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Effects of Carboxymethyl Chitosan on the Biochemical and Microbial Quality of Dried Anchovy

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Abstract

Carboxymethyl chitosan (CMCH) is one of the water soluble derivatives of chitosan that can be used as an edible coating for food products. However, its use in fish and fish products is not well established. So, in the present study, the effects of CMCH as an edible coating for preserving the quality of dried anchovy were evaluated. Anchovies were dipped in 0.5 and 1% (w/v) solutions of CMCH for 5 min, drained and dried under sun. Control samples were prepared by dipping in potable water and drying under sun. TVB-N and TMA-N of the treated samples were significantly ($p<0.05$) lower than control over 135 days storage period. At the end of 135 days, TVB-N and TMA-N levels were 121.95, 115.63 and 106.72 mg% and 47.13, 38.24 and 35.43 mg% for control, 0.5% CMCH treated and 1% CMCH treated samples, respectively. Microbial growth and fungal growth were lower in 1% CMCH treated sample. Aerobic plate count at the end of 135 days reached 4.89, 4.80 and 4.70 log10 cfu g^{-1} respectively, for control, 0.5% CMCH and 1% CMCH treated anchovies. Fungal count in control reached 1.93 log10 cfu g^{-1} whereas that of 1% CMCH treated anchovy was 1.50 log10 cfu g^{-1} at the end of 135th day. CMCH treatment has also controlled lipid oxidation during storage of dried anchovy as the TBARS of control was higher (2.91 mg MDA Kg^{-1}) than 0.5% CMCH (2.17 mg MDA Kg^{-1}) and 1% CMCH treated (1.92 mg MDA Kg^{-1}) anchovy on 135th day. In brief, the edible coating of CMCH has effectively controlled the spoilage changes in dried anchovy during storage.

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Introduction

Edible coatings and films have received significant role in food packaging because of their advantages compared to traditional synthetic packaging. Majority of edible film coating agents are contributed by underutilized edible polysaccharides, lipids and protein, which can act as carriers of bioactive components (Umaraw et al., 2020). The most important advantage of these materials is that they can be consumed along with the packaged food and hence, will not cause any environmental threat. The increased attention towards edible coating is driven by increased interest in safe, ready to eat convenient and stable food and improved awareness on the environmental issues created by synthetic packaging materials (Dehghani et al., 2018). Fish is highly susceptible to spoilage and drying is a traditional method employed to preserve the fish for a longer period at room temperature. Nevertheless, dried fish are prone to microbial and fungal attack and fat oxidation during the course of storage which deteriorates its quality. Packaging of dried fish is a crucial factor determining its quality during storage. The most important requirement of dried fish is a moisture and oxygen proof packaging material to control the migration of moisture and air to alter the properties of dried product (Rahman, 2007). Low density polyethylene (LDPE), polypropylene (PP) or polyester polyethylene laminated materials are used as the common packaging materials for consumer pack of dried fish. Though these materials have good tensile strength and are readily available, they have high oxygen and water vapour transmission rates (Gopal et al., 1998).

Among the sources used for edible packaging, polysaccharides have a vital role as it is widely available and cost effective. Chitosan extracted from the shell of crustaceans is a biocompatible and biodegradable polysaccharide having strong antimicrobial, antioxidant and antifungal properties (Li et al., 2013). Chitosan is approved as GRAS by USFDA in 2001 and has received wide attraction as a potential food preservative (Sagoo et al., 2002). Efficacy of chitosan to improve the quality of seafood during refrigerated or chilled storage is well established by numerous researchers. (Gunlu & Koyun, 2013; Nowzari et al., 2013; Soares et al., 2013). Nevertheless, the poor solubility of chitosan at neutral pH poses practical difficulties in its applications to a wide variety of foods. To overcome this technological demerit, various chemical modifications have been suggested by several researchers to alter the solubility of chitosan (Binsi et al., 2019). Among these, carboxymethylation has achieved a great success in imparting better water solubility to chitosan (Wang et al., 2009). The unique properties of CM-chitosan such as large hydrodynamic volume, high viscosity, film- and gel-forming capabilities, and antibacterial properties, besides its excellent solubility at neutral pH increases its use in processing and preservation of wet and dry commodities. However, the application of CMCH as an additive in fish processing has not been well exploited.

Anchovy is a small, common forage fish of the family *Engraulidae*. Anchovies are usually classified as oily fish and they are used in small quantities to flavor many dishes. In India, dried anchovy is a delicacy in many of the coastal states. The aim of the present study was to evaluate the efficacy of a CMCH coating on the quality of dried anchovy stored under ambient conditions.

