Effect of Chitosan and Chitosan-Nanoparticles as Active Coating on Microbiological Characteristics of Fish Fingers

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Abstract

The effect of different concentrations of chitosan and chitosan nanoparticles as active coating on microbiological characteristics of fish fingers during frozen storage at -18°C were studied. Results indicated that, uncoated fish fingers (T1) and that coated with commercial edible coating (T2) had higher total bacterial count (TBC), psychrophilic bacteria, and coliform bacteria, proteolytic bacteria when compared with fish fingers coated with either chitosan or chitosan nanoparticles. Moreover, the lowest counts of abovementioned microorganisms were recorded for chitosan nanoparticles treatments during frozen storage at – 18 C up to 6 months. The flow behavior of the edible coating solutions prepared from chitosan and chitosan nanoparticles was studied; it was observed that all samples of edible coating solutions exhibited non-Newtonian pseudoplastic behavior for all concentrations studied.

Key Words: Extraction, Chitosan, Nanoparticles, Antimicrobial, Fish Fingers, rheological properties.

1. Introduction

Ideal biobased and biodegradable polymers are defined as materials that are produced from renewable resources and completely degraded to carbon dioxide and water by the action of micro-organisms. Chitosan is a linear copolymer composed of β (1→4)-linked 2-acetamido-2-deoxy-β-d-glucopyranose and 2-amino-2-deoxy-β-d-glucopyranose units. It occurs as a component of the cell wall of some fungi but it is generally produced by carrying out the deacetylation of chitin, an abundant polysaccharide found in the shells of crustaceans, particularly crabs and shrimps. It is a biocompatible, biodegradable and antimicrobial polymer.

Nanotechnology may be able to create many new materials with a vast range of applications. The interesting and sometimes unexpected properties of nanoparticles are largely due to the huge surface area of the material accompanied usually by an increase in stability and improved functionality which dominates the contributions made by the small bulk of the material. Chitosan nanoparticles have many applications in medical and pharmaceutical uses they have been used successfully in drug delivery systems to control the releasing process of the drug (Du et al, 2009; Krishna et al, 2010; Sangeetha et al., 2010; Rafeeq et al, 2010; Kim et al, 2010; Allemann et al, 1993)

Fish fingers produced from minced fish flesh as a battered and breaded product, are commonly stored and marketed in the frozen state. Nevertheless, frozen storage does not completely inhibit microbial and chemical reactions that lead to quality deterioration of fish (Reddy & Srikar, 1996). Moreover, fish and its products such as fish burgers, fish fingers, fish balls, frankfurters and sausages can undergo undesirable changes during frozen storage that lead to deterioration which may limit their storage time.

These undesirable changes result from protein denaturation (Fijuwara et al, 1998;Benjakul et al, 2005), and lipid oxidation (Sarma et al, 2000; Richards & Hultin, 2002).

Polysaccharides, proteins, and lipids edible coatings can extend the shelf life of foods by functioning as solute, gas, and vapour barriers. Generally, meat and other foods are covered with dry particles (breaded) or dipped in liquid solutions of these particles which called battering (Kilincceker et al, 2009; Ojagh et al, 2010).

Chitosan has functional properties that make it useful in nutrition (Gallaher et al., 2002). These include its antimicrobial activity and ability to form protective films (Cuero, 1999;Jeon et al, 2002), texturizing (Benjakul et al, 2003), binding action (No et al, 2000); and its antioxidant activity (Kamil et al 2002). Many investigators have studied chitosan as edible coating material for fishery products to enhance microbiologicalquality and extend the shelf life (Alishahi et al, 2011; Augustini& Sedjati, 2007; Fan et al, 2009;Mohan et al, 2012)In spite of numerous studies, which have indicated that improving the quality and extension the shelf life of fish through the use of chitosan, there are very little or no reports on the effect of chitosan coating or chitosan nanoparticles coating on quality of fish fingers during frozen storage.

Rheological properties of coating solutions, which are concentration (C) dependent, are important for the use of these solutions in edible coatings, (Kislenko et al, 2006). The knowledge of rheological properties of coating solutions is necessary for a successful product formulation and engineering scale up, (Bhandari et al., 2002 and Rao et al., 1984).

The flow behavior of the edible coating solutions needs to be studied since the viscosity of the film forming solution is the key to control the desirable thickness of the coating (Chen, 1995). The viscosity of the coating solutions is also important for decreasing the dewetting process which prevents the creation of a continuous layer around food, making it necessary that the magnitude of the viscous forces be greater than that of the interfacial ones (Mate& Krochta, 1996). The aim of this study was first to prepare and characterize chitosan and chitosan nanoparticle solutions then using these solutions as edible coating to improve microbiological quality and extend the shelf-life of fish fingers.

2. Material and Methods

2.1. Materials

2.1.1.Fish sample

Carp fish varying from 500 to 900 gm in weight, were purchased from the private sector shop in the local market at Giza, Egypt. Fish were transferred to the laboratory in an ice box within 30 min.

