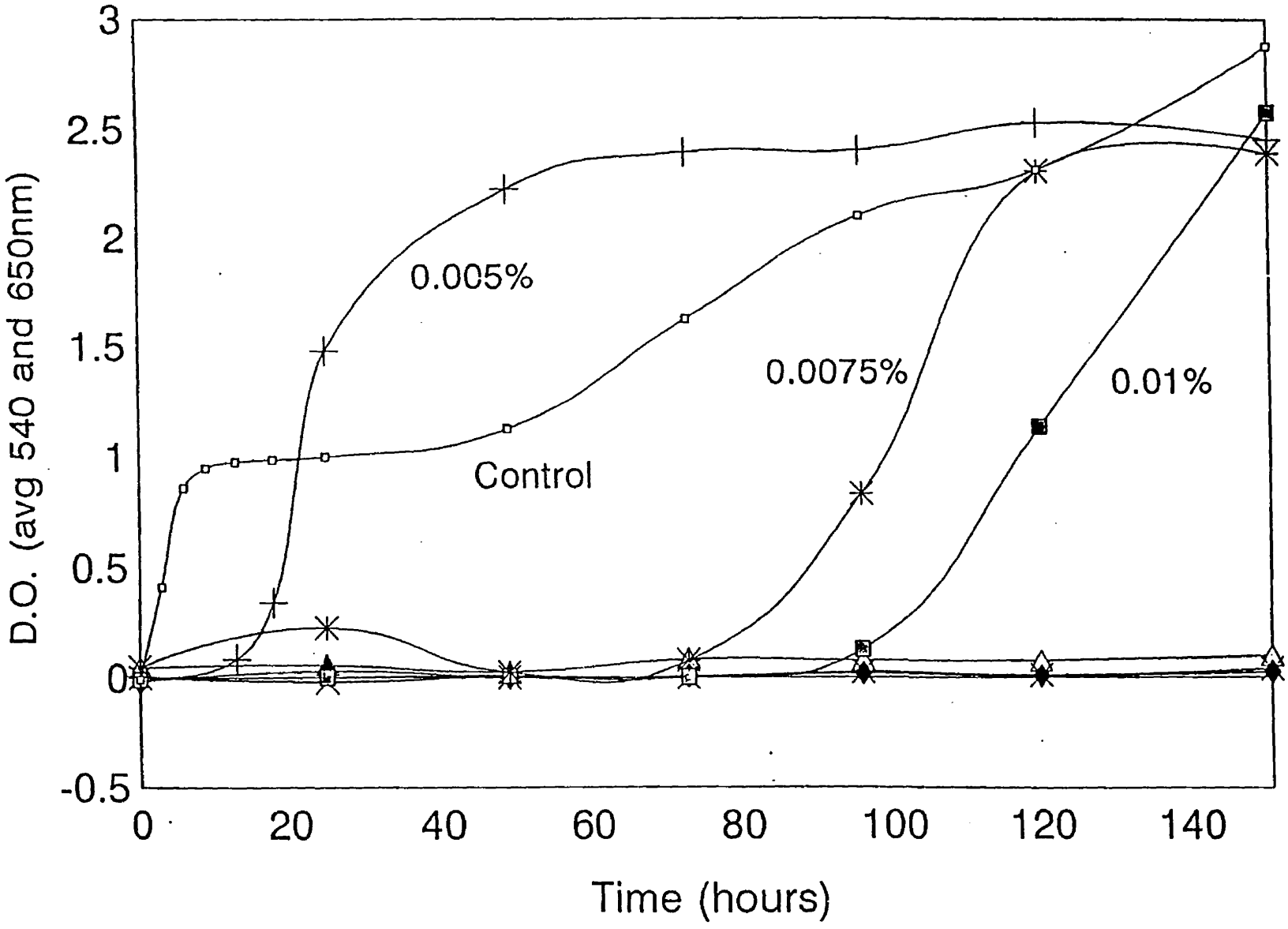

D.O. (avg 540 and 650nm)
3
2.5
2
1.5
1
0.5
0
-0.5
0
20
40
60
80
100
120
140
Time (hours)
0.005%
0.0075%
0.01%
Control

Fig. 20 Inhibition of *E. coli* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◊ 0.05%; △ 0.1%).

