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CONTROL OF SPOILAGE BACTERIA IN TUNA FISH BY MARINE LACTIC ACID BACTERIA AND THEIR BACTERIOCINS

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Abstract: The increasing demand for high quality, safe, processed foods has created a niche for preservatives of biological origin. Lactic acid bacteria and their exocellular antimicrobial metabolites have potential as natural biopreservatives to control the growth of spoilage and pathogenic bacteria in processed foods. The application of LAB and Cell Free Filtrates (CFF) in controlling spoilage bacteria in Tuna (*Thunnus* sp.) fish was determined in the present study. Tuna is a much sought fish in the international market and is mostly sold in the form of chunks and stored at refrigerated temperatures. The study on the effect of different temperatures on the bacterial quality of Tuna flesh indicated that there was a drastic reduction in the total count of bacteria when the fish was stored at chilling temperatures of 4^0C and -20^0C. Two Lactic Acid Bacteria *viz*., *Lactococcus lactis* and *Lactobacillus brevis* and their antimicrobial metabolite bacteriocin were checked for their antagonistic activity against four bacterial isolates, namely, *Arthrobacter* sp., *Acinetobacter* sp., *Bacillus brevis* and *Bacillus pumilus* from Tuna fish. The LAB strains showed a wide spectrum of inhibition against three Gram positive and one Gram negative fish isolates, though the activity differ for both. The comparative analysis of the whole cells and the CFF showed higher activity in the case of antimicrobial metabolites. The study emphasized the importance of LAB and its application in preservation of seafood to ensure its quality and safety especially within the context of increasing demand for minimally processed aquatic food products.

Key words: Lactic acid bacteria, Biopreservation, Bacteriocins, Antibacterial activity

INTRODUCTION

The empirical use of microorganisms and their natural products for the preservation of foods (biopreservation) has been a common practice in the history of mankind. As a measure of preserving a variety of foods by inhibiting pathogens, LAB are believed to be an alternative to food preservatives since their cellular metabolites including various organic acids, H_2O_2 and bacteriocins inhibit the growth of common pathogens and spoilage and spoilage organisms contaminating food (Altuntas, 2013, Francoise, 2010). Bacteriocins generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death. The incorporation of bacteriocins as a biopreservative ingredient into model food systems has been studied extensively and has been shown to be effective in the control of pathogenic and spoilage microorganisms. Biopreservation de facto, extends the shelf-life and enhances the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products namely seafood (Calo Mata *et al*., 2008) Sea food has traditionally been an important part of people's diet in many parts of the world. Also, the fishery products with extended shelf-life are gaining popularity, because they are advantageous for marketing and distribution. About one-third of the world's food production is lost annually as a result of microbial spoilage (Agbabiaka *et al*., 2016) including fresh sea food. In the last years, the traditional customary methods applied to seafood like salting, smoking and canning have depreciated of mild technologies involving lower salt content, lower

cooking temperature and vacuum (VP) or modified atmosphere packing (MAP). The treatments are usually not sufficient to destroy microorganisms causing psychrotolerant pathogenic and spoiling bacteria to develop during the extended shelf-life of these products (Ghanbari and Jami., 2013; Saranraj *et al*., 2013). Tuna is much sought fish in the international market and is mostly sold in the form of chunks and stored at refrigerated temperatures. The present study is conducted to investigate the changes in the microbiological quality of tuna at different temperatures of storage and finally to check the potential of Lactic acid bacteria and its bacteriocin in controlling the spoilage bacteria in the fish.

MATERIALS AND METHODS

Isolation and screening of LAB for antimicrobial activity

Lactic Acid Bacteria isolated from marine perch fish (*Perca flavescens*) and marine Bat fish (*Platax* sp.) were used in the present study. The strains were propagated in MRS (de Mann Rogosa Sharpe) agar (Hi-Media) supplemented with 1% w/v; NaCl for 72 h at 30°C The Cell free supernatants sterilized by passing through 0.22µm membrane filter (Millipore, India) and evaluated for antimicrobial activity by agar well diffusion assay (BSI, 1968) against the indicator organisms.

