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Effects of chitosan and nano-chitosan as coating materials on the quality properties of large scale tongue sole *Cynoglossus arel* during super-chilling storage

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Abstract

The present study examined the effects of chitosan (Ch) and chitosan combined with sodium tripolyphosphate (Nch) nanoparticles as coating materials for *Cynoglossus arel* fillets during superchilling storage. Treatments included the following: C (untreated, control samples), AC (treated with 1% glacial acetic acid, acid control), Ch (treated with 2% w/v chitosan solution, Chitosan) and Nch (treated with 2% w/v chitosan and 2% sodium tripolyphosphate, Nanochitosan). Nch and Ch coatings significantly were effective on total mesophilic counts (TMC), psychrotrophilic bacteria (PTC) and Enterobacteriaceae. Production of total volatile bases nitrogen (TVBN) and trimethylamine (TMA) for Nch and Ch samples was significantly lower than in control samples during storage. The thiobarbituric acid (TBA) and free fatty acids (FFA) exhibited an increasing trend (p<0.05), whereas the total sulfhydryl (SH) decreased (p<0.05) with increasing storage time. The results indicated that Nch and Ch can be used for preservation of quality properties of fish samples. The Nch was better than ones Ch in reducing lipid oxidation of fillets and bacterial contamination.

Keywords: *Cynoglossus arel*, Edible coating, Chitosan, Nano-chitosan, Shelf life

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Introduction

Active packaging is one of the methods for natural preservation in order to delay the degradation and maintain the quality of the products longer (Nowzari *et al*., 2013). Among the materials proposed for food active packaging, chitosan have received increasing attention because of their outstanding physical and biological properties. Chitosan, a linear polysaccharide of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is a biocompatible polysaccharide obtained from de-acetylation of chitin found widely in nature, such as in shrimp, crab cuticles and fungi. In food industry, chitosan coatings have been used successfully because of some advantages such as edibility, biodegradability, aesthetic appearance and barrier properties, being nontoxic and non-polluting, as well as being carrier of foods additives (i.e.: antioxidants, antimicrobials). Therefore, theses coatings can retain quality of raw, frozen and processed foods including fish items by preventing bacterial growth and delaying lipid oxidation.

Natural or artificial polymers of nanoparticles have one or more dimensions of the order of 100 nanometers (nm) or less (Sinaha and Okamoto, 2003). Unique physical and chemical features were displayed by nanoparticles due to effects such as the quantum size effect, mini size effect, surface effect and macro quantum tunnel effect (Ramezani *et al*., 2015). Recently, the use of chitosan nanoparticles as food packaging materials has increased due to their advantages over other traditional materials (Ramezani *et al*., 2015). The major differences between nano-materials and other materials are the changes in physicochemical properties. Nano-chitosan can be prepared by using several methods including the ionotropic between chitosan and sodium tripolyphosphate. The cationic amino groups of chitosan interact with negatively charged of metals or small multiple-charged anionic molecules, such as sulphates, citrates, and tripolyphosphate is reported to be the most popular (Shu and Zhu, 2000). Nanoparticles of chitosan tripolyphosphate can be mainly used for the purpose of controlled-released drug carrier, therapeutic effects enhancement and targeted drug delivery (Prabaharan and Mano, 2005). Moreover, Chitosan nanoparticles inhibited the growth of bacteria in food due to antimicrobial properties of nano-chitosan (Du *et al*., 2009). The antimicrobial activities of nano-chitosan was reported by Ramezani *et al*. (2015), when comparing the effectiveness of chitosan and nano-chitosan coatings on silver carp fillets in refrigerated storage. In addition, the use of chitosan-tripolyphosphates nanoparticles retains antioxidant activity in vitro using a free radical scavenging activity test and reducing power test (Zhang *et al*., 2008).

During storage of fish fillet in refrigerator, major changes occur in proximate, microbiological, chemical and sensory composition. These activities decrease shelf life of seafood.

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The large-scale tongue-sole (*Cynoglossus arel*) is the most popular fish in Iran with the highest economic value. This fish is mainly offered on the Iranian market as skinned and boneless fillets. The use of chitosan might increase the hurdles for microbial growth, thereby retarding quality changes of fish fillet more effectively. Therefore, the aim of the present assay is to investigate a comparative basis antimicrobial and antioxidant effect of chitosan and nano-chitosan coatings on quality of large-scale tongue-sole fillets under superchilling conditions (-3 ˚C).

Materials and methods

Preparation and characterization of chitosan nanoparticles

Chitosan solution was prepared with 2% (w/v) chitosan (Sigma Chemical Co., medium molecular weight, viscosity 200-800 cP) in 1% v/v acetic acid (Ojagh *et al*., 2010). To achieve complete dispersion of chitosan, the solution was stirred at room temperature to dissolve completely. Glycerol was added at 0.75 ml g^{-1} concentration as a plasticizer and stirred for 10 mins (Ojagh *et al*., 2010).