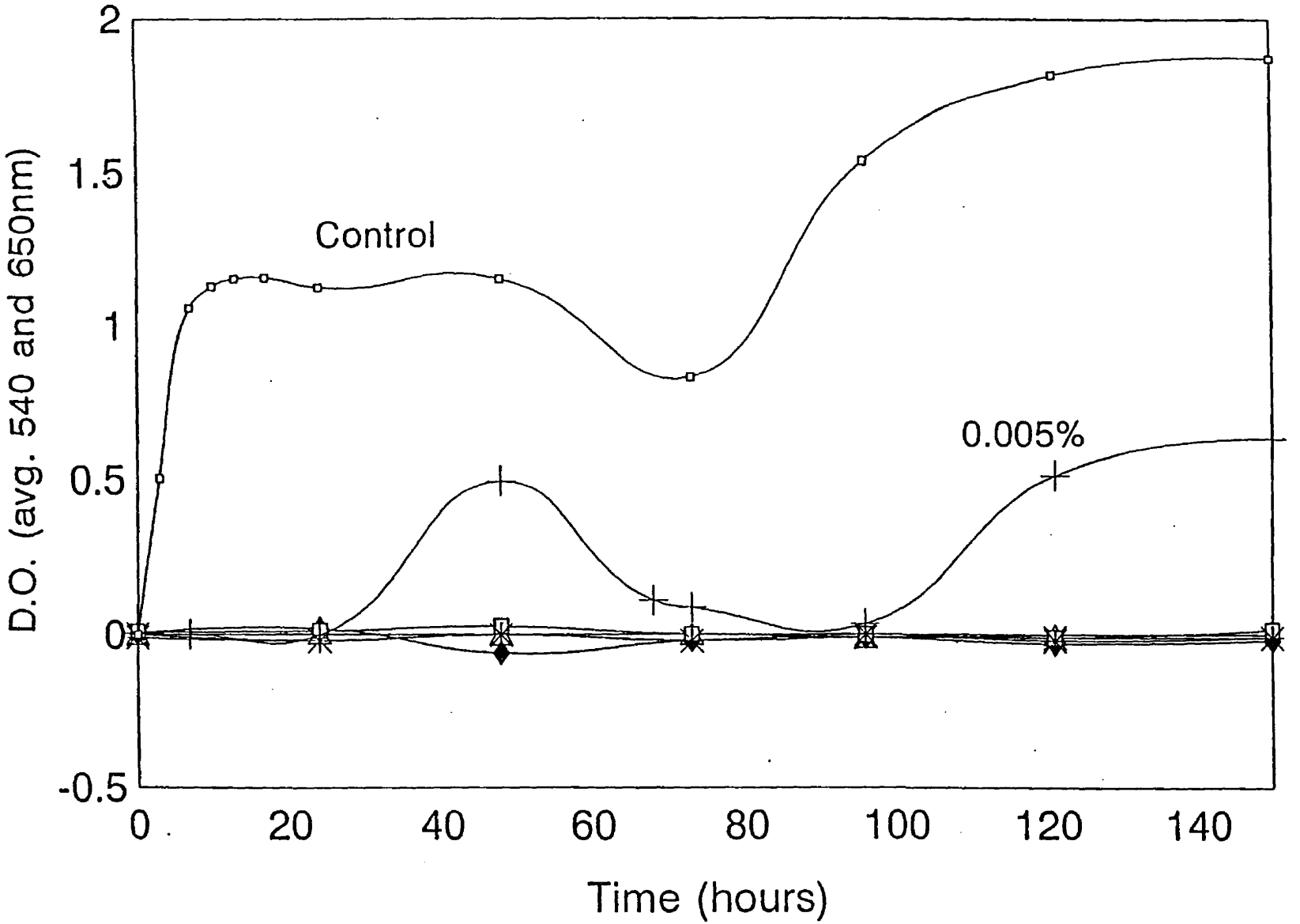

2
1.5
1
0.5
0
-0.5
D.O. (avg. 540 and 650nm)
Control
0.005%
0
20
40
60
80
100
120
140
Time (hours)

Fig. 21 Inhibition of *S. typhimurium* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◊ 0.05%; △ 0.1%).