2.1.2. Other ingredients

Food grade sodium tripolyphosphate (99.5% purity) was obtained from El-Gomhoria for chemicals Co., Egypt. Salt, sugar, wheat and corn flour, cumin, onion, garlic powder, black pepper, thyme, egg and skimmed milk were purchased from local market at Giza, Egypt.

2.2. Methods

2.2.1. Extrication of chitosan

Chitosan was extracted frommarine shrimp shells. The exoskeleton of the shrimp were crushed and treated in the usual way with HCl, NaOH 1-2 M then with 40% NaOH to extract the chitosan (Abdou et al, 2008). The degree of deacetylation (DDA%) of chitosan determined by potentiometric titration (Domard & Rinaudo, 1983), and the molecular weight was calculated using the value of intrinsic viscosity (Ravindra et al, 1998) measured by an Ubbelohde viscometer. The value of (DDA%) and molecular weight of chitosan were 85% and 3.98×10^4 gm/mol respectively.

2.2.2. Preparation of chitosan nanoparticles

Nanoparticles were produced based on ionic gelation of tripolyphosphate (TPP) and chitosan as described elsewhere (Calvo et al., 1997). Nanoparticles were spontaneously obtained upon the addition of 2%, 2.8% and 4% solutions of TPP aqueous basic solution to 2%, 2.8% and 4% of the chitosan acidic solution respectively (the ratio of TTP to chitosan was 1:1) under magnetic stirring at room temperature.

2.2.3. Scanning Electron Microscopy:
The surface morphology of chitosan nanoparticles was investigated using Transmission Electron Microscope (TEM)polymer sample was suspended in acetone for 20 min.then, adrop of the suspension was placed on a grid and letting the solvent evaporate prior to imaging

2.2.4. Preparation of commercial edible coating
Commercial edible coating was prepared by mixing 94% corn flour, 2% egg yolk, 2% skimmed milk, 1.8 % salt and 0.2 % cumin with water by 1: 3 (w:w).

2.2.5. Rheological properties of edible coating solutions
Rheological parameters (shear stress, shear rate, viscosity) of chitosan and chitosan nanoparticales were measured at different temperatures using Brookfield Engineering labs DV-III Ultra Rheometer. The samples were placed in a small sample adapter and a constant temperature water bath was used to maintain the desired temperature. The viscometer was operated between 10 and 50 rpm and shear stress, shear rate, viscosity data were obtained directly from the instrument, the SC4-21 spindle was selected for the measurement.

2.2.6. Preparation of fish fingers
Upon arrival, fish were washed with chilled water (4ºC), beheaded, gutted, washed again with chilled water, and then filleted. The fillets were minced with meat mincer using a 4.5 mm diameter holes plate. Carp fish fingers were prepared by the following recipe according toLong et al (Long et al, 1983) US Department of Agriculture (USDA, 2001) Minced fish meat and the other ingredients were mixed for 3 min by using laboratory mixer (Hobbart Kneading machine, Italy). The obtained mixture was spread in thin layer (1.5 cm) in stainless steel trays and formed to fingers using a kitchen knife (9.0 × 2.0 cm) then stored in freezerat -18ºC for 24 hr.

The frozen fish fingers were divided to eight different batches. As seen in **table (1)** every batch was immersed or dipped into the corresponding edible coating for about 30 sec.

All fish fingers treatments were packaged in a foam plates, wrapped with polyethylene film and stored at -18 ºC for six months. Samples were taken for analysis every month periodically.

Table (1) The composition of different edible coatings used

Sample	Coating composition
T1	Without coating (control)
T2	commercial edible coating
T3	2% chitosan solution
T4	2.8% chitosan solution
T5	4% chitosan solution
T6	Chitosan nanoparticles solution (2%chitosan+2% TPP)
T7	Chitosan nanoparticles solution(2.8%chitosan+2.8% TPP)
T8	Chitosan nanoparticles solution(4%chitosan+4% TPP)

2.2.7. Microbiological methods

2.2.7.1. Sample preparation:
Ten gram of a representative fish fingers sample were mixed with 90 ml of sterile buffered 0.1 % peptone water in a sterile blender, under sterile conditions, to give 1/10 dilution. Serial dilutions were prepared to be used for counting total bacteria count,coliform bacteria, psychrophilic bacteria proteolytic bacteria, *staphylococcus aureus* and yeast & mold counts.

2.2.7.2. Bacteriological methods
Total bacterial count (TBC), *Staphylococcus aureus*, Coliform bacteria, proteolytic bacteria, Psychrophilic and yeast & mold counts of fish fingers were determined according to the procedures described by Difco Manual (1984). Incubations were carried out at 37ºC/48hr for TBC; at 37ºC/24hr for *Staphylococcus aureus* and Coliform: at 30ºC for 48 hr for proteolytic bacteria; at 7ºC/10 day for Psychrophilic and 25ºC/5 day for yeasts & molds count. Moreover, the presence or absence of salmonella was determined according to the methods described by FAO (1979)

2.2.7.3. Data analysis

Data were subjected to analysis of variance (ANOVA). The least significant difference (LSD) procedure was used to test for difference between means (significance was defined at ($p < 0.05$) as reported by Snedecor and Cochran (1995).