Indicator strains

The antimicrobial activity of the isolates were verified using the indicator organisms viz., *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, and Vibrio fischeri* obtained from the laboratory stock.

Isolation and enumeration of bacteria from Tuna flesh

Tuna (*Euthynnus affinis*) procured from fish landing centre located in Colachel, Tamil Nadu, India were immediately brought to the laboratory in polythene bags, and washed in potable water, beheaded and eviscerated. They were allowed to bleed for 15 minutes and washed again in potable water. Tuna flesh weighing approximately 20 g were cut from the whole Tuna of about 2 kg and stored under different temperatures (-20°C, 4°C, 12°C and 28-30°C) in sterile polythene bags. The samples were drawn at periodic intervals for microbiological quality analyzes by the enumeration of Total Viable Count (TVC) of bacteria. The Tuna flesh was aseptically removed from the package and the fish muscle (1 g) homogenized in 40 ml of chilled sterile peptone-phosphate buffer using sterile mortar and pestle. Ten fold dilutions were made using the same diluents for respective bacterial analyzes. Appropriate dilutions of tuna homogenate were spread plated onto nutrient agar for enumeration of TVC. The plates were incubated at room temperature for 5 days and the colonies were counted and expressed as CFU/g.

Determination of Total Count of Lactic Acid Bacteria

The isolated LAB strains were inoculated in 100 ml MRS broth and after 72 h of incubation the number of bacteria per ml of the broth was detected by serial dilution followed by spread plating in MRS agar medium.

Antibacterial activity of isolated LAB against fish isolates

Antagonistic activity of the different LAB strains against the fish isolates were detected by the soft agar lawn method (Ko and Ahn, 2000) and confirmed using well-diffusion assay (BSI, 1968) In soft agar lawn method, overnight culture of the different LAB were spot inoculated onto the surface of MRS agar plate, incubated at 30°C for 24-48 h to allow colonies to appear and then overlaid with 7 ml of soft agar (nutrient broth and 0.75% agar) containing 0.5 ml of the overnight culture of the indicator strains. After additional overnight incubation at 37°C, formation of a clear zone around the colonies was checked. For well diffusion assay, fresh culture of fish isolate from nutrient agar slant was inoculated into 100 ml of nutrient broth and incubated at 37°C for 24 h and 0.5 ml of fresh nutrient broth culture was mixed with 20 ml of molten and cooled nutrient agar medium and poured into Petri dishes. After setting the agar at room temperature for 15 minutes, it was hardened by placing the plates in refrigerator (4-8°C) for 15 min and wells were made with sterile cork borer (6 mm). 100µl of either the culture

broth or the cell free culture filtrate of bacteriocin producing bacterial strains was transferred into the wells with the help of micropipette. Uninoculated MRS medium was used as the control. The plates were incubated at 37°C for 24 h without inversion. At the end of incubation, diameter of zones of inhibition formed around the well was measured with the help of a divider and a scale.

RESULTS AND DISCUSSION

Detection of the antimicrobial activity of LAB

The LAB strains were screened for its inhibitory potential against the indicator bacteria. Both of them showed maximum inhibitory activity against most of the indicator strains (Table 1). On biochemical characterization, the organism isolated from marine Perch was identified as *Lactococcus lactis* and the Bat Fish isolate as *Lactobacillus brevis.*

Enumeration of Spoilage bacteria from Tuna flesh

The Tuna flesh stored at different temperatures (-20°C, 4°C, 12°C and 27±2 °C) was analysed for the total viable count at an interval of 24 h for 4 days the results of which are depicted in fig. 1.

Initially (Day zero), the total bacterial count of tuna was 10^7 CFU/g. According to Huss (1995), the tropical fish normally contain high bacterial population. However, there was a drastic reduction, in the total count of bacteria when the fish is stored at chilling temperature (-20^{0}C). The load of bacteria remained more or less constant for 2 days of storage at -20^{0}C, and reduced by a log by the 3rd day. But, as observed by Raja *et al.* (2014), the growth of recoverable aerobic bacteria was not hindered by low temperature. In case of the fish stored at room temperature, the load increased progressively to reach a level of 10^{12} cfu/g by the 4th day, which made the fish unacceptable.

