Fishery Technology 55 (2018) : 104 - 113

Shelf life Extension of Tuna Fillets by Gelatin and Chitosan Based Edible Coating Incorporated with Clove Oil

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Abstract

The present study aimed to develop bioactive edible coating (BEC) solutions from gelatin and chitosan, incorporated with different concentrations of clove oil as a natural preservative and evaluate their effect on shelf life of tuna fillets. The antibacterial activity against 11 fish spoilage and fish-borne bacteria were tested by agar well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Gram positive bacteria were more susceptible than gram negative bacteria. Among all the tested bacteria, *Bacillus cereus* and *Staphylococcus aureus* were most sensitive to BEC solutions. The tuna fillets were dipped in BEC solutions with different concentrations *viz.,* 1% Acetic acid, 1% Chitosan (C), 1% Gelatin + 1% Clove oil (GC), 1% Chitosan + 1% Clove oil (CC), 1% Gelatin + 1% Chitosan + 1% Clove oil (GCC) and changes in APC, TBARS, TVB-N and sensory values were studied during storage under refrigerated condition (4°C). The fillets without dip treatment was considered as control. Dip treatment of BEC solutions significantly delayed the rate of microbial spoilage and extended the shelf life of tuna fillets by six days during refrigerated storage. Solutions incorporated with clove oil, especially fillets treated with 1% gelatin + 1% chitosan + 1% clove oil (GCC) solution had better *in-vitro* antimicrobial properties and showed excellent preservative action on tuna fillets. The BEC solutions incorporated with clove oil demonstrated its potential as an excellent natural antibacterial agent which can be used as an effective alternative to synthetic antibacterial agents and could be used for packaging of tuna and other fishery products.

Keywords: Antimicrobial agent, minimum inhibitory concentration, tuna fillets, sensory evaluation

Introduction

Fish is a highly perishable commodity due to its nutritional composition and it gets spoiled rapidly due to enzymatic autolysis, lipid oxidation and microbial deterioration. These processes lead to a reduction shelf life of fish and fishery products (Arashisara et al., 2004; Sathish et al., 2014). Microbial growth on the food surface is one of the major causes of food spoilage (Siragusa & Dickson, 1992). Antimicrobial edible coating is one of the new approaches in controlling microbial growth and improving safety and delaying spoilage of meat, fish and poultry products (Umaraw & Verma, 2017). Edible coatings, which are thin, continuous layers of edible material, applied on or between food components, play an important role on food preservation and are finding great application in recent times (Falguera et al., 2011). The edible coating can be a barrier against moisture, oil, gases, improve mechanical properties and retain volatile compounds and also preserve the colour and texture of products (Flores et al., 2007). The coating materials commonly used are polysaccharides, proteins and lipids and the possibility of incorporating active compounds (antimicrobials, antioxidants, nutraceuticals, flavours and colourants) in polymeric matrices is one of the main advantages of coatings (Sanchéz-Gonzáles et al., 2010).

Among the different biopolymers, gelatin and chitosan are hydrophilic with good affinity and compatibility to form composite and bilayer films (Pereda et al., 2011). Gelatin can be applied as an

Received 18 September 2017; Revised 16 January 2018; Accepted 09 March 2018

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outer covering to protect food against drying, light and oxygen which makes it more suitable for production of biodegradable packaging materials (Gómez-Guillén et al., 2011). However, gelatin does not have ideal water vapour barrier and mechanical properties (Chiou et al., 2008). Therefore, addition of other biodegradable material with gelatin can enhance film forming properties and is one of the effective strategies to improve the water barrier and mechanical properties. Chitosan is another biopolymer that has several advantages such as biocompatibility, biodegradability and can be used as active antimicrobial coatings and films due to its antimicrobial properties and film-forming ability (Sathivel et al., 2007). The combination of gelatin with chitosan could produce high performance biocomposite, bilayer films with improved mechanical and physical properties which are suitable for designing food coatings and packagings (Caner, 2005).

Incorporation of essential oils into chitosan films or coatings may not only enhance the film's antimicrobial and antioxidant properties but also reduce the water vapour permeability (Yanishlieva et al., 1999). Edible films also serve as carriers of essential oils that are released into the food surface and can control microbial growth and extend the shelf life (Lee, 2010). Clove (*Syzygium aromaticum*) is a plant, widely cultivated in India and is used in various food preparations and as flavour enhancers in herbal medicine (Dorman & Deans, 2000). Clove oil has potential to act as antimicrobial agent and kills many gram-positive, gram-negative organisms and some fungi (Gislene et al., 2000).

Growing concern over the safety of synthetic chemical preservatives has led to the increased utilization of natural preservatives in food industry, which is having antioxidant and antimicrobial properties (Viuda-Martos et al., 2010). Bioactive edible coatings or films from natural preservatives with antioxidant and antibacterial properties, prolong the shelf life of fish and fish products (Zahra et al., 2015). The main advantage is that the edible film helps in the reduction of environmental pollution (Bourtoom, 2008). Hence, the aim of the present study is to develop a gelatin – chitosan based bioactive edible coating solution incorporated with clove oil and evaluate its antimicrobial activity and preservative effects on tuna fillets during storage under refrigerated condition (4°C).