Nanoparticles were achieved by the cross linking of chitosan (95% de-acetylated, MW: 1000kDa)-sodium tri-polyphosphate solution. Chitosan (2%) was dissolved in 1% acetic acid solution to form chitosan solution. Sodium tri-polyphosphate solution (2%, w/v) dissolved in distilled water. Under magnetic stirring at room temperature, 4 ml of tri-polyphosphate solution was added into 100 ml of chitosan solution. The mixture was stirred for 60 mins, then, treated with sonication at 1.5 kW for 10 mins before being used for further analysis (Du *et al*., 2009). Particles size and zeta potential were measured using a Zeta sizer Nano-ZS-90 (Malvern Instruments). The analysis was performed at a scatting angle of 90 º at 25 ºC. For zeta potential measurements, samples were dispersed in water and measured under the automatic mode.

Sample preparation

Large-scale tongue-sole fish, *C. arel*, was purchased from a local fish market in Abadan city, Khuzestan province, Iran. Fish were freshly caught and completely free of additives. The fish were kept in the ice with a fish/ ice ratio of 1:2 (w/w) and transported to the seafood processing laboratory with 1 h. Upon arrival, fish were washed in cold water. Each fish was carefully filleted by hand. Two fillets were obtained from each fish after removing the head and bone. The fillets had an average weight of 100 g. All treatments are listed as follows:

1) Uncoated control (C): a dip treatment in distilled water for 20 min

2) Acid control (AC): a dip treatment in 1% glacial acetic acid for 20 min

3) Chitosan (Ch): a dip treatment in 2% chitosan solution for 20 min

4) Nano-chitosan (Nch): a dip treatment in 2% nanochitosan solution for 20 min

Then the fillets were removed and allowed to drain for 2 hrs at ambient temperature (20 ºC) on a pre-sterilized metal net under a biological containment hood in order to form the edible coatings. All treatments

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maintained at super-chilling storage (-3 ºC) and were taken for microbiological, physicochemical, sensorial analyses every 4 days for up to 16 days.

Microbiological analysis

Samples were collected aseptically. The samples (25 g) were placed in a stomacher bag containing 225 ml of 0.85% saline water. After mixing for 1 min in a stomacher blender, further serial dilution was done using the same diluent. Thereafter, 0.1 ml of appropriate dilution was used for microbiological analysis by spread plate method. The media and condition used were: Plate Count Agar (PCA, Merck, Denmark, Germany) incubated for psychrotrophic bacteria count at 4 °C for 10 days and for mesophilic bacteria at 30 °C for 24-48 hrs and eosin methylene blue agar incubated at 37 ºC for 24 hrs for Enterobacteriaceae count. The microorganism value was expressed as $\log_{10}$ CFU g^{-1} (Sallam, 2007).

Physicochemical analysis

Determination of total volatile base nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) value was estimated by the micro-diffusion method (Goulas and Kontominas, 2005). The micro diffusion method was determined by distillation after the addition of MgO to homogenised fish sample. The distillate was collected in a flask containing aqueous solution of boric acid and methyl red as an indicator. Afterward, the boric acid solution was titrated with 0.1 N sulphuric acid (H_2SO_4) solutions. The TVB-N value (mg N 100g of fish) was determined according to the consumption of sulphoric acid. The constant 14 was used to calculate the TVB-N number using Eq. 1.

TVB-N value=14×V (1)

V=ml of sulphuric acid (H_2SO_4) solution for titration

Determination of pH

The measurement of pH was carried out on 10 g of sample homogenised in distilled water (1/10 sample/ water). The pH value of the sample was determined using a digital pH meter (Suvanich *et al*., 2000).

Determination of TMA

One hundred grams of fish muscle were deproteinized and the filtrate was collected. TMA was assayed as the picrate salt by colorimetry. One milliliter of filtrate and 3 ml of water were added to a test tube. Three other test tubes received 1, 2 and 3 ml of a standard TMA solution (concentration= 0.01 mg ml^{-1}) and 3, 2 and 1 ml of distilled water, respectively. The TMA stock solution was prepared by adding 1 ml of HCl to 0.682 g of TMA and making up to a final volume of 100 ml with distilled water. To prepare the TMA standard solution, 1 ml of the stock solution was mixed with 1 ml of HC1 and diluted to 100 ml with water. A 5th tube containing 4 ml of water was used as a blank for colorimetry. One milliliter of a 20% formaldehyde solution, 10 ml of toluene and 3 ml of saturated potassium carbonate solution were placed in the 5 tubes. The 20%

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formaldehyde solution was prepared as follows: 100 g of magnesium carbonate and 1 L of commercial formaldehyde (40%) were shaken and filtered. One hundred milliliters of this stock solution were diluted to 200 ml with water. The 5 tubes were shaken vigorously for 40 s; 8 ml of the toluene phase were then transferred to a tube containing 0.2 g of anhydrous sodium sulfate and shaken until dehydrated. Five milliliters of the dehydrated toluene phase were mixed in another tube with 5 ml of picric acid solution prepared by diluting 1 ml of a picric acid stock solution to 100 ml with toluene. The picric acid stock solution was prepared by dissolving 2 g of picric acid in 100 ml of toluene and spectrophotometer at a wavelength of 410 nm.