Materials and Methods

Anchovy (average length 6.5 cm and weight 30 g) was purchased from Visakhapatnam Fishing harbor and brought to laboratory within half an hour in chilled condition. Carboxymethyl chitosan was procured from fish processing division of ICAR-Central Institute of Fisheries Technology, Kochi. Chemicals used in the study were purchased from Merck and Himedia, India. Glass wares used were purchased from Borosil, India.

Anchovies were washed thoroughly in potable water and drained over a mesh tray. It was then portioned in to three batches. One batch was sun dried without CMCH coating (here in as control). Another batch was coated with 0.5% (w/v) CMCH solution by dipping it in CMCH solution @ 1:1 ratio (w/v) for 5 min, drained and sun dried. For sun drying, the samples were spread on the rack placed on raised bamboo rack and allowed to dehydrate under sun for a period of two days. The third batch was coated with 1% (w/v) CMCH solution by dipping @ 1:1 ratio (fish: solution, w/v) for 5 min, drained and sun dried. During two days of sun drying, an average temperature of 34°C, 67% humidity and 21 km h^{-1} wind velocity was observed. The samples were allowed to dry till the moisture content was found to be sufficiently below 20%, by visual observation. It was then packed in polyethylene pouches and stored at room temperature. Sampling was done at 45 days interval for a period of 135 days to analyse the changes in biochemical and microbial quality parameters.

Proximate composition of fresh and dried anchovies was evaluated by AOAC (1990) methods. pH of the samples was measured by a digital pH meter (LABMAN, LMPH-10) after homogenizing the sample in distilled water (1:5 w/v). Total volatile base nitrogen (TVB-N) and Trimethylamine (TMA-N) content were estimated by the microdiffusion method (Conway, 1950). Peroxide Value (PV) was determined following the method of Yildiz et al. (2003) and Thiobarbituricacid reactive substances (TBARS) was measured by the protocol given by Tarladgis et al., 1960 with slight modifications. Microbial count at 37°C and fungal count was determined by BAM (2011) method.

Results and Discussion

Proximate composition of fresh and dried anchovies is depicted in Table 1. The raw anchovy used in our experiment had a moisture content of 76.52% and the corresponding protein, fat and ash contents were 17.54, 2.06 and 3.52%, respectively. Protein content of fresh anchovy suggests anchovy as a high protein fish. Dewi (2002) categorised Japanese anchovy and Indonesian anchovy as a high protein fish, previously. A considerable variation in proximate composition of an individual fish is attributed to the variation in factors like nutrition, size, sex, age, environment and season (Huss, 1995). Moisture content of anchovy was reduced to 13.96-13.03% after drying (Table 1). Similar moisture content in dried anchovy was reported by Karim et al. (2017). Removal of moisture to a greater extent has led to

Table 1. Proximate composition of the dried anchovies

Parameter	Fresh anchovy	Control	0.5% CMC	1% CMC
Moisture (%)	76.52^{b}±1.02	13.03^{a}±0.43	13.86^{a}±0.5	13.96^{a}±0.74
Protein (%)	17.54^{a}±0.62	58.66^{b}±1.52	57.08^{b}±1.48	58.14^{b}±1.42
Fat (%)	2.06^{a}±0.20	6.94^{b}±0.48	6.72^{b}±0.36	6.98^{b}±0.54
Ash (%)	3.52^{a}±0.33	20.29^{b}±0.92	21.34^{b}±0.83	21.73^{b}±0.16

*values are mean (n=3) ± standard deviation. Different superscript letters in the same row indicate the values are statistically different

a significant increase ($p<0.05$) in concentration of other components in dried anchovy. Protein content had increased from 17.54% in fresh fish to 57-58% in dried fish. A significant increase ($p<0.05$) in ash and fat content was also noticed after drying as the values increased from 3.52% (fresh)- 20-21% (dried) and 2.06 (fresh) to 6.7-6.9% (dried), respectively. The ash content of dried anchovy in our experiment was in agreement with the ash content reported by Karim et al. (2017) which varied from 18-23% using different drying methods. Though the effect of CMCH coating on the protein, fat and ash of dried anchovy was not significant, ash content of the dried CMCH coated samples was slightly higher than uncoated sample. Presence of salt in CMCH which was used while modifying chitosan to CMCH might have attributed to the higher ash content in CMCH coated samples.