3. Results and discussion

3.1. Scanning Electron Microscopy

Three different concentrations with the same ratio (1:1) of chitosan/TPP are used. Transmittance electron microscope was used for the determination of the particle size and the morphological structure of the prepared polymer matrix. It was found that chitosan/TPP (T8) has average particle size of 10 nm. **Figure (1)** shows the scanning electron microscopy of chitosan nanoparticales.

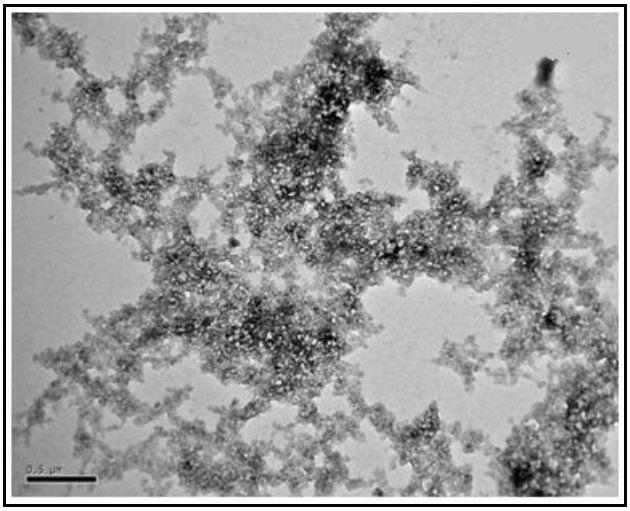


Fig. 1. Scanning electron microscopy of chitosan nanoparticales (T8)

3.2. Rheological properties of edible coating solutions

Figures (2) show the relation between shear rate and apparent viscosity for chitosan and corresponding chitosan/tripolyphosphate solutions. The results indicate that the solutions exhibited non-Newtonian pesudoplastic behavior, since the viscosity decreased with increasing the shear rate. These results are in accordance with the findings of Abdou et al, (2009).

The relation between shear rate and viscosity was fitted well to the power law equation:

$$\tau = k\ \gamma^{n} \quad \text{or} \quad \mu = k\ \gamma^{n-1}$$

Where, τ is the shear stress, Pa, k is the consistency index, γ is the shear rate, sec^{-1}, n is the flow behavior index.