Materials and Methods

Gelatin and chitosan were extracted from fish skin and shrimp shell waste respectively in Fish Processing Division, ICAR-Central Institute of Fisheries Technology, Cochin, India. Clove oil was procured from Hi Media, Mumbai, India. All the chemicals and media used in the study were of analytical grade.

For preparing bioactive edible coating (BEC) solutions, clove oil with different concentrations (1 to 5%) were added into distilled water, followed by Tween 20 (0.2 % v/v of the film forming solution) as emulsifying agent and vortexed till clove oil dissolved in distilled water. For single solution, 1% Chitosan (C) solution was prepared by dissolving 1 g of chitosan in 100 ml of 1% acetic acid solution and stirred overnight at room temperature to achieve complete dispersion. The resultant chitosan solution was filtered using a Whatman No.1 filter paper. 1% Gelatin (G) solution was prepared by dissolving 1 g of gelatin in 100 ml^{-1} distilled water with constant stirring. For double complex gelatin or chitosan-clove oil solutions, clove oil was added to gelatin (GC) (1 g 100 ml^{-1} distilled water) or chitosan (CC) solutions (1 g 100 ml^{-1} of 1% acetic acid) at five different concentrations of 1 to 5%. For triple complex gelatin-chitosan-clove oil solutions (GCC), clove oil (1%) was added to gelatin (1 g 100 ml^{-1} of distilled water) and chitosan solution (1 g 100 ml^{-1} of 1% acetic acid) and further tween 20 (0.2% v/v of the film forming solution) was added to BEC solutions, 1% acetic acid solution was used as acid positive control and 10% Dimethyl sulfoxide (DMSO) as negative control.

Standard bacterial strains (MTCC, Chandigarh, India) and a few lab isolates were used for the study. The details of the bacterial isolates are as follows: *Aeromonas hydrophila* (lab isolate), *Salmonella enteritidis* (lab isolate), *Klebsiella* sp. (lab isolate), *Bacillus firmus* (lab isolate), *Bacillus cereus* (lab isolate), *Micrococcus* sp. (lab isolate), *Escherichia coli* (MTCC-40), *Salmonella paratyphi* (MTCC-3220), *Vibrio cholera* (MTCC- 3904), *Salmonella typhi* (MTCC-733) and *Staphylococcus aureus* (MTCC-87). All the cultures were maintained in nutrient agar slants at 4°C. The bacterial concentration was adjusted to 0.5 McFarland standards (10^8 cfu ml^{-1}) by turbidity measurement at 600 nm in UV-spectrophotometer. A 1:10 dilution of cell suspension (10^7 cfu ml^{-1}) was used for the test.

For evaluating the antimicrobial activity, a modified method of Bagamboula et al. (2004) was used for agar well diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of BEC solutions was determined by Micro broth dilution method (NCCLS, 1999) using a 96 well tissue culture plate.

Little tuna (*Euthynnus affinis*) fillets were dipped in BEC solutions (1% Acetic acid, 1% chitosan (C), 1% GC, 1% CC, 1% GCC) for 1 min and the excess liquid was drained off. Fillets without dip treatment were considered as control. Both the control and treatment samples were then placed in sterile polythene (LDPE) bags and stored under refrigerated temperature (4°C). Three samples were randomly removed from each treatment group periodically (0, 3, 6, 9, 12 days) and the microbial, biochemical and sensory characteristics were determined.

The aerobic plate count (APC) was estimated by spread plate technique (Hitching et al., 1995) and expressed as cfu g^{-1} of the sample. Thiobarbituric acid reactive substances (TBARS) was estimated according to Tarladgis et al. (1960) and results were expressed as mg malonaldehyde (MDA) kg^{-1} of sample. TVB-N was measured by steam-distillation of the TCA fish extract, using the modified method of Malle & Tao (1987) and expressed as TVB-N mg 100 g^{-1}. The sensory evaluation was done, based on the method prescribed by Meilgaard et al. (1999). An overall acceptance score was calculated as an average of all scores and score of 6 and above was considered as acceptable.

The results were expressed as mean ± standard error and One-way ANOVA was performed to compare the results of different treatments. The significant difference between the treatment was determined by Turkey' HSD test and the level of significance was set up at $p<0.05$. The statistical package, SPSS (Version 16, SPSS Inc, Chicago, IL) was used for data analysis.