Determination of 2-thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid (TBA) measurement was determined following the method of Siripatrawan and Noipha (2012). Ten grams of homogenized sample were added with 97.5 ml of distillated water and 2.5 ml of 4N HCl. The mixture was heated with steam distillation. Five ml of distillate was added to 5 ml of thiobarbituric reactive reagent containing 0.02 M TBA in 90% glacial acetic acid and incubated in boiling water for 35 min. After cooling, the absorbance of the pink solution was measured at 538 nm using a spectrophotometer. The constant 7.8 was used to calculate the TBA number using Eq.2 (Siripatrawan and Noipha, 2012). The TBA value is expressed as mgmalonaldehyde kg^{-1} sample.

$$\text{TBA value} = 7.8Abs_{538} \quad (2)$$

Determination of free fatty acid (FFA)

The free fatty acids value was determined in the lipid extract by the procedures of Woyewoda *et al*. (1986) according to the Eq. 3. Results were expressed in % of oleic acid.

Preparation of actomyosin

Fish muscle (0.5 g) was transferred to the appropriately labeled microfuge tube containing Laemmli sample buffer. The microtube was flicked 15 times with finger to mix the fish tissues into the sample buffer. Alternatively, the sample is vortexed for a few seconds. The samples were incubated for 5 minutes at room temperature to extract and solubilize the proteins. The buffer containing the extracted proteins was pipetted into a new 1.5 ml screw cap microtube. Fish protein samples were boiled (about 100ºC, 5 mins), as well as the purified actin and myosin samples. Additionally, protein standards (PageRuler^{TM} Plus Prestained Protein Ladder, 10 to 180 kDa) were boiled to denature the proteins in preparation for electrophoresis.

Determination of total sulfhydryl content

One milliliter of actomyosin (0.4 g ml^{-1}) solution was added to 9 ml of Tris-HcL buffer (0.2 M), pH 6.8, containing urea (8 M), SDS (2 g $100ml^{-1}$) and EDTA (10 mM). To a 4 ml aliquot of the mixture, 0.4 ml of DTNB (0.1 g 100 ml^{-1}) solution was added. The mixture was incubated at 40 ºC for 25 mins and the absorbance was measured at 412 nm with a spectrophotometer. A blank was

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prepared by replacing the sample with KCl using the molar extinction coefficient of 13600 M^{-1} cm^{-1} and was expressed as mol 10^{-5}g protein (Masniyom *et al*., 2005).

Sensory evaluation

Samples were prepared by steaming for 60 mins at 80 °C. Salt (1.5%) was added. The cooked samples were evaluated by 15 panelists from the Department of seafood processing with the ages of 23-28 (16 females and 4 males), using the 5-point hedonic scales where 5: like extremely; 3: neither like or nor dislike; 1: dislike extremely. Panelists were regular consumers of fish and had no allergies to fish. All panelists were asked to evaluate for texture, odor, flavor and likeness.

Statistical analyses

All experiments were performed in triplicate and a completely randomised design was used. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range tests. P values less than 0.05 were considered statistically significant.

Results

Particle size and zeta potential of chitosan nanoparticles

According to Muller *et al*. (2001), the size (including size distribution) and zeta potential are essential characteristic parameters for nano-suspensions. Size distribution profile of the chitosan nanoparticles is shown in Fig. 1A. The mean particle size (nm) of Ch-TPP nanoparticle was 120.3 with a narrow size distribution (width: 57.68 nm; polydispersity index1.00). The chitosan nanoparticles had a zeta potential of + 28.90 mV (Fig. 1B). It was observed that Ch-TPP nanoparticles were stable because all of the Ch-TPP nanoparticles had a zeta potential >30 mV. Zeta potential values reflect the density of the particle surface charge (Gan *et al*., 2005).

Microbial analysis: total mesophilic counts (TMC), psychrotrophilic bacteria (PTC) and Enterobacteriaceae

Variations in the values of total mesophilic counts (TMC), psychrotrophilic bacteria (PTC) and Enterobacteriaceae during super-chilling storage are presented in Table1. Values of TMC, PTC and Enterobacteriaceae increased during the storage period. But, the PTC multiplied at a higher rate than TMC and dominated the bacterial flora of the fish fillets stored at super-chilling condition. The increase in bacteria count of the fish samples is accompanied with the formation volatile bases and reduces in sensory properties. Coating of fish samples with Ch and Nch caused a reduction in the bacterial count afterwards, the count of bacteria gradually increased, indicating the antimicrobial activity of chitosan and chitosan nanoparticles. However, on day 16, TMC and PTC of fillets treated with Nch were significantly lower than that treated with Ch (p<0.05). During the storage time, samples coated with nano-chitosan had the lowest Enterobacteriaceae, in comparison with the control, acid

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control and chitosan coated samples (p<0.05).