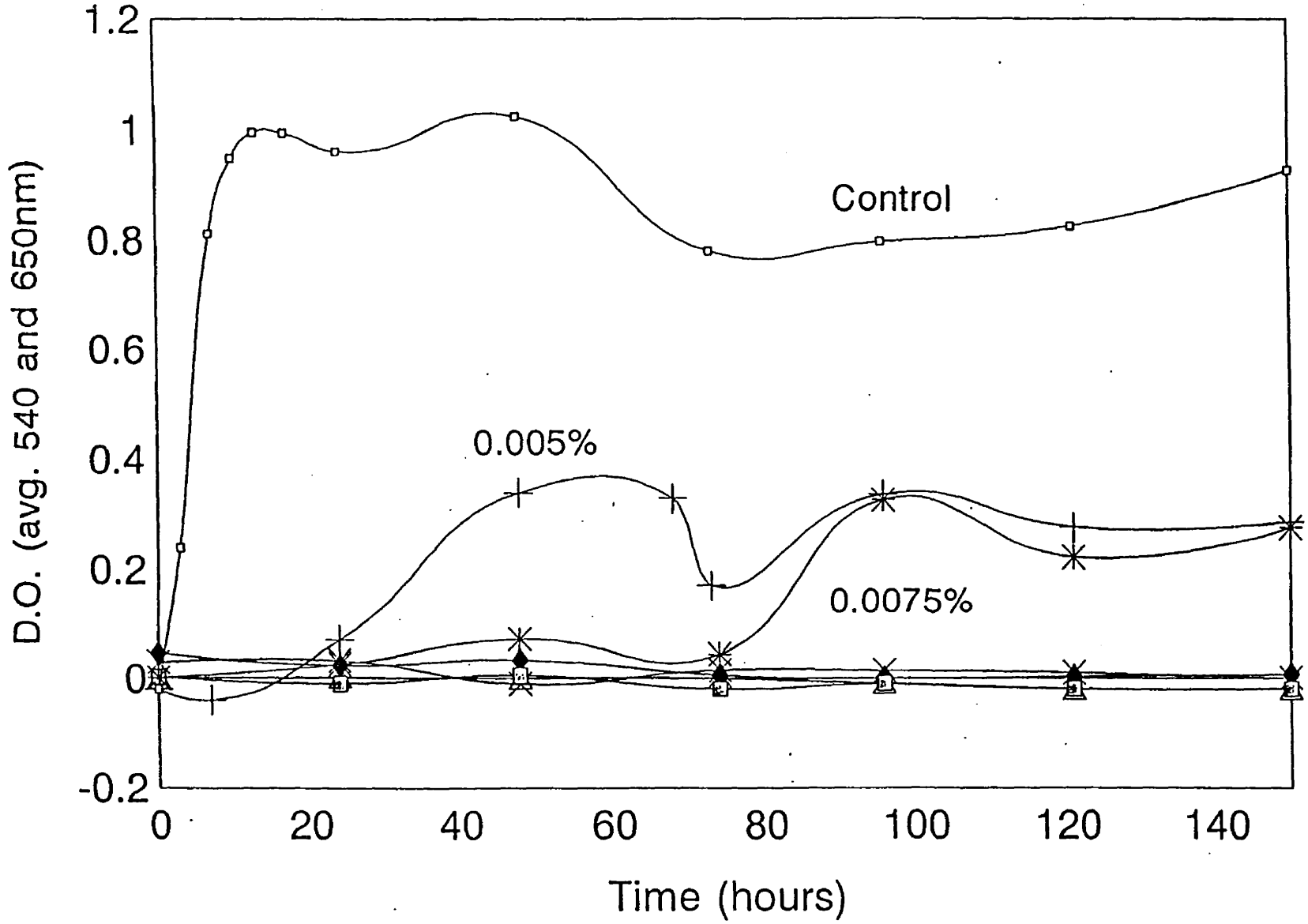

1.2
1
0.8
0.6
0.4
0.2
0
-0.2
D.O. (avg. 540 and 650nm)
Control
0.005%
0.0075%
0
20
40
60
80
100
120
140
Time (hours)

Fig. 22 Inhibition of *P. fluorescens* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◊ 0.05%; △ 0.1%).