Results and Discussion

Clove oil was found to be effective against all the foodborne microbes tested. In agar well diffusion method, among gram positive bacteria, *B. cerues* was found to be the most sensitive to 1 to 5% clove oil with inhibition zone of 30.33±0.33 mm diameter at 100 µl (Table 1) and among gram negative bacteria *Klebsiella* sp had the highest inhibition zone (21.66±0.33 mm Ø) at 100 µl (Table 2). However, the results revealed that the zone of inhibition increased as the concentration of clove oil increases and gram positive bacteria were more sensitive, compared to gram negative bacteria (Table 1 and 2) as already documented (Mahfuzul Hoque et al., 2008). *Bacillus cereus* was highly sensitive against 5% clove oil, had MIC value of 31.5 µl ml^{-1} (Table 3). Gram positive bacteria such as *B. cereus, S. aureus, B. firmis* and gram negative bacteria like *E. coli, A. hydrophila, S. typhi, S. enteritidis, Klebsiella* sp had the MBC value of 62.5 µl ml^{-1} (Table 4), revealing their susceptibility. Clove oil has been reported to contain high level of eugenol, responsible for its strong biological and antimicrobial activities which denature proteins and react with cell membrane phospholipids changing their permeability and inhibiting the gram-negative and gram-positive bacteria (Chaib et al., 2007).

The effectiveness of 1% chitosan against gram-positive and gram-negative bacteria has shown different levels of sensitivities (Table 1, 2, 3 and 4). Among the gram-positive bacteria, *Staphylococcus aureus* had the maximum zone of inhibition (16.00±0.00 mm Ø), followed by gram-negative bacteria such as *S. paratyphi, S. typhi* and *S. enteritidis* (14.33±0.33 mm Ø) at 100 µl (Table 1 and 2). The results showed that the antimicrobial activity was stronger against the gram-positive bacteria than the gram-negative bacteria which is similar to results of Dutta et al. (2009). It has been demonstrated that the hydrophilicity of gram-negative bacteria is significantly higher than gram-positive bacteria, making them most sensitive to chitosan.

The antimicrobial activity of gelatin - clove oil against all the tested gram positive and gram negative bacteria exhibited different levels of sensitivities (Table 1, 2, 3 and 4). In agar well diffusion method, gelatin (1%) solution did not show any antimicrobial activity, whereas the mixture of gelatin-clove oil results was similar to effects of the clove oil which indicated that gelatin didn't contribute to any antimicrobial activity of its own (Table 1 and 2). The results of MIC and MBC values of gelatin-clove oil solutions against the tested bacterial strains were in the range of 31.2 to 250 µl ml^{-1} and 62.5 to 1000 µl ml^{-1} respectively. Gelatin has been widely used to manufacture edible films for its excellent film forming ability and biodegradability, however, it has poor mechanical property and water resistance. Incorporation of natural polysaccharide such as chitosan into gelatin film is an effective way to improve its film forming properties (Gomez-Guillen et al., 2011).

Table 1. Effect of bioactive coating solutions against Gram positive bacteria (Zone of inhibition in mm)

Bioactive coating solutions	*Staphylococcus aureus*		*Bacillus cereus*		*Bacillus firmis*		*Micrococcus sp*	
	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl
1% Clove oil	10.00±0.57	11.66±0.33	10.33±0.33	12.33±0.33	11.00±0.57	13.33±0.33	9.66±0.33	11.00±0.57
2% Clove oil	11.33±0.33	13.66±0.33	12.33±0.33	16.33±0.33	12.33±0.33	15.66±0.33	10.33±0.33	11.66±0.33
3% Clove oil	12.33±0.33	15.33±0.33	15.33±0.33	18.33±0.33	15.33±0.33	19.00±0.57	11.33±0.33	12.33±0.33
4% Clove oil	14.33±0.33	17.66±0.33	17.33±0.33	20.33±0.33	19.66±0.33	25.66±0.33	11.66±0.33	14.33±0.33
5% Clove oil	17.33±0.33	20.33±0.33	19.66±0.33	30.33±0.33	23.33±0.33	29.00±0.57	13.33±0.33	16.33±0.33
1% gelatin	-	-	-	-	-	-	-	-
1% gelatin + 1% clove oil	10.33±0.33	12.00±0.00	10.66±0.33	12.33±0.33	10.66±0.33	13.33±0.33	10.00±0.00	11.33±0.33
1% gelatin + 2% clove oil	11.33±0.33	13.66±0.33	12.33±0.33	16.33±0.33	12.33±0.33	13.33±0.34	10.66±0.33	11.66±0.33
1% gelatin + 3% clove oil	12.33±0.33	15.33±0.33	15.33±0.33	18.33±0.33	15.33±0.33	13.33±0.35	11.33±0.33	12.66±0.33
1% gelatin + 4% clove oil	14.33±0.33	17.33±0.33	17.66±0.33	20.33±0.33	19.66±0.33	13.33±0.36	12.00±0.00	14.33±0.33
1% gelatin + 5% clove oil	17.66±0.33	20.33±0.33	20.00±0.00	30.33±0.33	23.33±0.33	13.33±0.37	13.66±0.33	16.66±0.33
1% chitosan	11.33±0.33	16.00±0.00	9.33±0.33	11.00±0.57	10.33±0.33	11.66±0.88	10.33±0.33	12.33±0.33
1% chitosan + 1% clove oil	14.33±0.33	19.00±0.57	11.33±0.33	12.66±0.33	11.33±0.33	13.66±0.33	10.66±0.33	12.33±0.33
1% chitosan + 2% clove oil	16.33±0.33	20.66±0.33	11.66±0.33	13.66±0.33	13.00±0.57	16.33±0.33	11.33±0.33	12.66±0.33
1% chitosan + 3% clove oil	17.66±0.33	23.33±0.33	13.33±0.33	15.66±0.33	13.66±0.33	17.66±0.33	13.33±0.33	16.00±0.57
1% chitosan + 4% clove oil	19.66±0.33	25.66±0.33	15.33±0.33	17.00±0.57	15.33±0.33	21.00±0.57	14.33±0.33	17.66±0.33
1% chitosan + 5% clove oil	23.00±0.57	27.33±0.33	15.66±0.33	18.33±0.33	19.33±0.66	26.66±0.33	16.00±0.57	20.33±0.33
1% gelatin + 1% chitosan + 1% Clove oil	17.66±0.33	23.66±0.33	13.66±0.33	16.00±0.57	13.33±0.33	17.66±0.33	14.00±0.57	16.66±0.33
1% Acetic Acid	8.66±0.33	9.66±0.33	7.66±0.33	8.00±0.57	8.33±0.33	9.66±0.33	7.00±0.57	9.66±0.33
10% DMSO	-	-	-	-	-	-	-	-