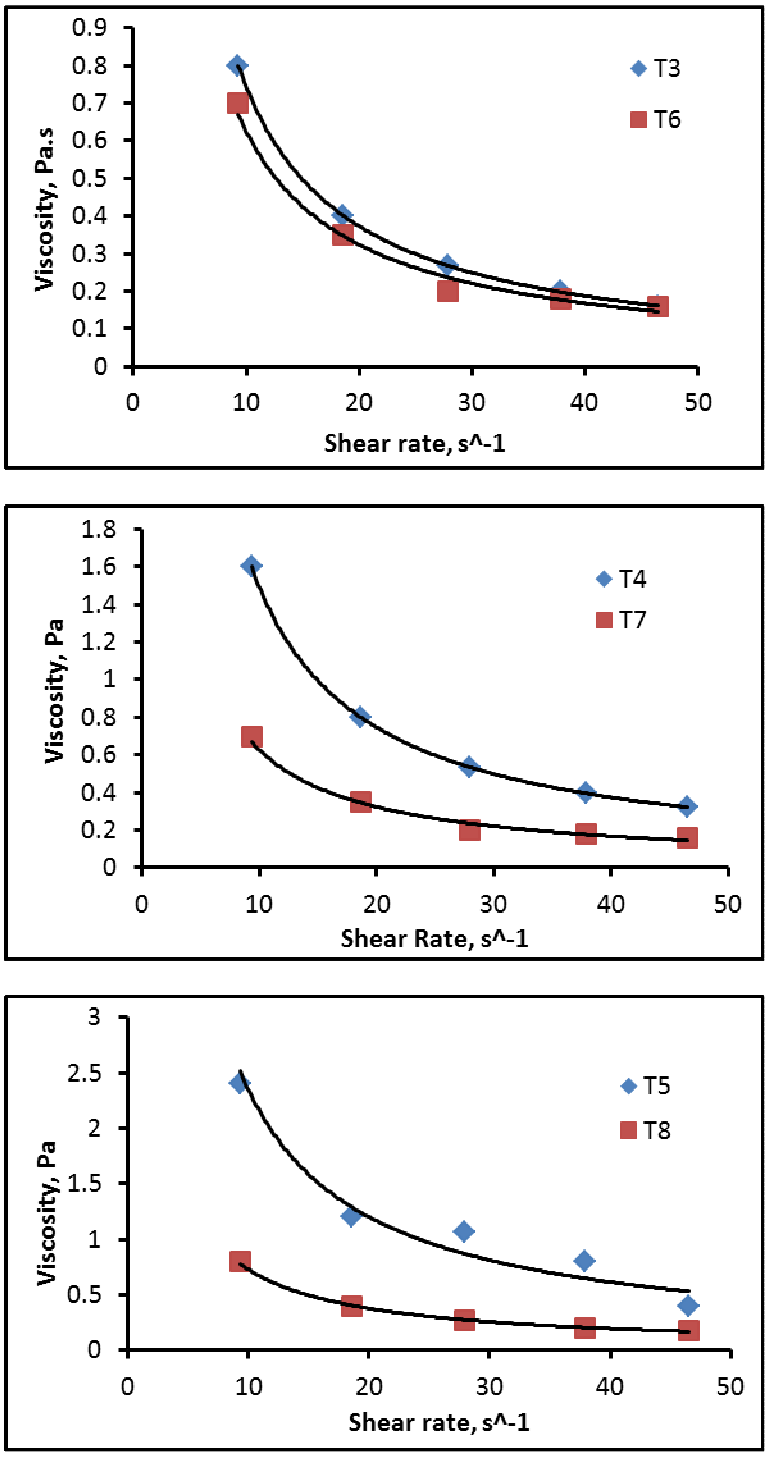


Fig. 2. Effect of shear rate on apparent viscosity of chitosan and chitosan nanoparticales solutions at different concentrations.

From **Figure (2)** it was observed that the spacing between the curves of chitosan and chitosan nanoparticles solutions was increased by increasing the concentration chitosan and sodium tripolyphosphate, this may be due to the structural change in samples as shown in Figure (1).

Table (2) presents the parameters obtained by fitting to the power law model for chitosan and chitosan nanoparticles solutions**.** The consistency indexes (k) and flowbehaviour index (n) of chitosan decreased with the addition of sodium tripolyphosphate.

Table (2) Parameters of Power law model for chitosan and chitosan nanoparticles solutions

Sample	k (consistency index)	n (flow behavior index)
T3	7.3479	0.34
T6	1.785	0.005
T4	14.696	0.053
T7	5.5295	0.005
T5	13.118	0.052
T8	6.4796	0.035

3.2.1. Effect of concentration on apparent viscosity of chitosan and chitosan nanoparticles solutions

Figure (3-a) shows the effect of concentrations of different chitosan solutions on the apparent viscosity. The results indicated that by increasing the concentration of chitosan the apparent viscosity increased at all shear rates studied.

Therefore, in this section, the effect of soluble solid contents on the apparent viscosity of chitosan solutions at different shear rates (9.3, 18.6, 27.9, 37.9 and 46.5 sec^{-1}) were studied. In order to quantify the dependence of apparent viscosity on soluble solid contents, power-law equation was applied.

$$\mu = AC^{B}$$

Where, A, B are constants, and C is the concentration in wt%, and μ is the viscosity of the samples of chitosan, Pa.s

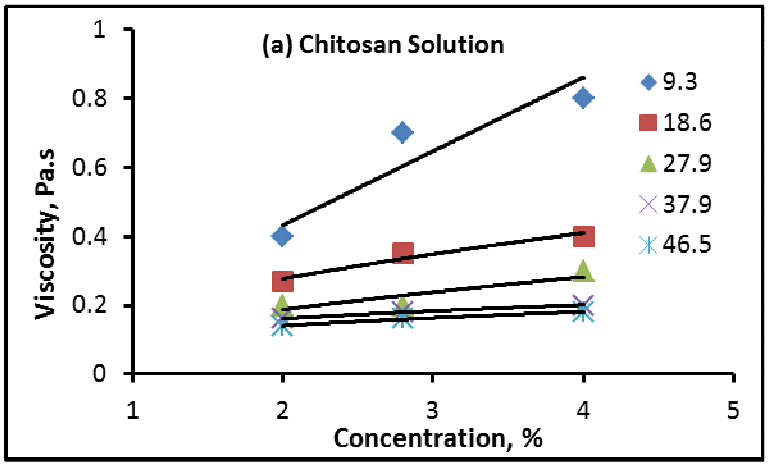
Figure (3-b) shows the effect of concentrations of different chitosan nanoparticles solutions on the apparent viscosity at different shear rates and it can be seen that the same trend was obtained.

The results indicate that the apparent viscosities of chitosan nanoparticles decreased than the apparent viscosity of chitosan solutions this is because TPP-crosslinked chitosan moleculesturned into more dense particles whose hydrodynamic volumeswere smaller than pure chitosan chains. Fewer free chitosan chainsand more crosslinked chains result in a decrease of total hydrodynamic volumes of chitosan. When the total hydrodynamic volume of chitosan is smaller than the solution volume, chitosan chains donot entangle with each other. (Li, Huang, 2012)

The results were fitted well to power law equation as previously discussed above

$$\mu = A_1 C^{B_1}$$

Where, A_1, B_1 are constants and C is the concentration in wt%, and μ is the viscosity of chitosan, Pa.s.