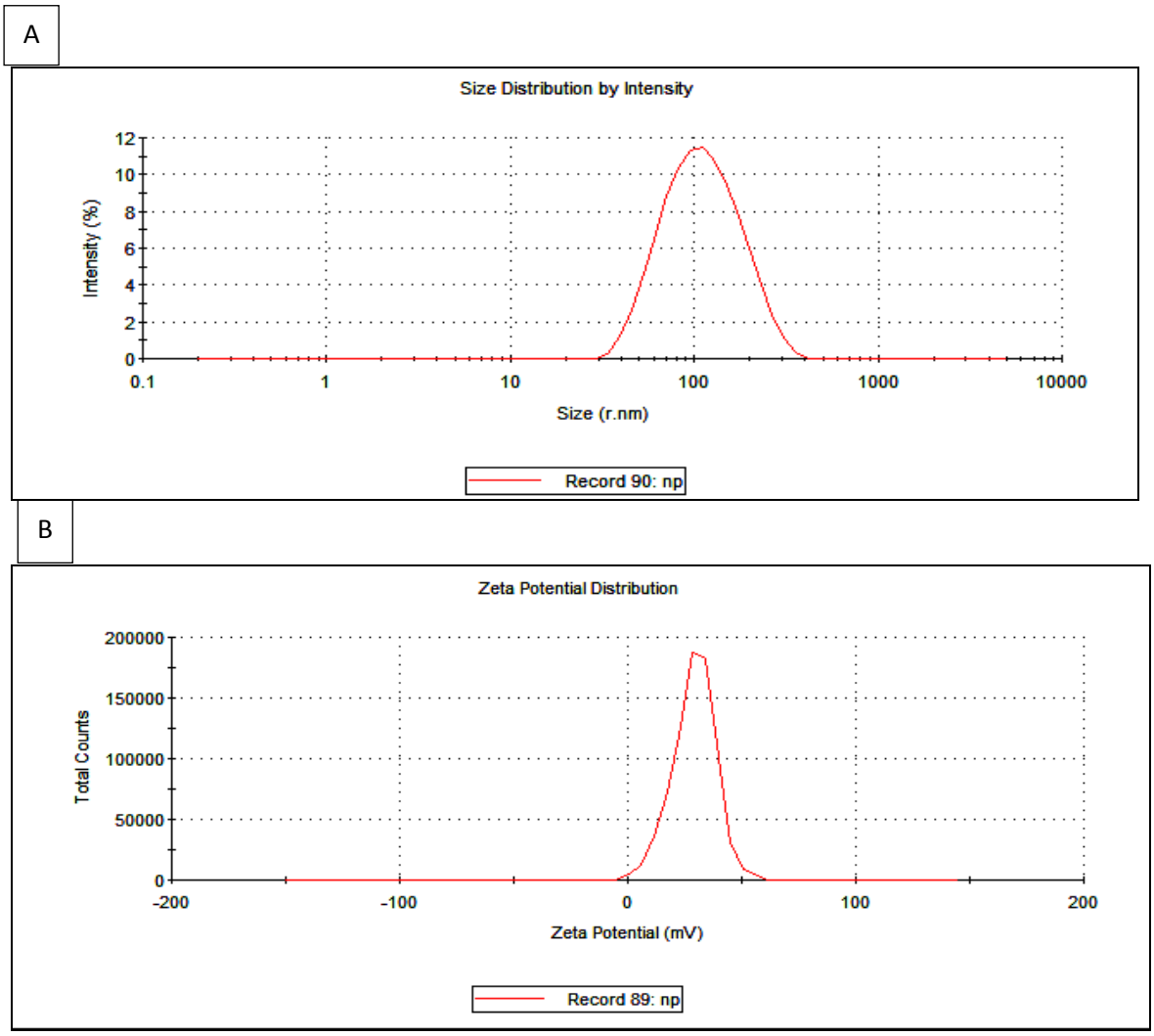


Figure 1: Particles size (A) and zeta potential distribution (B) of chitosan nanoparticles.

Table 1: Bacterial changes of *Cynoglossus arel* fillets coated by chitosan and nanochitosan.

Days of storage		0	4	8	12	16
TVC (log_{10} CFU g^{-1})	Control	2.83 ± 0.01^{Ae}	3.28 ± 0.07^{Ad}	4.55 ± 0.02^{Ac}	5.41 ± 0.08^{Ab}	6.13 ± 0.07^{Aa}
	AC	2.83 ± 0.01^{Ae}	2.95 ± 0.02^{Bd}	3.50 ± 0.14^{Bc}	4.61 ± 0.06^{Bb}	5.09 ± 0.25^{Ba}
	Nch	2.83 ± 0.01^{Ae}	2.52 ± 0.07^{Cb}	2.70 ± 0.01^{Db}	3.13 ± 0.04^{Da}	3.30 ± 0.1^{Da}
	Ch	2.83 ± 0.01^{Ae}	2.79 ± 0.04^{Bcd}	2.97 ± 0.5^{Cc}	3.73 ± 0.14^{Cb}	4.00 ± 0.05^{Ca}
PTC (log_{10} CFU g^{-1})	Control	3.37 ± 0.01^{Ae}	4.22 ± 0.05^{Ad}	4.82 ± 0.03^{Ac}	5.29 ± 0.1^{Ab}	6.67 ± 0.07^{Aa}
	AC	3.37 ± 0.01^{Ae}	3.86 ± 0.04^{Bd}	4.55 ± 0.02^{Bc}	4.95 ± 0.01^{Bb}	5.41 ± 0.09^{Ba}
	Nch	3.37 ± 0.01^{Ae}	3.45 ± 0.02^{Dd}	3.65 ± 0.03^{Dc}	3.93 ± 0.02^{Db}	4.25 ± 0.1^{Da}
	Ch	3.37 ± 0.01^{Ae}	3.66 ± 0.02^{Cd}	3.92 ± 0.1^{Cc}	4.28 ± 0.05^{Cb}	4.82 ± 0.05^{Ca}
Enterobacteriaceae (log_{10} CFU g^{-1})	Control	2.45 ± 0.05^{Ae}	3.31 ± 0.09^{Ad}	3.96 ± 0.07^{Ac}	4.36 ± 0.03^{Ab}	5.45 ± 0.16^{Aa}
	AC	2.45 ± 0.05^{Ae}	2.99 ± 0.008^{Bd}	3.40 ± 0.008^{Bc}	4.00 ± 0.03^{Bb}	4.29 ± 0.01^{Ba}
	Nch	2.45 ± 0.05^{Ae}	2.65 ± 0.04^{Cc}	2.75 ± 0.03^{Dbc}	2.85 ± 0.01^{Dab}	2.95 ± 0.03^{Da}
	Ch	2.45 ± 0.05^{Ae}	2.91 ± 0.1^{Bc}	2.96 ± 0.03^{Cc}	3.13 ± 0.06^{Cb}	3.38 ± 0.05^{Ca}