Results are mean ± standard error (n=3)

The antimicrobial activity of chitosan – clove oil solutions showed high antimicrobial activity against all bacteria and was superior compared to all the bioactive coating solutions tested (Table 1, 2, 3 and 4). Among the gram positive bacteria *S. aureus* was the most susceptible, had the maximum zone of inhibition (27.33±0.33 mm Ø) followed by *B. firmis* (26.66±0.33 mm Ø) against 1% chitosan + 5% clove oil at 100 µl (Table 1). Among gram negative bacteria, *E. coli* was more susceptible to 1% chitosan + 5% clove oil with the inhibition zone of 25.66±0.88 mm Ø at 100 µl (Table 2). The results showed that, chitosan - clove oil mixture is more effective against gram positive bacteria compared to gram negative bacteria (Table 1 and 2). This could be due to the cell wall structure of bacteria as gram-positive bacteria are more sensitive to such agents (Nychas, 1995). The results of MIC and MBC values of chitosan - clove oil against the employed bacterial strains were in the range of 3.9 to 7.8 µl ml^{-1} and 7.8 to 15.6 µl ml^{-1}, respectively and the inhibition increased with increasing concentrations.

GCC mixture at 1% level was more effective against test organisms than those of 1% GC and 1% C (Table 1, 2, 3, 4). This effect could be due to the fact that gelatin and chitosan are highly reactive molecules establishing ionic and hydrogen bonding (Taravel & Domard 1995). Among Gram positive bacteria, *S. aureus* was the most susceptible to 1% GCC, had

Table 2. Effect of bioactive coating solutions against Gram negative bacteria (Zone of inhibition in mm)