Variation in the moisture content of the dried samples during storage is shown in Fig. 1a. All the three samples displayed a significant increase ($p<0.05$) in moisture content during storage. Though the control sample had a moisture content comparable to the CMCH coated counterparts immediately after drying, its moisture content increased at a higher rate than other samples, indicating a moisture barrier effect of CMCH on the dried anchovy. On day 90 and afterwards, moisture content of control sample was significantly higher ($p<0.05$) than that of 1% CMCH coated anchovies. The increase in moisture content of dried samples during storage is on account of absorption of water from packaging environment. Olayemi et al. (2011) observed increased moisture content in smoke dried catfish stored in different packaging materials. Our findings suggest that coating with CMCH can control the moisture absorption in dried fish during storage. Jeyakumari et al. (2016) observed a low rate of moisture absorption in chitosan coated dried Bombay duck. In their study, moisture content of uncoated Bombay duck increased from 11.5-13% over a period of 3 months storage while chitosan coated sample registered only 1% increase in moisture at the end of storage period.

Total volatile base nitrogen (mainly comprises ammonia, primary, secondary and tertiary amines and trimethyl amines) is chiefly used as an index of biochemical spoilage in fish during storage (Fan et al., 2009). Variations in the mean TVB-N values of the sample are depicted in Fig 1b. Fresh fish had a TVB-N value of 11.75 mg%, indicating its good quality based on the acceptable limit (30-35 mg%) established for fresh fish (EC, 2008). After drying, TVB-N had significantly ($p<0.05$) increased in all the samples consequent up on the increased concentration of protein content in dried fish followed by moisture removal. During storage, control sample recorded the maximum content of volatile bases which was significantly ($p<0.05$) higher among its counterparts coated with CMCH, reaching a value of 121.95 mg% at the end of 135 days of storage. Since volatile bases are generated by microbial degradation of protein, the results of present study point out that coating of CMCH had controlled the spoilage of protein by microorganisms. The effect of coating significantly ($p<0.05$) increased with increase in concentration of CMCH.

There are many studies reporting the positive effect of chitosan in controlling the TVB-N accumulation in fish and fish products (Yu et al., 2017; Wu et al., 2018). Chitosan coating reduced the TVB-N accumulation by 51% during refrigerated storage in liquid smoked Tilapia fillet, demonstrating the effectiveness of chitosan coating to maintain fish quality (Silva Santas et al., 2017). Effect of chitosan and carboxy methyl chitosan coating (1 and 1.5%) on preserving the quality of vannamei shrimp was investigated by Hunag et al. (2012) and the authors reported that TVB-N development was slower in

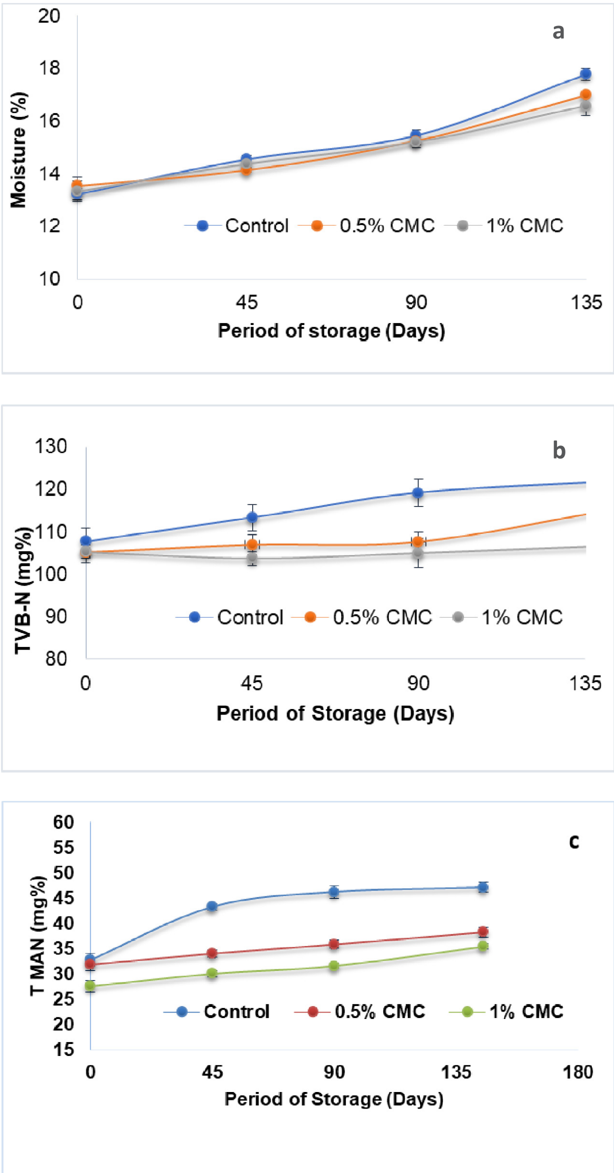


Fig. 1. Changes in moisture (a), TVB-N (b) and TMA-N (c) of dried anchovies during storage (n=3, mean ± SD)