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Physicochemical analysis

Changes in total volatile basic nitrogen (TVB-N) value

Table 2 showed the variation of TVB-N value of large-scale tongue-sole fish during storage. The initial TVB-N varied from 7.00 mg N $100g^{-1}$ of fish indicated that the large-scale tongue-sole fish was of good quality. The TVB-N level gradually increased along with the time of storage in all samples ($p< 0.05$), but the increasing rate varied with treatments. The rate of TVB-N enhancement was significantly slower in chitosan and nano-chitosan coated samples than the control samples.

Table 2: Physicochemical changes of *Cynoglossus arel* fillets coated by chitosan and nanochitosan.

Days of storage		0	4	8	12	16
TVBN (mg N $100g^{-1}$ muscle)	Control	7.00 ± 0.80^{Ae}	11.2 ± 0.8^{Ad}	20.2 ± 0.4^{Ac}	25.9 ± 1.21^{Ab}	35.00 ± 0.8^{Aa}
	AC	7.00 ± 0.80^{Ad}	9.8 ± 0.8^{ABc}	16.7 ± 0.86^{Bb}	19.40 ± 0.2^{Ba}	21.6 ± 0.26^{Ba}
	Nch	7.00 ± 0.80^{Ab}	7.7 ± 0.4^{Bb}	11.9 ± 0.4^{Ca}	12.6 ± 0.8^{Da}	13.30 ± 0.4^{Da}
	Ch	7.00 ± 0.80^{Ac}	10.86 ± 0.87^{Ab}	12.6 ± 0.8^{Cb}	16.10 ± 0.4^{Ca}	16.40 ± 0.5^{Ca}
pH	Control	6.13 ± 0.15^{Ac}	6.62 ± 0.13^{Ab}	6.58 ± 0.11^{Ab}	6.84 ± 0.06^{Aab}	7.13 ± 0.02^{Aa}
	AC	6.13 ± 0.15^{Abc}	5.89 ± 0.1^{Cc}	5.82 ± 0.23^{Bc}	6.49 ± 0.05^{Bab}	6.83 ± 0.07^{Ba}
	Nch	6.13 ± 0.15^{Aa}	6.31 ± 0.03^{ABa}	6.19 ± 0.06^{ABa}	6.35 ± 0.03^{Ba}	6.47 ± 0.14^{Ca}
	Ch	6.13 ± 0.15^{Ab}	6.24 ± 0.15^{Bb}	6.24 ± 0.1^{ABb}	6.32 ± 0.15^{Bb}	6.71 ± 0.04^{BCa}
TMA (mg N $100g^{-1}$ muscle)	Control	2.32 ± 0.51^{Ad}	4.52 ± 0.76^{Bc}	7.62 ± 0.51^{ABb}	8.81 ± 0.33^{Bb}	10.72 ± 0.25^{Ba}
	AC	2.32 ± 0.51^{Ad}	8.06 ± 0.25^{Ac}	8.95 ± 0.25^{Ac}	11.12 ± 0.53^{Ab}	$12.93\pm.51^{Aa}$
	Nch	2.32 ± 0.51^{Ad}	3.46 ± 0.25^{Bcd}	4.52 ± 0.76^{Cbc}	5.41 ± 0.25^{Dab}	6.29 ± 0.25^{Da}
	Ch	2.32 ± 0.51^{Ad}	4.96 ± 0.5^{Bc}	6.29 ± 0.76^{BCbc}	7.18 ± 0.25^{Cab}	8.06 ± 0.25^{Ca}
TBA mg MDA kg^{-1} muscle	Control	0.15 ± 0.04^{Ac}	0.27 ± 0.02^{Bc}	0.58 ± 0.06^{Ab}	0.74 ± 0.02^{Aa}	0.58 ± 0.02^{Bb}
	AC	0.15 ± 0.04^{Ac}	0.46 ± 0.08^{Ab}	0.70 ± 0.04^{Aa}	0.66 ± 0.02^{Aa}	0.66 ± 0.02^{Aa}
	Nch	0.15 ± 0.04^{Ab}	0.11 ± 0.02^{Bb}	0.30 ± 0.03^{Ba}	0.31 ± 0.04^{Ca}	0.27 ± 0.02^{Db}
	Ch	0.15 ± 0.04^{Ab}	0.23 ± 0.04^{Bc}	0.34 ± 0.02^{Ba}	0.42 ± 0.02^{Ba}	0.42 ± 0.02^{Ca}
FFA (% oleic acid)	Control	0.48 ± 0.03^{Ae}	0.89 ± 0.13^{Ad}	1.44 ± 0.03^{Ac}	1.83 ± 0.82^{Ab}	2.46 ± 0.04^{Aa}
	AC	0.48 ± 0.03^{Ad}	0.90 ± 0.07^{Ac}	1.07 ± 0.06^{Bbc}	1.35 ± 0.03^{Bb}	1.99 ± 0.15^{Ba}
	Nch	0.48 ± 0.03^{Ac}	0.68 ± 0.15^{Ac}	0.92 ± 0.03^{Cb}	1.19 ± 0.04^{Bab}	1.24 ± 0.03^{Ca}
	Ch	0.48 ± 0.03^{Ad}	0.70 ± 0.08^{Ac}	0.97 ± 0.51^{BCb}	1.35 ± 0.03^{Ba}	1.41 ± 0.00^{Ca}
SH (mol $10^5 g^{-1}$ protein)	Control	4.63 ± 0.49^{Aa}	3.45 ± 0.02^{Bb}	3.83 ± 0.16^{Aab}	2.1 ± 0.25^{Cc}	1.75 ± 0.13^{Aa}
	AC	4.58 ± 0.3^{Aa}	3.73 ± 0.14^{ABa}	4.08 ± 0.21^{Aa}	2.81 ± 0.39^{BCb}	1.50 ± 0.38^{Ba}
	Nch	4.31 ± 0.24^{Aa}	4.16 ± 0.17^{Aa}	4.33 ± 0.33^{Aa}	4.09 ± 0.1^{Aa}	3.21 ± 0.21^{Ca}
	Ch	4.18 ± 018^{Aa}	3.80 ± 0.15^{ABab}	4.24 ± 0.19^{Aa}	3.48 ± 0.26^{ABb}	2.78 ± 0.21^{BCa}