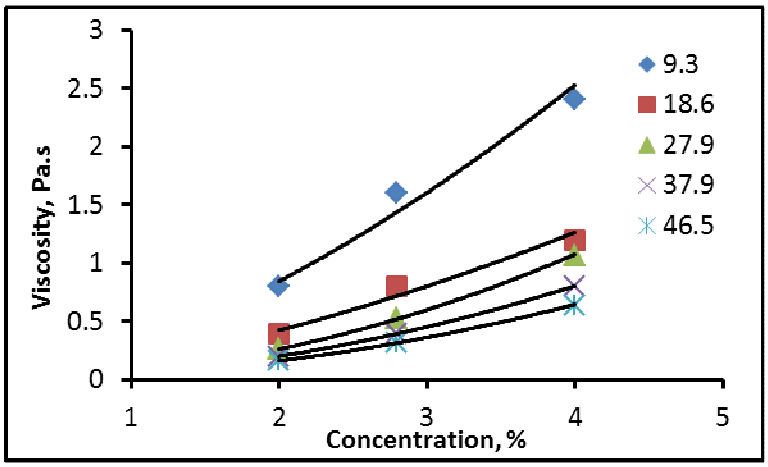


Fig. 3. Effect of Concentration on the apparent viscosity of chitosanand chitosan nanoparticles solutions at different shear rates.

Table (3) The values of the parameters of the power-law equation for different concentrations of chitosan and chitosan nanoparticles solutions at different shear rates.

Shear rate	Chitosan nanoparticles		Chitosan	
	A_1	B_1	A	B
9.3	0.2168	0.9937	0.2826	1.5805
18.6	0.1868	0.5651	0.1413	1.5805
27.9	0.1242	0.5905	0.064	2.0362
37.9	0.1284	0.3217	0.0504	1.9994
46.5	0.1093	0.3622	0.0403	1.9994

3.3. Microbiological evaluation of different fish fingers

The microbial load of fish fingers depends upon the microbial load of the raw fish meat, sanitary conditions, time and temperature of storage as well as other ingredients which are used in preparation of fish fingers such as salt, sugar, wheat and corn flour, cumin, onion, garlic, spices, egg and skimmed milk. The last mentioned components have been found to contain high numbers of bacteria.

3.3.1. Total bacterial count (TBC)

Figure (4) indicate the total bacterial count (TBC) of different fish fingers treatments during frozen storage at -18°C up to 6 months. From this figure it is obvious that, the initial TBC of different fish fingers treatments ranged from 3.66–4.72 log cfu/g. These results agree with findings of Cakli, et al., (2005).who found that fish fingers made from different fish species contained bacterial concentration ranging from 4.50 to 4.61 log cfu/g immediately after processing.

The initial TBC of fish fingers coated with commercial edible coating (T2) was slightly higher than uncoated fish fingers (T1), this may be due to that commercial edible coating containing high numbers of bacteria. Moreover, different concentration of chitosan lowered the TBC in fish fingers treatments with the range 0.27 to 0.43 log cfu/g and 0.39 to 0.55 log cfu/g when compared with (T1) and (T2) respectively. The highest reduction in TBC was recorded for fish fingers coated with different concentration of chitosan nanoparticles with range 0.63–0.94 cfu/g and 0.75–1.06 cfu/g when compared with (T1) and (T2), respectively immediately after processing.

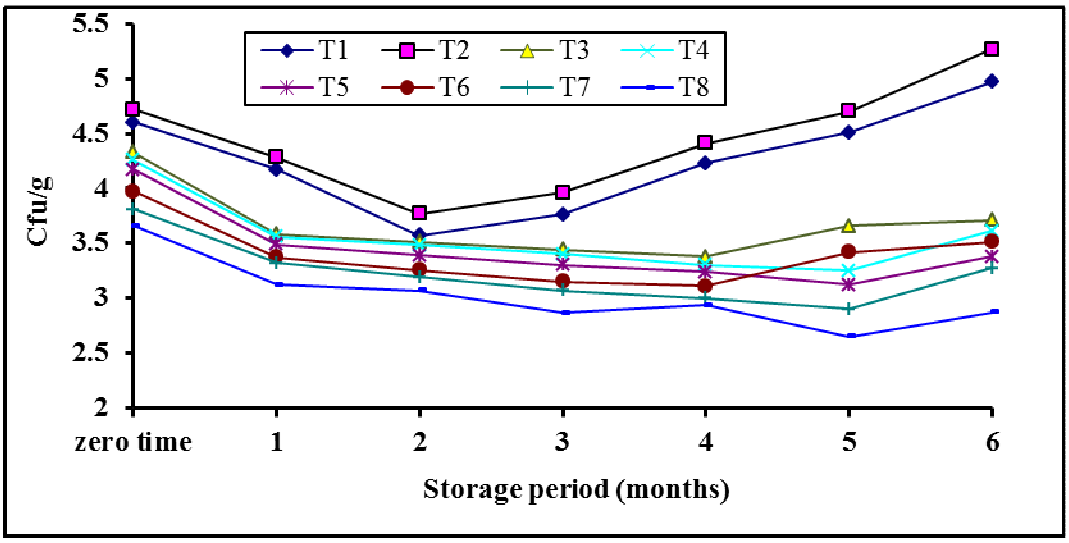


Fig. 4. Changes in TBC of different fish fingers treatments during frozen storage.