Bioactive coating solutions	*Escherichia coli*		*Salmonella paratyphi*		*Salmonella typhi*		*Salmonella enteritidis*		*Aeromonas hydrophila*		*Vibrio cholera*		*Klebsiella sp*	
	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl
1% Clove oil	10.66±0.33	12.33±0.33	9.33±0.33	10.66±0.33	9.66±0.33	11.66±0.33	9.33±0.33	10.66±0.33	7.66±0.33	9.33±0.33	9.33±0.33	10.33±0.33	10.66±0.33	12.66±0.33
2% Clove oil	12.33±0.33	13.66±0.33	10.33±0.33	11.66±0.33	10.33±0.33	12.66±0.33	10.33±0.33	11.66±0.33	9.33±0.33	10.66±0.33	10.33±0.33	11.33±0.33	12.33±0.33	13.66±0.33
3% Clove oil	13.33±0.33	14.33±0.33	11.33±0.33	12.66±0.33	11.33±0.33	12.66±0.33	11.33±0.33	12.66±0.33	10.33±0.33	12.33±0.33	11.33±0.33	12.66±0.33	13.33±0.33	14.66±0.33
4% Clove oil	14.66±0.33	16.33±0.33	12.33±0.33	13.66±0.33	12.66±0.33	14.66±0.33	12.33±0.33	13.66±0.33	11.33±0.33	13.66±0.33	11.66±0.33	14.66±0.33	14.66±0.33	16.33±0.33
5% Clove oil	17.33±0.33	21.00±0.57	13.33±0.33	14.66±0.33	14.66±0.33	15.66±0.33	13.33±0.33	14.66±0.33	12.66±0.33	15.33±0.33	13.66±0.33	16.33±0.33	17.33±0.33	21.66±0.33
1% gelatin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 % gelatin + 1% clove oil	10.33±0.33	11.66±0.33	9.00±0.00	10.33±0.33	9.66±0.00	10.66±0.33	9.00±0.00	10.33±0.33	8.33±0.33	9.33±0.33	9.00±0.00	10.66±0.33	10.33±0.33	11.66±0.33
1 % gelatin + 2% clove oil	11.66±0.33	13.33±0.33	10.33±0.33	11.66±0.33	10.33±0.33	11.66±0.33	10.33±0.33	11.66±0.33	9.33±0.33	10.33±0.33	10.33±0.33	11.33±0.33	11.66±0.33	13.33±0.33
1 % gelatin + 3% clove oil	13.33±0.33	14.00±0.00	11.00±0.00	12.66±0.33	11.33±0.33	12.66±0.33	11.00±0.00	12.66±0.33	10.00±0.00	12.33±0.33	11.00±0.00	12.33±0.33	13.33±0.33	14.33±0.33
1 % gelatin + 4% clove oil	14.66±0.33	15.66±0.33	12.33±0.33	13.33±0.66	12.33±0.33	13.66±0.33	12.33±0.33	13.33±0.66	11.33±0.33	13.33±0.33	11.66±0.33	14.66±0.33	14.33±0.33	15.66±0.33
1 % gelatin + 5% clove oil	17.33±0.33	20.33±0.33	12.66±0.33	15.33±0.33	14.33±0.33	15.66±0.33	12.66±0.33	15.33±0.33	12.66±0.33	15.33±0.33	13.33±0.33	16.66±0.33	17.33±0.33	20.33±0.33
1% chitosan	7.33±0.33	11.66±0.33	11.33±0.33	14.33±0.33	11.33±0.33	14.33±0.33	11.33±0.33	14.33±0.33	6.33±0.33	9.33±0.33	6.33±0.33	10.33±0.33	8.33±0.33	11.66±0.33
1 % chitosan + 1% clove oil	7.66±0.33	14.66±0.33	12.66±0.33	16.33±0.33	12.66±0.33	16.33±0.33	12.66±0.33	16.33±0.33	8.66±0.33	10.33±0.33	9.66±0.33	10.33±0.33	9.66±0.33	14.66±0.33
1 % chitosan + 2% clove oil	10.00±0.57	15.66±0.33	14.33±0.33	17.66±0.33	14.33±0.33	16.66±0.33	14.33±0.33	17.66±0.33	10.33±0.33	11.66±0.33	10.66±0.33	11.66±0.33	11.00±0.57	15.66±0.33
1 % chitosan + 3% clove oil	10.66±0.33	18.00±0.57	15.66±0.33	20.33±0.33	16.66±0.33	20.66±0.33	15.66±0.33	20.33±0.33	10.66±0.33	12.66±0.33	11.33±0.33	13.00±0.57	12.66±0.33	18.00±0.57
1 % chitosan + 4% clove oil	13.00±0.57	17.33±0.33	18.00±0.57	21.66±0.33	18.66±0.33	21.66±0.33	18.00±0.57	21.66±0.33	11.66±0.33	13.66±0.33	11.66±0.33	15.33±0.33	13.00±0.57	17.33±0.33
1 % chitosan + 5% clove oil	14.00±0.57	25.66±0.88	20.33±0.33	23.33±0.33	21.33±0.33	23.66±0.33	20.33±0.33	23.33±0.33	13.33±0.33	16.33±0.33	12.66±0.33	17.33±0.33	14.00±0.57	18.66±0.88
1% gelatin + 1% chitosan + 1% clove oil	11.33±0.33	18.33±0.33	16.33±0.33	21.00±0.57	17.33±0.33	21.66±0.33	16.66±0.33	21.33±0.33	10.66±0.33	12.33±0.33	10.66±0.33	13.00±0.57	11.66±0.33	16.33±0.33
1% Acetic Acid	-	-	8.33±0.33	11.00±0.57	-	9.66±0.33	6.66±0.33	8.33±0.33	6.66±0.33	7.33±0.33	6.33±0.33	7.00±0.57	-	6.33±0.33
10% DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Results are mean ± standard error (n=3)

maximum zone of inhibition of 23.66±0.33 mm Ø followed by *B. firmis* (17.66±0.33 mm Ø) at 100 µl (Table 1) and among the gram negative bacteria, *S. typhi* (21.66±0.33 mm Ø) was more susceptible (Table 2). Further, it was observed that, GCC mixture was more effective against gram-positive bacteria than gram-negative bacteria (Table 1 and 2). This could be due to the presence of an additional relatively impermeable external membrane surrounding the cell wall in gram-negative bacteria which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Sanchez-Gonzalez et al., 2011). The results of MIC and MBC values of mixed GCC solution against the employed bacterial strains were in the range of 3.9 to 7.8 µl ml^{-1} and 7.8 to 15.6 µl ml^{-1} (Table 3 and 4), which was similar to those of the CC solution, confirming that gelatin didnot add to the antimicrobial activity. However, the results showed that the incorporation of clove oil into chitosan edible film effectively increased the antimicrobial activity compared to chitosan (1%) without clove oil.