Changes in pH value

Changes in the pH of *C. arel* muscles during storage are presented in Table 2. The initial pH of all samples was found 6.13. After day 12, the pH value increasing was lower in fish coated with Nch, compared with the Ch ($p<0.05$).

Changes in trimethylamine (TMA) value

The initial TMA value of control samples was 2.32 mg N 100g sample which increased up to 10.72 mg N 100g sample at the end of the storage period (Table 2).

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Changes in TBA value

The TBA index has been widely used as an indicator of lipid oxidation degree. TBA values of fish stored in super-chilling are presented in Table 2. At 0 day, TBA value of all samples was found 0.15 mg malonaldehyde kg^{-1} muscle. In this study, from day 8 of storage, no significant difference was found among the Ch and Nch, however, on the last day of storage, the TBA value of the control sample was significantly higher than Ch and Nch. Nch had lower TBA values than the other treatments.

FFA

Both the primary and secondary oxidation products have been assessed to consider the complexity of the lipid oxidation process. The initial FFA value was 0.48 % of oleic acid (Table 2). As it was concluded from FFA values, chitosan and nano-chitosan coatings protect *C. arel* fillets so would reduce production of free fatty acids.

Total sulfhydryl (SH)

The changes in total SH content of actomyosin extracted from *C. arel* fillets under different conditions are shown in Table 2. In all fish samples, the values of SH decreased during super-chilling storage in samples. According to the results, it was suggested that SH groups in *C. arel* muscles underwent oxidation to the highest extend when coated in acetic acid, especially as the storage time increased. The rate of oxidation was lower in coated sample under chitosan and nanochtosan. After 12 days of storage, coated samples under nanochitosan the highest SH content.

Sensory evaluation

The result of the sensory evaluation was correlated with the microbial and chemical analysis (Tables 1,2). The results of the sensory evaluation (odor and flavor) of cooked *C. arel* fillets are presented in Table 1. The sensory evaluation results showed that texture, odor, flavor and likeness scores decreased with increasing storage time. For control samples, the deterioration occurred after 4 days of storage as evidenced by strong fishy and putrid odor. Also the deterioration in favor occurred after 4 days during storage at super-chiling condition.

Discussion

The initial TMC, PTC and Enterobacteriaceae (log10 CFU g^{-1}) in the large-scale tongue-sole fillet were 2.83, 3.37 and 2.45 log_{10} CFU g^{-1}, respectively, indicating a high quality of the fish fillet (Sikorski *et al*., 1990). Treatment of fish coated with Nch could retard the growth of total viable bacteria and psychrotrophilic bacteria more effectively, compared with Ch due to the higher antimicrobial activity of nano-chitosan compared to chitosan due to their higher surface area per unit volume and charge density which provides dreater interaction with the anionic bacteria cell membrane (de Azeredo, 2013). A microbiological acceptability limit is 7 log CFU g^{-1} for fresh water and marine species that is suitable for human consumption (ICMSF, 1986). All samples coated with Ch and Nch and uncoated did not

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achieve this count to the end of storage time.