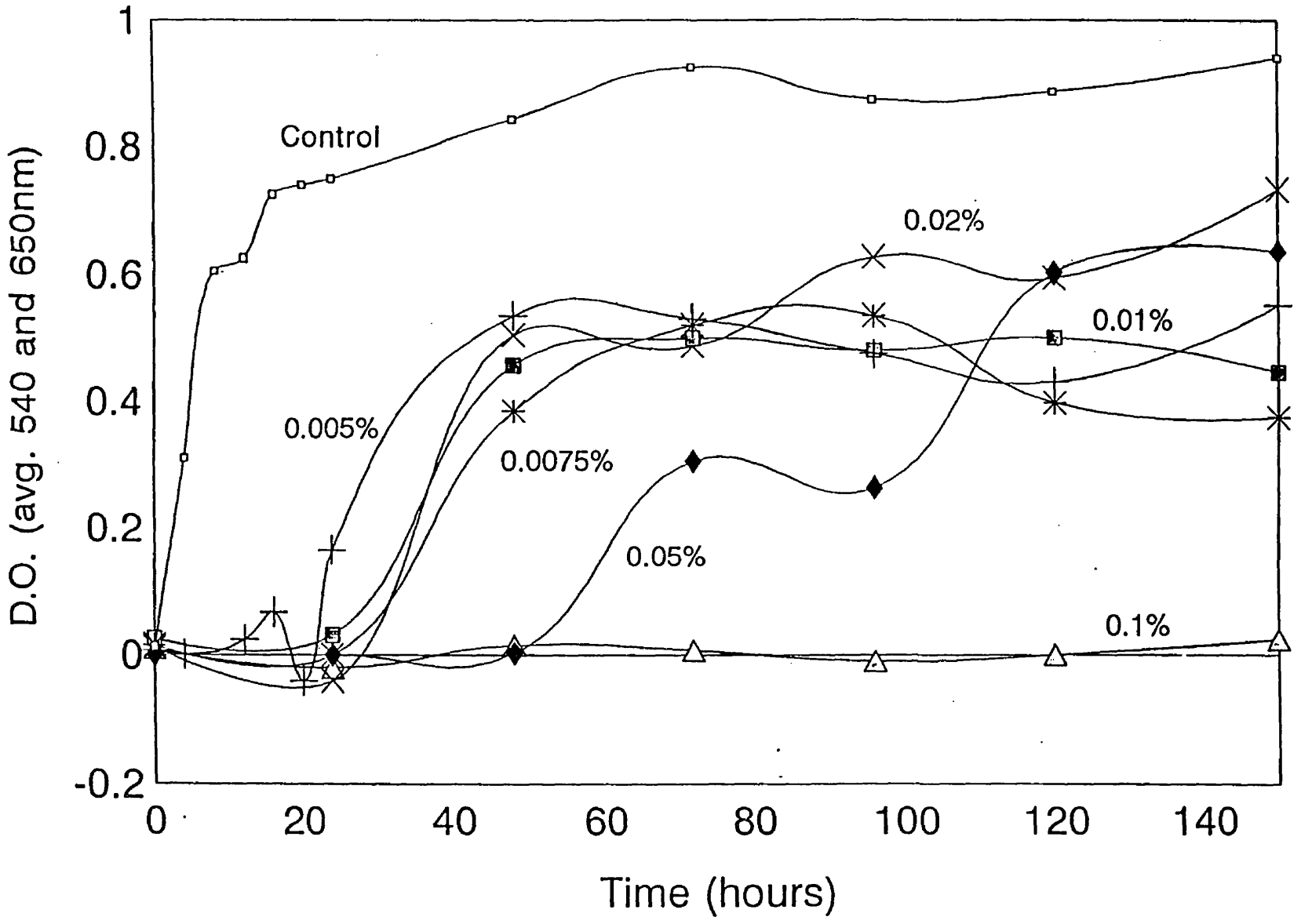

1
0.8
0.6
0.4
0.2
0
-0.2
D.O. (avg. 540 and 650nm)
0
20
40
60
80
100
120
140
Time (hours)
Control
0.02%
0.01%
0.005%
0.0075%
0.05%
0.1%

Fig. 23 Inhibition of *P. vulgaris* growth by lab prepared chitosan (▪ control; + 0.005%; * 0.0075%; □ 0.01%; x 0.02%; ◊ 0.05%; △ 0.1%).