Antimicrobial activities depend on the concentration of essential oil used in the BEC solutions. The *in-vitro* results have shown that antimicrobial activities increased with increasing concentration of clove oil (Table 1, 2, 3 and 4). Though the highest concentration of clove oil had the maximum antimicrobial activity against the tested bacterial strains, but it affected the sensory properties of food products and masked the products original flavours.

Table 3. Minimum Inhibition Concentration (MIC) of BEC solution against foodborne and fishborne spoilage bacteria (µl/mL)

Bioactive coating solutions	*Escherichia coli*	*Salmonella paratyphi*	*Staphyloco-ccus aureus*	*Bacillus cereus*	*Vibrio cholerae*	*Micrococcus sp*	*Aeromonas hydrophila*	*Bacillus firmis*	*Salmonella typhi*	*Salmonella enteritidis*	*Klebsiella sp*
Clove oil (crude)	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48
1% Clove oil	250	500	250	250	250	250	250	250	250	250	250
2% Clove oil	125	250	125	125	250	250	250	250	250	250	250
3% Clove oil	125	250	125	125	250	125	125	250	125	125	250
4% Clove oil	125	250	62.5	125	125	125	250	125	125	250	125
5% Clove oil	62.5	250	62.5	31.2	125	62.5	62.5	62.5	62.5	62.5	62.5
1% gelatin	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
1% gelatin + 1% clove oil	125	250	125	250	250	250	250	250	250	250	250
1% gelatin + 2% clove oil	125	250	125	125	250	250	250	250	250	250	250
1% gelatin + 3% clove oil	125	250	125	125	125	125	125	250	125	125	250
1% gelatin + 4% clove oil	125	125	62.5	62.5	125	125	125	125	125	125	125
1% gelatin + 5% clove oil	62.5	125	62.5	31.2	125	62.5	62.5	62.5	62.5	62.5	62.5
1% chitosan	15.6	31.2	15.6	15.6	15.6	15.6	15.6	15.6	31.2	31.2	15.6
1% chitosan + 1% clove oil	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
1% chitosan + 2% clove oil	15.6	15.6	15.6	15.6	15.6	15.6	7.8	15.6	15.6	7.8	15.6
1% chitosan + 3% clove oil	15.6	15.6	7.8	15.6	15.6	15.6	7.8	7.8	15.6	7.8	7.8
1% chitosan + 4% clove oil	7.8	15.6	7.8	7.8	15.6	15.6	7.8	7.8	15.6	7.8	7.8
1% chitosan + 5% clove oil	7.8	7.8	3.9	3.9	7.8	7.8	3.9	7.8	7.8	7.8	7.8
1% gelatin + 1% chitosan + 1% clove oil	7.8	7.8	3.9	3.9	7.8	7.8	3.9	7.8	7.8	7.8	3.9

Hence, the lowest concentration of clove oil (1%) different BEC solutions which also had antimicrobial activity against all the tested bacterial strains was taken for evaluation of its shelf life extension on tuna fillets in order to keep natural flavour of the product.

Changes in total bacterial counts during storage are shown in Fig. 1A. The APC of fresh tuna samples was within the acceptable limit of 5 x 10^5 cfu g^{-1} which indicated acceptable fish quality (ICMSF 1986). During the storage, the APC gradually increased with the progression of storage period in all treatments (Fig. 1A). The APC control fish fillets reached 6.81±0.00 on 6^{th} day of storage, whereas APC of samples treated with BEC solutions was slightly over 6 log cfu g^{-1} only on 12^{th} day of storage. This has clearly shown that the tuna fillets treated with BEC solutions significantly delayed the rate of microbial spoilage and extended the shelf life of tuna fillets by at least 6 days during storage at 4°C. Among the BEC solutions, sample treated with a mixture of 1% GCC showed very high antimicrobial activity in *in-vitro* study and had the lowest APC of 5.97±0.01 log cfu g^{-1} followed by samples treated with 1% CC solution (6.26±0.02 log cfu g^{-1}) at the end of 12 days of storage. The gelatin-chitosan based BEC solution enriched with clove oil showed excellent antimicrobial properties and could extend the shelf life of food as reported in cold-smoked sliced sardine (Gómez-Estaca et al., 2007).