Generally, fish fingers coated with different concentration of chitosan coatings (T3, T4 and T5) had slightly higher TBC than those coated with chitosan nanoparticles (T6, T7 and T8) at any time of frozen storage. This may be due to chitosan nanoparticles had higher antimicrobial effect than chitosan. In this concern, Zhang et al., (2007). reported that the antimicrobial activity of nanoparticles increased with decreasing particle size. Also, from the same figure, it could be noticed that, TBC in fish fingers was decreased with increasing chitosan or chitosan nanoparticles concentrations at any time of frozen storage.

TBC decreased in all fish fingers treatments during the frozen storage period increment up till 2 months of frozen storage for T1 and T2, 4 months for T3 and T6 and up to 5 months for other treatments (T4, T5, T7 and T8) and then TBC slightly increased relatively until the end of frozen The reduction of TBC during frozen storage may be caused by the intra and extra cellular ice crystals formed during the freezing process that induce the irreversible damage to both the outer and cytoplasmic membranes of bacteria (Uijas & Ingham, 1999) on the other hand, the increase of TBC at the abovementioned months of frozen storage may be due to increase in simple nitrogen compounds (amino acids and nucleoides) and fatty acids which were produced by hydrolysis of protein and fat by natural fish enzymes which consequently leads to suitable conditions for bacterial growth.

The TBC in T1 and T2 increased from 4.60 and 4.72 log cfu/g at zero time to 2.97 and 5.27 log cfu/g at the end of frozen storage (6 months) respectively. On the other hand, T3, T4, T5, T6, T7 and T8 lowered the TBC during the frozen storage and kept it lower than 3.71 and 3.51 log cfu in fish fingers coated with chitosan and chitosan nanoparticles respectively at the end of frozen storage. The lowest TBC (2.87 log cfu/g) was recorded for T8 followed by T7 (3.27 log cfu/g), T5 (3.38 log cfu/g), T6 (3.51 log cfu/g), T4 (3.61 log cfu/g) and T3 (3.71 log cfu/g) at the end of frozen storage.

According to many studies (Helander et al, 2001; Yi et al, 2003;Xue et al, 2006) the antibacterial activity of chitosan under acidic environment may result from its polycationic structure due to the protonation of $-NH_2$ on the C-2 position of the D-glucosamine repeat unit. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells. Chitosan also inhibits the microbial growth by the chelation of essential metals and nutrients, spore components, as well as the penetration of the nuclei of the microorganisms, which leads to the interference with protein synthesis by binding with DNA. Furthermore, chitosan coatings act as an oxygen barrier and thus inhibit the growth of aerobic bacteria (Shahidi et al, 1999;Devlieghere et al, 2004).

3.3.2. Psychrophilic bacteria

Changes in psychrophilic bacteria counts of different fish fingers treatments during frozen storage at -18°C up to 6 months are shown in **figure (5)**.

From these results, it could be noticed that, the initial psychrophilic bacteria counts ranged from 2.81 to 3.36 log cfu/g for all fish fingers treatments. Psychrophilic bacteria counts of T1 were higher as compared to the coated fish fingers treatments at zero time and throughout the frozen storage period. Also, T2 had higher psychrophilic bacteria counts than T3, T4 T5,T6, T7 and T5. Moreover, fish fingers coated with chitosan nanoparticleshad lower psychrophilic bacteria count when compared to that coated with chitosan. Chitosan coatings lowered psychrophilic bacteria counts in T3, T4 and T5 with 0.18, 0.24 and 0.39 log cfu/g reduction when compared with T1. Also, chitosan nanoparticles coating lowered psychrophilic population in T6, T7 and T5 with 0.39, 0.44 and 0.55 log cfu/g respectively at zero time.

Psychrophilic bacteria counts gradually decreased during frozen storage at -18°C in all fish fingers up to 3 months for treatments T1, T2, T3, T4 and T5, 4 months for treatments T6 and T7 and 5 months for T8, after that slightly increased relatively until the end of frozen storage. At the end of frozen storage (6 months), the highest psychrophilic bacteria count (3.34 log cfu/g) was recorded for T1 followed by T2 (3.29 log cfu/g). On the other hand, the lowest count of psychrophilic bacteria (2.52 log cfu/g) was recorded for T8.