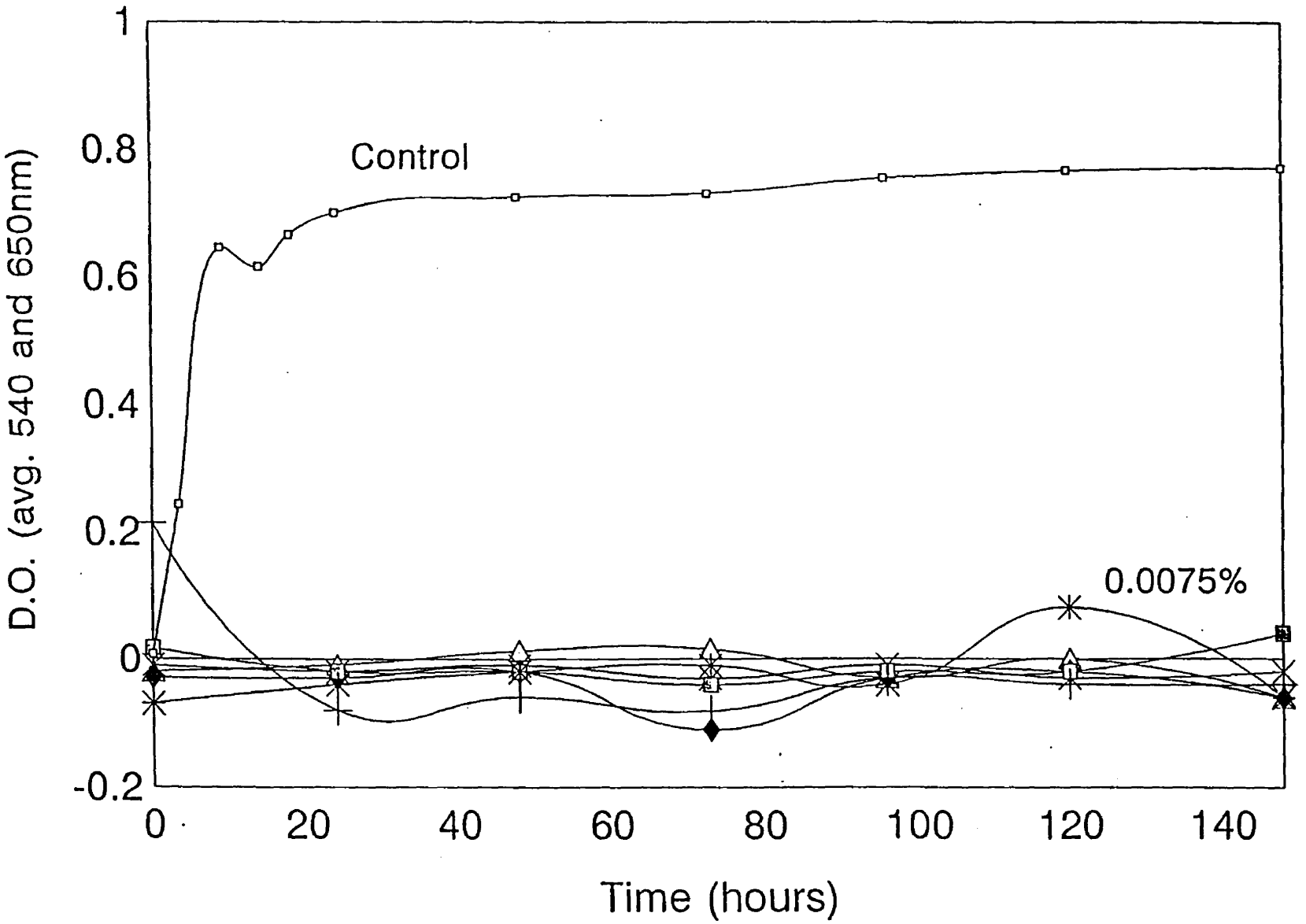

1
0.8
0.6
0.4
0.2
0
-0.2
D.O. (avg. 540 and 650nm)
Control
0.0075%
0
20
40
60
80
100
120
140
Time (hours)

TABLE 8. Inhibitory action of selected compounds compared to chitosan.