CMCH coated shrimp than those coated with chitosan and in control sample. Researchers suggest that chitosan's inhibitive action on microorganisms reduce the microbial activities responsible for oxidative deamination of non-protein nitrogen compounds leading to formation of volatile bases (Sun et al., 2017). Minh & Nghia (2019) studied the inhibitory effect of chitosan coating at different concentrations on the proteolytic changes as measured by TVB-N in dried spiny eel for a period of 12 months storage at ambient temperature. The results revealed that TVB-N content decreased with increase in concentration of chitosan coating.

Marine fishes contain trimethylamine oxide (TMA-O), the compound responsible for the unique fishy flavour in marine fish. Due to microbial/enzymatic activities in fish or fish products during storage, TMAO is decomposed to ammonia, formaldehyde and trimetyl amine (Viji et al., 2016). With the intensification of spoilage, a subsequent reduction of TMA-O to TMA takes place. Progress of TMA-N accumulation in dried fish is shown in Fig.1c. In the present study, the fresh anchovy had 5.5 mg% TMA-N. A significant increase ($p<0.05$) in TMA-N content was observed in all the three samples as the storage period progressed. Similar to TVB-N, after drying and throughout the storage, the control sample had significantly higher ($p<0.05$) TMA-N value compared to 0.5% and 1% CMCH coated samples. Among the CMCH treated samples, TMA-N value of 0.5% CMCH coated anchovies was significantly higher ($p<0.05$) than 1% CMCH coated sample since 45^{th} day of storage. Freshly caught fish naturally contains 5-10 mg N/100 g TMA-N (Connel, 1995) and the maximum acceptable limit in dried fish is 48-50 mgN 100 g^{-1}. Increase of TMA-N during storage of dried fishes has been reported previously (Relekar et al., 2014). Yu et al., 2017 suggested that being an antimicrobial compound, chitosan can also strongly inhibit the development of TMA-N which is produced by microbial deamination of trimethylamine oxide.

Oily fishes and products are likely to lipid oxidation during processing and storage. The primary products of lipid oxidation are peroxides which can be measured and expressed as peroxide value. Impact of CMCH coating on the peroxide value of dried anchovy is shown in Fig. 2a. Peroxide value increased with storage time in control samples till 90 days and decreased at the end of 135 days of storage. In CMCH coated anchovies, PV started to decline after 45^{th} days of storage. Peroxide value of samples didn't vary significantly ($p>0.05$) during the initial period while significantly higher ($p<0.05$) PV was recorded in control anchovies compared to 1% CMCH coated anchovies from 45 days onwards. As suggested by many researchers, the reduction in PV can be due to oxidation of hydroperoxides into secondary lipid oxidation products. The amount of hydroperoxides generated correlated well with lipid oxidation during early stages of lipid oxidation (Rodriguez-Turienzo et al., 2011). The effect of

CMCH coating on PV of dried anchovies became significant from 90 days of storage onwards. Coating with 1% CMCH showed a wider range of variation from control than that showed by 0.5% CMCH coating, indicating that 1% CMCH was more effective to retard lipid oxidation of anchovy. Previously, Kim et al. (2017) demonstrated a suppressing effect of fish gelatin hydrolysate-based film on lipid oxidation in boiled-dried anchovy. In another study, chitosan coating has suppressed lipid oxidation at a dose dependent manner in dried spiny eel during 12 months of storage (Minh & Nghia, 2019). Suppressing effect of chitosan coating on lipid oxidation of dried fish is also demonstrated by Jeyakumari et al. (2016). Reports suggest that chitosan efficiently act as a secondary antioxidant chiefly by chelating metal ions accelerating lipid oxidation (Olatunde & Benjakul, 2018). The antioxidant activity of chitosan also owes to the capacity of its residual free amino groups to stabilize the free radical by producing stable macromolecular radicals and ammonium groups (Yuan et al., 2016). Zhao et al. (2011) reported that presence of hydroxyl and amino groups in its polymeric chain contributes the antioxidant properties of carboxymethyl chitosan.