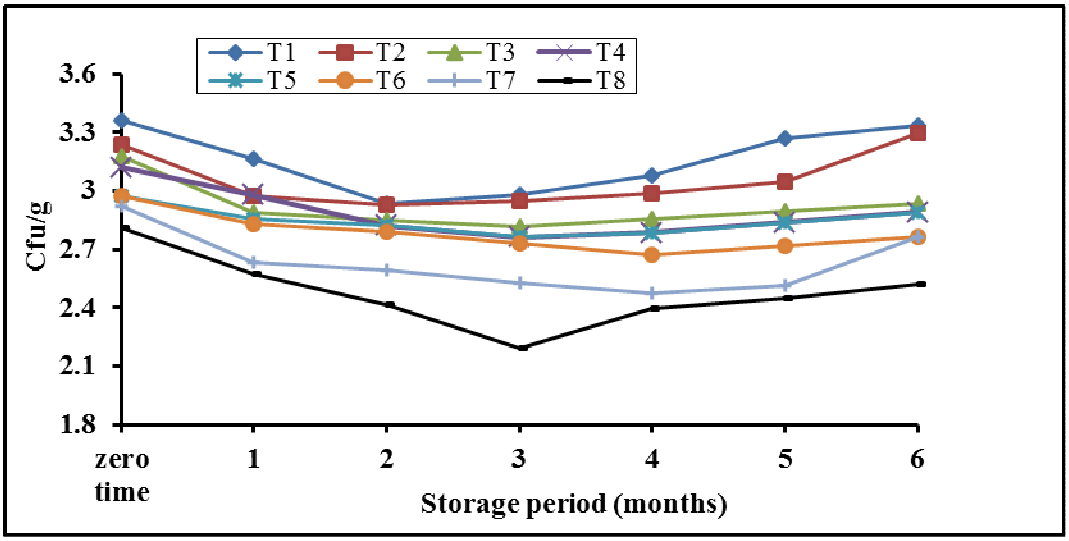


Fig. 5. Changes in psychrophilic bacterial counts of different fish fingers treatments during frozen storage.

3.3.3. Coliform bacteria

The initial coliform bacteria counts of different fish fingers treatments ranged from 2.46 to 2.87 log cfu/g. Changes in coliform bacteria counts of different fish fingers treatments during frozen storage at -18°C up to 6 months are presented in **Figure (6)**. The population of coliform bacteria in T1 and T2 decreased from 2.72 and 2.87 log cfu/g respectively immediately after processing (zero time) to 2.61 and 2.68 log cfu/g, respectively after 3 months of frozen storage, but these values increased to 2.88 and 2.96 log cfu/g, respectively at the end of frozen storage.

At any time of frozen storage, fish fingers which coated with chitosan nanoparticles had the lowest coliform bacterial count when compared to other treatments. Moreover, coliform bacterial counts of fish fingers coated with different concentrations of chitosan were lower as compared to T1 and T2.

Also, from the same figure, it could be noticed that, counts of coliform bacteria gradually decreased with increasing the storage period up to 6 month in all fish fingers treatments coated with different concentration of chitosan or chitosan nanoparticles. On the other hand, coliform bacteria of T1 and T2 gradually decreased with increasing the storage period up to 3 month of frozen storage and then increased until the end of frozen storage.
The population of coliform bacteria in T3, T4 and T5 decreased from 2.64, 2.56 and 2.53 log cfu/g at zero time to 2.40, 2.26 and 2.21 log cfu/g, respectively at the end of frozen storage. These populations of coliform bacteria in T6, T7 and T8 also decreased from 2.59, 2.51 and 2.46 log cfu/g at zero time to 2.14, 2.51 and 1.88 cfu/g, respectively at the end of frozen storage. Generally, the lowest counts of coilform bacteria were recorded for T8 followed by T7 at any time of frozen storage.

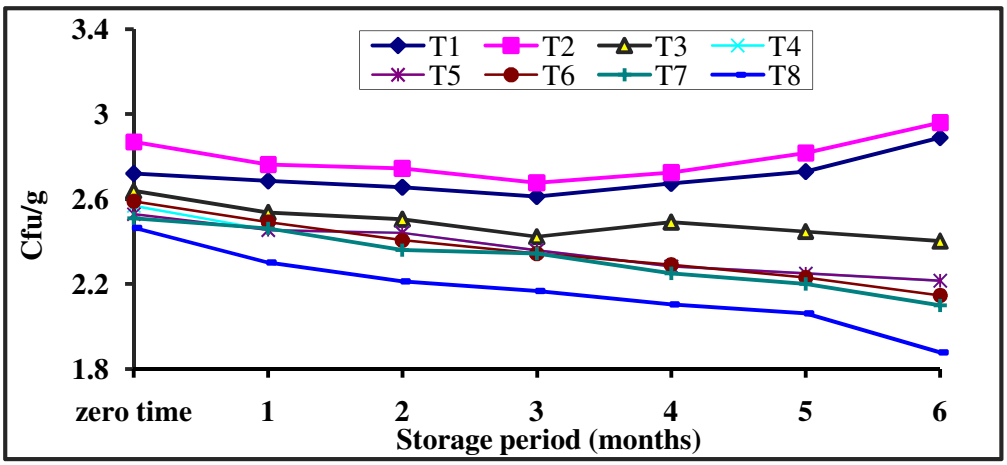


Fig. 6 Changes in coliform bacterial counts of different fish fingers treatments during frozen storage