Chitosan coating has been reported to be effective as antimicrobial agent (Jeon, 2002; López-Caballero *et al*., 2005; Ojagh *et al*., 2010). The antimicrobial effects of chitosan are thought to be related to the presence of the positive charge on the NH_3^+ group of glucosamine monomer in chitosan molecules which interact with negatively charged macromolecules on the microbial cell surface, leading to the leakage of intracellular constituents of the microorganisms. Moreover, the chitosan action mechanism appears to be related to disruption of the lipopolysaccharide layer of the outer membrane of gram-negative bacteria (Pereda *et al*., 2011), and also to its function as a barrier against oxygen transfer (Jeon *et al*., 2002). Ojagh *et al*. (2010) demonstrated that and edible antimicrobial coating solution incorporating chitosan and cinnamon oil was effective in controlling the total mesophilic and psychrophilic counts of fresh rainbow trout during refrigerated storage. Similarly, Nowzari *et al*. (2013) reported that chitosan-gelatin coating and film in rainbow trout fillets extended the shelf life of fillets during refrigerated storage.

In comparison of antimicrobial activity of chitosan and nano-chitosan, studies on the antimicrobial activity of chitosan nanoparticles are still limited. Shi *et al*. (2006) reported that chitosan nanoparticles exhibit higher antibacterial efficacy against *E.coli*, *S.aureus*, and *S.typhimurium* than chitosan based on the special character of the nanoparticles such as nanoparticle´s longer surface area and higher affinity with bacteria cell for a quantum-size effect. Ramezani *et al*. (2015) reported that nano-chitosan coating is more appropriate than chitosan coating to extend the shelf-life and delay the deterioration of fresh silver carp fillets during refrigerated storage. Ibrahim *et al*. (2015) reported that chitosan nanoparticles exhibit higher antibacterial activity against gram-positive bacteria than gram-negative bacteria. But Sadeghi *et al*. (2008) showed that chitosan nanoparticles have less inhibition effect on *S. aureus* than polymer of chitosan in free soluble form because nanoparticles have less positive charge available to bind to the negatively charges of bacterial cell. In this study, it can be clearly observed Nch showed little antibacterial activity even at the higher quality of sample compared to Ch.

The TVB value, an indicator of spoilage, usually include calculation of trimethylamine, dimethylamine, ammonia and other volatile bases, which impart characteristic off-flavors to fish (Goulas and Kontominas, 2005). TVB is products of bacterial spoilage such as *S. putrefaciens* and *P. phosphoreum*, autolytic enzymes and endogenous enzymes, which used as index to assess the keeping quality and shelf life of seafood products. A level of 25 mg N $100g^{-1}$ muscle has been considered the highest acceptable level (Kilincceker *et al*., 2009). At the day 16^{th} of storage, TVBN level of AC, Nch and Ch was less than 25 mg N $100g^{-1}$

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muscle, indicating that the fillets of fish maintained at a good quality during storage. At the end of the storage, nano-chitosan samples had significantly (p<0.05) lower TVB-N values than other treatments. The longer storage period of chitosan treated samples compared to untreated samples may have been due to a lower microbial counts which breakdown compounds like trimethylamine oxide (TMAO), peptides, amino acids, etc., resulted in an decrease in the basic nitrogen fraction for. To our knowledge, no information is available in the literature on the effects of nanochitosan coating on TVBN production in *C. arel*. Zarei *et al*. (2015) found that treated of silver carp with Nano-chitosan could retard the TVBN content increasing compared other treatments. Based on these TVB-N values, control large-scale tongue-sole fillet samples are expected to have a shelf-life of 8 days. The other treatments extended the large-scale tongue-sole's shelf-life to 16 days.

During the storage time, the pH values gradually increased, presumably due to accumulation of basic compounds generated from both autolytic processed by endogenous enzymes and microbial enzymatic actions (Nirmal and Benjakul, 2011), although it could also be associated with the increase in bacterial counts especially psychrtrophic bacterial counts. At the day 16th of storage, pH values of samples treated with Nch and Ch were lower than control (p>0.05), due to its property of inhibiting the growth of bacteria, yeasts and moulds (Shahidi *et al*., 1999). The similar pH value was observed for samples coated with Nch and Ch at day 12 of super-chilling storage (p>0.05). These results were in agreement with the lower microbial growth in fish treated with Nch. A similar observation was made by Ramezani *et al*. (2015) that lowered increasing rate of pH was observed in the fish treated with Nch, compared with the control and those treated with AC and Ch. Thus, the lowered increase in pH value was more likely related with the lower level of volatile basic amines in the muscle tissue during super-chilling storage. The acceptable upper limit for the pH of fish is 6.8-7. In this study, the pH values of all samples except control sample did not exceed this limit.

The present study revealed that TMA value of control *C. arel* fillets increased during storage but Nano-chitosan-coated and chitosan-coated of fillet retarded the decomposition of trimethylamine N-oxide caused by bacterial spoilage and enzymatic activities. This reduction in TMA production when using chitosan-coated samples in fish has also been reported by Günlü and Koyun, 2. Acceptability limits of TMA for various fish species are different: sea bass (5 mg N 100g^{-1}) (Masniyom *et al*., 2002); sardines (5-10 mg N 100g^{-1}) (Özogul *et al*., 2004); hake (12 mg N 100 g) and 10-15 mg N 100g^{-1} as a general limit for fish (Connell, 1990). Such variations in the limit values of fish may be related to the fish species, season, initial bacterial count and storage conditions (Connell, 1990).