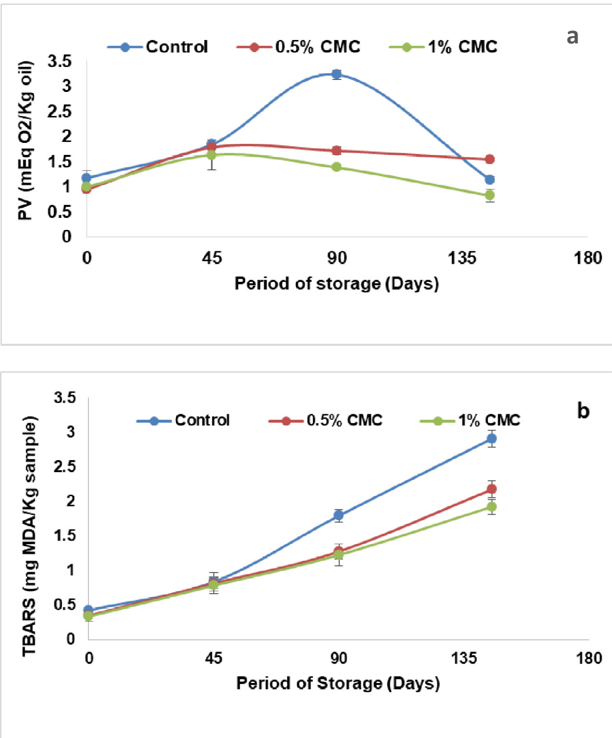
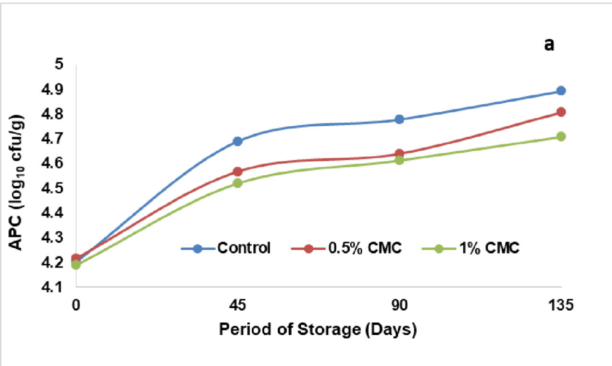


Fig. 2. Changes in peroxide value (a) and TBARS (b) of dried anchovies during storage (n=3, mean ± SD)

Thiobarbituric acid reactive substances give a measure of secondary lipid oxidation products, especially malonaldehyde content. As depicted in Fig. 2b, TBARS of all samples increased significantly ($p<0.05$) during storage. Unlike PV, the effect of CMCH coating on secondary oxidation was significant ($p<0.05$) immediately after drying and during the entire storage, as the control samples registered highest amount of TBARS value. The protective effect of CMCH on secondary oxidation could be due to the antioxidant effect of chitosan and its oxygen barrier properties on the surface of coated fish. Weist & Karel (1992) explained that primary amino group of chitosan can make a stable fluorosphere with volatile aldehydes such as malonaldehyde, making it unavailable for reaction. It is also reported that chitosan coating on the surface of fish products act as a barrier against the transition of oxygen from surrounding atmosphere to the surface of fish, resulting in reduced availability of oxygen for lipid oxidation (Silva Santos et al., 2017; Binsi et al., 2013). In a previous study, Yu et al. (2013) developed active packaging film from carboxymethyl chitosan grafted with caffeic acid as antioxidant. The film was used to test oxidation in menhaden oil-in-water emulsion and has proven successful inhibition of oxidation. In a recent study by Bonilla et al. (2019), treatment of cat fish fillet in a water soluble chitosan solution could reduce the lipid oxidation by 70% compared to control fillet during refrigerated storage up to 20 days. The authors explained that greater mobility of chitosan molecule in water soluble chitosan might have contributed more amino group for antioxidant reaction and thereby controlled lipid oxidation in catfish fillet.