Preservative	Minimum inhibitory concentration (%)				
	E. coli	*S. aureus*	*Pseudomonas* spp.	*Bacillus* spp.	*Salmonella* spp.
Chitosan	0.0075-0.02 (pH 5.6)	0.0075-0.05 (pH 5.6)	0.01-0.1 (pH 5.6)	0.005-0.02 (pH 5.6)	0.005-0.02 (pH 5.6)
Nitrite	0.4 (O_2) 0.2 (no O_2)	0.04 (pH 5.8) 0.008 (pH 5.05)			
Sulfur dioxide	0.01-0.02	0.008	0.005	0.005	
Acetic acid		0.03 (pH 5.0)		0.04 (pH 4.9)	
Propionic acid	0.1 (pH 6.6)				
Sorbic acid	0.005-0.01 (pH 5.2-5.6)		0.01 (pH 6.0)	0.005-0.1 (pH 5.5-6.3)	0.005-0.1 (pH 5.0-5.3)
Benzoic acid	0.005-0.012 (pH 5.2-5.6)		0.02-0.048 (pH 6.0)	0.05 (pH 6.3)	
P-hydroxybenzoic acid esters	0.0012-0.01 (pH 5.5-7.0)		0.004-0.01 (pH5.5-7.0)	0.001-0.01 (pH 5.5-7.0)	0.0033-0.007 (pH 5.5-7.0)

Ref. Lueck (1980).

efficiency we generally observed in this study.

5.5. Conclusion

Generally, chitosan curtailed the growth of all the bacteria investigated and increased the lag phase of growth in most cases. However, it may be necessary to increase the period of observation, since it seems that growth may be retarded by more than 150 hr for some organisms. There is no apparent difference in chitosan's effect regarding the Gram type, but the age of the strain has a marked effect on its susceptibility to chitosan. With an antimicrobial activity in the slightly acidic range, and its functional properties as a thickening agent, chitosan could be used advantageously in sauces, marinades, pickled foods, and many other products, with the advantage of being a natural product having a broad spectrum of activity against microorganisms.

Chapter 6

General conclusions and recommendations for further work.

6.1. Overall conclusion

The recovery of chitin and chitosan has become more sophisticated over the years, resulting in highly purifed and diversified products which suits differents needs, in agriculture, medical fields, or cosmetic fields, to name a few. However, the recovery process itself could still be improved, and this study showed that the use of proteolytic enzymes is feasible, and advantageous in many ways. It responds to the need of obtaining a product least degraded, and it facilitates the recovery of proteins from the waste material.

Chitosan, the most important derivative product obtained from chitin, is showning a great potential in the food industry. Beside the use of it as a flocculating or a functional agent, chitosan as interesting properties as an antimicrobial agent with great effectiveness against a broad spectrum of microorganisms, as we demonstrated in this study. Shrimps microbial proliferation was reduced significantly in the presence of chitosan as a coating. The films obtained after drying of a chitosan layer serve as a microdiffusion barrier and acts as a modified atmosphere packaging. Thus, chitosan dips are an alternative to modified atmospheres and have demonstrated in this study their capacity at reducing melanosis and nucleotide degradation of fresh shrimps. Other types of product could benefit of the advantages of chitosan, such as sauces, bread and pastry, etc. Finally, chitin and chitosan acceptance by the FDA is the only limit to the spreading of their occurence in the food industry, but we can believe that this is going to change in the near future.

6.2. Recommendations for further study

Chitin preparation with enzymes is effective, simple, and economically advantageous. The properties of the product obtained by this method should be evaluated and compared to a product obtained with chemical methods, in order to demonstrate clearly the differences in molecular weight and degree of acetylation.

The preservation of shrimps using chitosan dips seems promising, as demonstrated in this study. The antimicrobial property of chitosan at slightly acidic pH has been demonstrated without a doubt. Since shrimps are often blanched soon after catch, it would be interesting

to study the use of chitosan to preserve shrimps in this form. Preliminary trials were made during this work and showed that the shelf life of blanched shrimps preserved with chitosan dips extend 20 days.

Further work is needed to determine precisely the mode of antimicrobial activity exhibited by chitosan. Although some clues have been given, the mechanism of action of chitosan has not been elucidated yet.