3.3.4. Proteolytic bacteria

Changes in proteolytic bacteria counts of different fish fingers treatments during frozen storage at -18°C up to 6 months shown in **figure (7)**. The initial proteolytic bacteria counts of different fish fingers treatments were ranged from 2.61 to 3.25 log cfu/g, and low initial proteolytic bacteria counts indicated good fish fingers quality. Proteolytic bacteria count of T1 was higher as compared to all coated fish fingers treatments. Also, T2had higher proteolytic bacteria than that coated with chitosan or chitosan nanoparticles at any time of frozen storage. Chitosan coatings reduced proteolytic bacteria count in T3, T4 and T5 with 0.36, 0.49 and 0.46 log cfu/g, respectively when compared with T1. Also, chitosan nanoparticles coatings reduced proteolytic bacteria count in T6, T7 and T8 with 0.49, 0.50 and 0.64 log cfu/g, respectively at zero time.

By advancement of frozen storage, proteolytic bacteria counts were decreased up to 2, 3,4 and 5 months of frozen storage for (T1), (T2), (T3, T4 and T5) and (T6 and T7) respectively and then increased relatively until the end of frozen storage. The counts of proteolytic bacteria in (T8) decreased up to 3 months of frozen storage followed by fluctuation trend until the end of frozen storage. The highest count of proteolytic bacteria (3.46 log cfu/g) was recorded for T1 followed by T2. On the other hand, the lowest count (1.60 log cfu/g) was recorded for T8 at the end of frozen storage. The reduction of proteolytic bacteria counts at the end of frozen storage were 0.71, 0.79, 0.94, 1.39, 1.59 and 1.86 log cfu/g in T3, T4, T5, T6, T7 and T8, respectively as compared with T1.

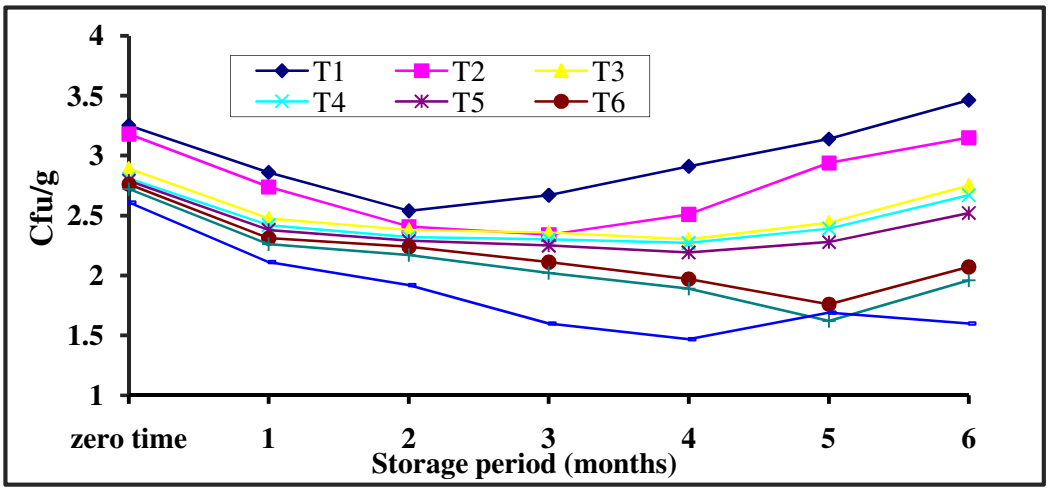


Fig. 7. Changes in proteolytic bacteria counts of different fish fingers treatments during frozen storage.

3.3.5. Salmonella, *staphylococcus aureus* and yeast & mold.

All fish fingers treatments whether the uncoated treatment or coated treatments with different coatings were completely free from *Salmonella*, *staphylococcus aureus* and yeast and mold at a zero-time and throughout the frozen storage.

Conclusion

Food packaging is the one sector of the industry where nanotechnology applications are beginning to live up to their promise.The results of our work indicated that using of chitosan and chitosan nanoparticales in different concentrations as edible coatings during frozen enhance microbiological quality of fish fingers and increase the shelf life up to 6 months. Results indicated that, uncoated fish fingers (T1) and that coated with commercial edible coating (T2) had highertotal bacterial count (TBC), psychrophilic bacteria, coilform bacteria, proteolytic bacteria when compared with fish fingers coated with either chitosan or chitosan nanoparticles.The rheological properties of the edible coating solutions was also studied; all samples exhibited non-Newtonian pseudoplastic behaviorfor all concentrations studied.

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