TBARS values of both control and treated fillets increased with storage (Fig. 1B). The initial TBARS

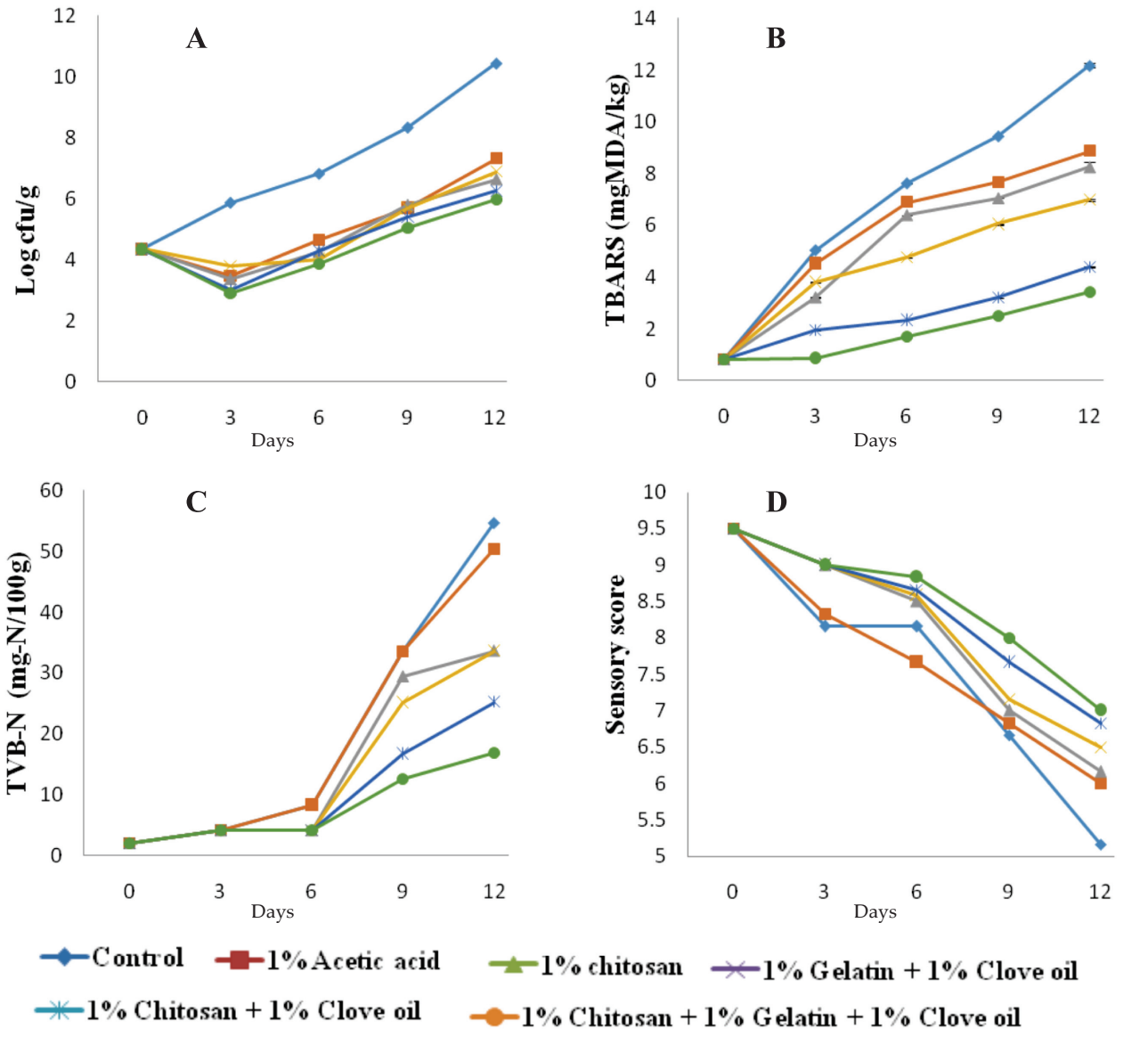

Fig. 1. Changes in quality parameters of tuna fillets dipped in BEC solutions during storage at 4°C.
A. Changes in the Aerobic plate count; **B.** Changes in TBARS value;
C. Changes in the TVB-N values; D. Changes in sensory quality

value of fresh tuna fillets was 0.81±0.005 mg MDA eq kg^{-1} muscle. Due to the rapid lipid oxidation in control sample, TBARS value increased to 7.63±0.011 and 12.18±0.087 mg MDA eq kg^{-1} on Day 6 and Day 12, respectively, whereas in the fillets treated with BEC solutions, the TBARS values increased gradually from 3.42±0.032 to 8.86±0.167 mg MDA kg^{-1} at end of storage (Fig. 1B), indicating that the fillets treated with BEC solutions effectively retarded lipid oxidation. Among all the treatments, TBARS values of fillets treated with 1% GCC solution, exceeded the acceptable limit of 2 mg MDA kg^{-1} (Connell, 1990) on 9th day of storage period, while the control exceeded (5.04±0.008 mg MDA kg^{-1}) on 3rd day and is clearly evident that, sample treated with 1% GCC solution effectively extended the shelf life of tuna fillets by 6 days. The BEC solutions applied on the surface of tuna fillets, may act as a barrier between the fillet and its surroundings due to its oxygen barrier properties, thus slowing down the diffusion of oxygen from the surrounding air to the surface of fillet and retarding lipid oxidation.