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IMAGE EVALUATION
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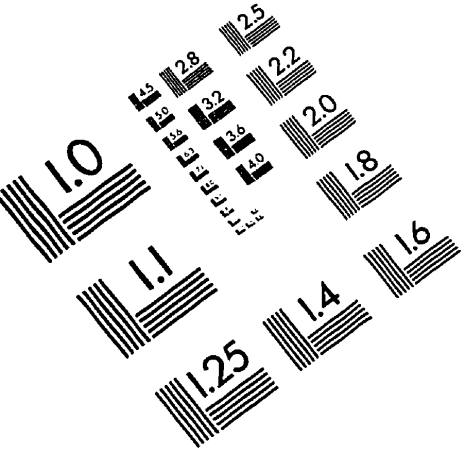

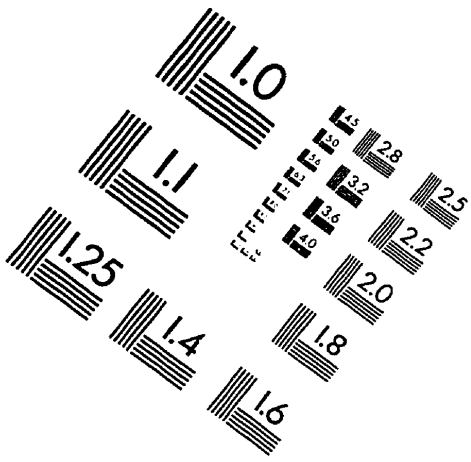

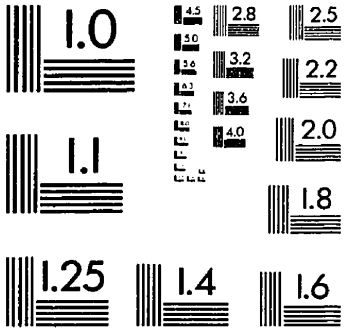

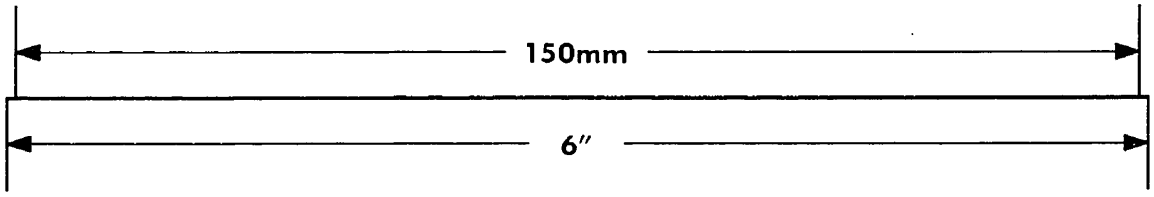

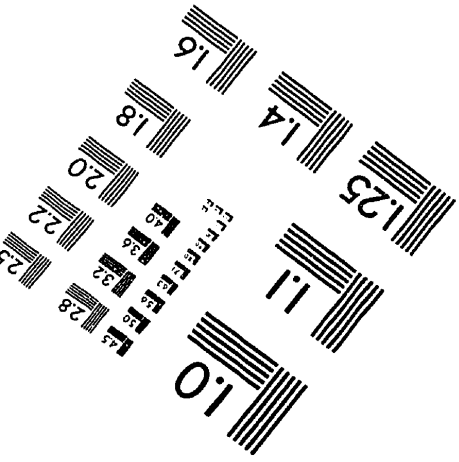

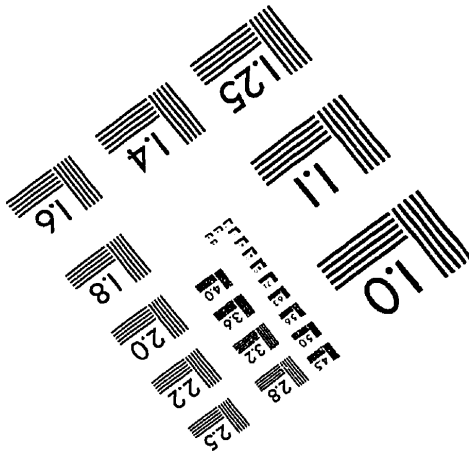

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