Spoilage bacteria generally cannot tolerate high levels of salt, but halophilic and some xerophilic bacteria can also stay at low water activity (Sen, 2005). In our study, there was no marked difference in the aerobic plate count (APC) of different samples immediately after drying. Whereas on day 45 and afterwards, control samples had remarkedly higher aerobicplate count than its counterparts coated with CMCH. Plate count of 1% CMCH coated samples remained 0.014 log10 cfu g^{-1} lower to that of control immediately after drying, whereas the difference has increased to 0.185 log10 cfu g^{-1} at the end of 135th day (Fig. 3a). Samples coated with 0.5% CMCH maintained 0.14-0.09 log10 cfu g^{-1} lower APC than that of control on and after day 45. The results revealed that coating with CMCH can control bacterial growth in dried fish. Reports says that antimicrobial action of chitosan is mainly due to interaction between positively charged chitosan and negatively charged cell membrane of bacteria, causing rapture and leakage of proteins and intracellular matter (Dutta et al., 2009). Another mechanism reported is that chitosan coating performs as an oxygen barrier and thus inhibits proliferation of aerobic bacteria (Devlieghere et al. 2004). Microbial quality of chitosan coated (0.5 and 1%) dry salted anchovy over a period of 8 weeks was investigated by Agustini & Sedjati (2007). A significant difference in TPC between control and chitosan treated samples was observed, but the TPC of 0.5% and 1% chitosan treatments was not differed significantly. In a similar study, Silva Santos et al. (2017) noticed the effectiveness of chitosan coating in controlling growth of bacteria in liquid smoked Nile tilapia during storage. Recently, Shariatinia (2018) has given a detailed review on the antibacterial properties of carboxymethyl chitosan. The author has reviewed the antibacterial potential of CMCH, quarternised CMCH and CMCH nanocomposites against different bacteria. The maximum acceptable limit established by FSSAI (Food Safety and Standard Authority of India) for APC in dried salted fishery products is 5 log10 cfu g^{-1} (FSSAI, 2017). In the present study, none of the samples crossed this limit during the storage period of 135 days.



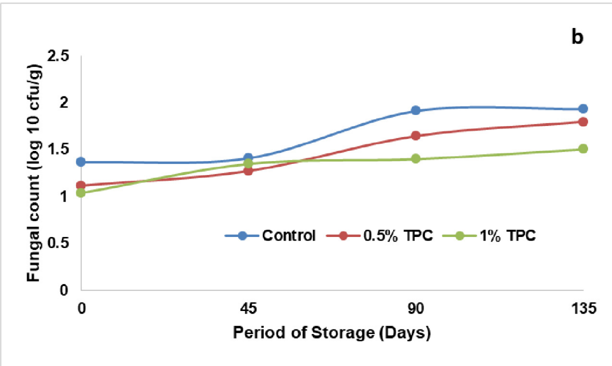


Fig. 3. Changes in aerobic plate count (a) and fungal count (b) during storage

During the process of sun drying, the fishes get easily contaminated by airborne moulds that proliferate during storage and deteriorate the quality of dried products. Moisture content and water activity are the prime factors determining growth of mould during storage. During storage period, the fungal (mould) growth in all the three samples showed lag phase up to 45^{th} day (Fig. 3b). After 45^{th} day, fungal count of control samples increased significantly and exponentially, reached1.93 log10 cfu g^{-1} at the end of 135 days. On the other hand, when a steady increase in fungal count was observed in 0.5% CMCH coated sample, only a mild increase was noticed in 1% CMCH coated sample. Chitosan and chitosan derivatives are proven to have antifungal activities (Virlee et al., 2017). Wrapping of dried anchovy in chitosan films incorporated with organic acid could significantly reduce the mould growth during storage (Vimaladevi et al., 2015). In a study, Chatterjee et al. (2020) investigated antifungal activity of water soluble chitosan designated as s-chitosan and the authors could observe an inhibition of growth of *Macrophomina phaseolina*, a fungi attacking crops by a fungistatic mode of action. Further, the rate of reduction in diameter of fungi mycelia was in parallel with s-chitosan concentration. In the review of Verlee et al. (2017), the authors say that chitosan makes the cell wall of fungi permeable by electrostatic interaction of positively charged chitosan and negatively charged components at the cell surface of fungal mycelia. This process leads to loss of intracellular components and rapture of cell wall, facilitating the entry of chitosan molecule into the cell depending on its molecular weight.

In the present study, preservative effect of carboxymethyl chitosan coating on dried anchovy was investigated. The results of the study revealed that CMCH acts a moisture barrier in dried anchovy in a dose dependent manner. The biochemical and microbial quality analysis carried out over a period of 135 days have also proven a promising effect of CMCH in delaying the spoilage changes during storage. Coating with CMCH significantly controlled lipid oxidation as well as microbial proliferation in a dose dependent manner. Hence, the findings of the present study suggest CMCH as a potential natural preservative in dried fish products.

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