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In the current study, TBA values for control sample, AC, Ch and Nch were 0.58, 0.66, 0.42 and 0.27 mg malonaldehyde kg^{-1} sample, respectively, at the end of the storage. These results indicated, using of chitosan and nano-chitosan coatings can reduced the degree of lipid oxidation in fish tissue during super-chilling storage at -3 ºC. This may be due to chitosan´s ability to scavenge hydroxyl radicals. Zhang *et al*. (2008) believed that the use of chitosan-tripolyphosphates nanoparticles retains antioxidant activity in vitro using a free radical scavenging activity test and reducing power test. This may be due to the small particle size and high surface area per unit volume of chitosan nanoparticles which improved the scavenging effect of OH radicals by chitosan. Ramezani *et al*. (2015) indicated that TBA content of fresh silver carp did not significant difference between Ch and Nch groups during refrigerated storage. The increase in TBA of samples during storage may be attributed to the partial dehydration of fish and interacting lipids with air oxygen (Kilincceker *et al*., 2009). These results suggested that oxidation of lipid in fish samples could be minimized by using of chitosan coating probably due to the antioxidant activity as well as its low oxygen permeability characteristic of chitosan. The antioxidant mechanism of chitosan could be through chelating action of ion metals and/or the combination with lipids of meat during storage (López-Caballero *et al*., 2005). TBA value of 5 to 8 mg malonaldehyde kg^{-1} muscle is an acceptable sensory limit (Sallam, 2007).

Due to hydrolysis of phospholipids and triglycerides because of lipases and phospholipases (Rostamzad *et al*., 2011), a gradual increase in FFA formation in all samples was observed, but FFA value of control samples was higher than treated samples, significantly (p<0.05). Rostamzad *et al*. (2011) showed that free fatty acids are known to undergo further oxidation to produce low molecular weight compounds that are responsible for off-flavor and undesirable taste of fish and fish products. This study showed that Nch can reduce FFA content in samples treated with nano-chitosan compared with other treatments due to lower microbial count.

The functional and textural characteristics of seafood depend mainly on myofibrillar proteins and actomyosin, which is the main protein in myofibrils. Changes in the composition of actomyosin result in changes to the functional groups, such as sulfhydryl groups and hydrophobic groups, and physicochemical properties such as ATP activity (Hayakawa and Nakai, 1985). The reduction of SH content may be explained by the denaturation and aggregation of muscle proteins as a result of cysteine thiol group oxidation, located at the catalytic center of the myosin head, or disulfide interchanges, leading to the formation of disulfide bonds (Hayakawa and Nakai, 1985). The reduction of SH content in control samples might be due to the sulfhydryl groups forming cross-linkages or the exposed sulfhydryl

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groups in protein interacting with additives or small molecular weight compounds in the water soluble protein fraction (Leelapongwattana *et al*., 2005). From the result, Nano-chitosan could retard the oxidation of SH group in muscle proteins, which might be associated with the denaturation of muscle proteins. In super-chilled fish, oxidation of sulfhydryl groups and the increase in TVBN were retarded by the effect of chitosan and nano-chitosan and this was coincidental with the decrease of disulfide bond formation.

Fresh *C. arel* fish fillets were generally considered to possess very high acceptability. Sensory attributes of fish were divided into 4 elements, whose preference levels were scored from 1 to 5, the higher preference level, the higher score. All samples started with score of 5. Upon the judgment made by the members of a sensory panel, odor and flavor were given unacceptable scores by the 16^{th} day. On the other hand, the sensory scores of fillets coated with nano-chitosan were in the range of 3.35- 3.85, after 16 days of storage. Among treatments, the highest score was recorded for the samples coated with nano-chitosan. Odor and taste showed a similar pattern of decreasing acceptability. The antioxidant and antimicrobial effects of chitosan and nano-chitosan coatings has been shown to prolong the shelf life of fish by 16 days as compared to the control sample. The result suggested that the chitosan and nano-chitosan had no significant effect in maintaining the quality the *C. arel* fillets.

Overall, it was generally observed, and particularly clear in results obtained in TVB-N and bacteriological analysis that both chitosan and nano-chitosan coatings were effective on bacterial contamination of *C. arel* fillets during super-chilling storage. However, chitosan nanoparticles had higher antimicrobial activity than chitosan during super-chilling storage. From the result, it was found that using a coating of chitosan and nano-chitosan had significant effects on reduced bacteria population or decreased capacity of bacteria or both. Furthermore, nano-chitosan treatment could minimize the microbial growth. These chitosan and nano-chitosan coatings also showed antioxidant effects, since TBA and FFA values was lower than control samples at the end of the storage time. Moreover, the protective effects of nano-chitosan against lipid oxidation were more than chitosan, because the migration of chitosan active agents is more available in solution form to present its antioxidant effect. This study demonstrated the potential of nano-chitosan solutions as active packaging that can be utilized as a safe preservative for fish under super chilling storage.

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