Table 4. Minimum Bactericidal Concentration (MBC) of BEC solution against foodborne and fishborne spoilage bacteria (µl/mL)

Bioactive coating solutions	*Escherichia coli*	*Salmonella paratyphi*	*Staphylococcus aureus*	*Bacillus cereus*	*Vibrio cholerae*	*Micrococcus sp*	*Aeromonas hydrophila*	*Bacillus firmis*	*Salmonella typhi*	*Salmonella enteritidis*	*Klebsiella sp*
Clove oil (crude)	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48	<0.48
1% Clove oil	500	1000	250	250	250	250	500	500	1000	1000	250
2% Clove oil	250	500	250	250	250	250	250	250	500	500	250
3% Clove oil	125	500	250	250	250	250	250	250	125	125	250
4% Clove oil	125	250	125	125	125	125	250	125	125	250	125
5% Clove oil	62.5	250	62.5	62.5	125	125	62.5	62.5	62.5	62.5	62.5
1% gelatin	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
1% gelatin + 1% clove oil	250	500	250	250	250	250	250	250	1000	1000	250
1% gelatin + 2% clove oil	250	500	250	250	250	250	250	250	500	500	250
1% gelatin + 3% clove oil	125	500	250	250	250	250	250	250	125	125	250
1 % gelatin + 4% clove oil	125	250	125	125	125	125	250	62.5	125	250	125
1% gelatin + 5% clove oil	62.5	250	62.5	62.5	125	62.5	62.5	62.5	62.5	62.5	62.5
1% chitosan	31.2	31.2	15.6	15.6	31.2	15.6	15.6	31.2	31.2	31.2	15.6
1% chitosan + 1% clove oil	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
1% chitosan + 2% clove oil	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
1% chitosan + 3% clove oil	15.6	15.6	15.6	15.6	15.6	15.6	7.8	7.8	15.6	7.8	7.8
1% chitosan + 4% clove oil	15.6	15.6	7.8	7.8	15.6	15.6	7.8	7.8	15.6	7.8	7.8
1% chitosan + 5% clove oil	7.8	7.8	7.8	7.8	15.6	7.8	7.8	7.8	7.8	7.8	7.8
1% gelatin + 1% chitosan + 1% clove oil	7.8	7.8	7.8	7.8	15.6	7.8	7.8	7.8	7.8	7.8	7.8

In the present study, TVB-N values of samples treated with BEC solutions was within the acceptable limit of 30–35 mg TVB-N 100 g^{-1} (Connell, 1995) during the entire period of storage (Fig. 1C). The fillet treated with 1% acetic acid and control samples crossed the rejection limit > 35 mg TVB-N on 12^{th} day of storage, which makes it evident that the fillets treated with BEC solutions had effectively delayed the spoilage of tuna fillets.

During storage, the sensory scores were decreased as storage days increased (Fig 1D). Tuna fillets dipped in 1% Acetic acid, 1% C, 1% GC, 1% CC, 1% GCC solutions had sensory scores more than acceptable level (>6), while the control fillets became unacceptable (5.38±0.11) at the end of storage period. Among the BEC solutions treated samples, 1% GCC had high sensory score (7.00±0.00) followed by 1% GC (6.50±0.28), due to the smell and appearance of tuna fillets. So, it is clear that, all BEC solutions could maintain the quality of tuna fillets and extend the shelf life of tuna fillets compared to control samples, especially fillets treated with clove oil enriched BEC solutions had better results when compared to single solutions.

From the *in-vitro* studies, it was observed that gram positive bacteria were more susceptible than gram negative bacteria to BEC solutions. Among all the tested bacteria, *B. cereus* and *S. aureus* were found most sensitive to BEC solutions, which demonstrated its potential as an antimicrobial agent. However, chitosan dissolved in 1% acetic acid cause acidic condition which also plays a role in antimi-

crobial activity. Among the BEC solutions, 1% GCC solution had better *in-vitro* antimicrobial properties and showed excellent preservative effects on tuna fillets kept under refrigerated temperature (4°C) by extending the shelf life by 6 days with good organoleptic acceptance. This clearly indicates their potential as natural antimicrobial agent which could replace commonly used synthetic antimicrobials and could also be used as active packaging film for fish products.

Acknowledgments

Authors are grateful to Dr. Ravishankar, C.N., The Director, ICAR-Central Institute of Fisheries Technology, Cochin and Prof. Baskaran Manimaran, Vice- Chancellor, Tamil Nadu Fisheries University, Nagapattinam, for providing valuable support in conducting this research work.

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