The plates were observed for the presence of morphologically distinct colonies and eight colonies named, MSU1 to MSU8 were isolated and stored for the further studies.

Control of the fish isolates by LAB

The isolated bacteria from Tuna fish were subjected to antibacterial assay using the cell free supernatants of the LABs. The inhibitory activity screening by soft agar lawn method (Table 2) and well diffusion method gave a clear indication that *L. lactis* and *L. brevis* inhibited the growth of most of the isolates with a clear zone of inhibition and have a wide spectrum of activity.

The number of bacteria present in 100 µl of the culture broth which produced the inhibition zone was determined by means of Total Plate Count. The well was loaded with approximately 1.8×10^7 and 9.2×10^6 cells of *Lactococcus lactis* and *Lactobacillus brevis*. The zone formation after the specific incubation period is shown in Table 3. Similarly, the cell free culture filtrate which is expected to contain the bacteriocin is also observed for its activity. The antimicrobial spectrum of the isolated LAB and the bacteriocin produced by them is listed in Table 3. The data indicates that the antimicrobial metabolites inhibited three Gram positive and one Gram negative fish isolate and had a broad spectrum of activity. A study by Abo-Amer (2007) discussed the antibacterial property of *L. plantarum* AA135 against a wide range of Gram-positive and Gram-negative pathogens.

The results obtained in the present study for both soft agar lawn method and the well-diffusion assay was almost similar with minimal difference. However, a study by Cadirci and Citak (2005) detailed the increased inhibitory activity of Lactic acid bacteria in spot on lawn method to that of well diffusion technique. But in the observations of Lewus (1991), only few strains that tested positive using spot-on-lawn method gave positive results in the well-diffusion assay. They considered that allowing some time for the bacteriocins to diffuse into the agar prior to incubation, or increasing the well size so that more sample could be applied, might increase the sensitivity of the assay. According to these authors, aggregation, non-diffusible bacteriocins, protease inactivation and concentration effects, can all lead to false negative results in the well-diffusion assay. The positive results obtained in the present study may be attributed to the fact that comparatively more amount (100 µl) of the test strains and its CFF was able to load in the well contrary to the earlier observations. Nevertheless it could be considered that the efficacy and spectrum of action of

Table 1. Activity spectrum of LAB

Indicator species	Activity of LAB	
	Lactococcus lactis	*Lactobacillus brevis*
Staphylococcus aureus	**++**	++
Escherichia coli	+	+
Pseudomonas aeruginosa	+	+
Bacillus subtilis	++	+
Vibrio fischeri	+	+

- No inhibition ++ Inhibition zone + Small inhibition

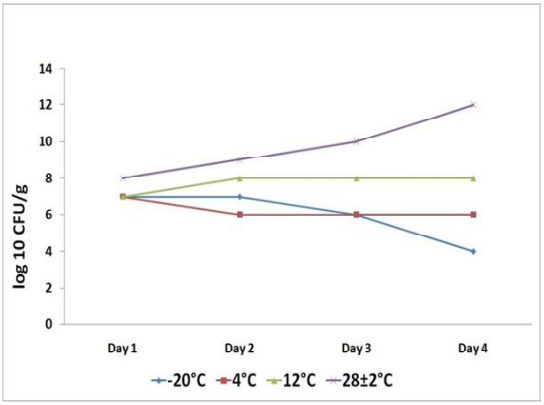

Fig. 1. Number of bacteria / g tissue of Tuna stored at different temperatures.

Table 2. Activity spectrum of LAB against fish isolates

Fish isolates	Activity of LAB	
	Lactococcus lactis	*Lactobacillus brevis*
MSUIS1	**++**	++
MSUIS2	++	-
MSUIS3	++	+
MSUIS4	+	+
MSUIS5	-	+
MSUIS6	+	+
MSUIS7	+	++
MSUIS8	++	+

- No inhibition ++ Inhibition zone + Small inhibition zone

Table 3. Inhibition of Fish isolates by LAB

Fish isolates	Zone of Inhibition (mm)			
	Lactococcus lactis		*Lactobacillus brevis*	
	FC	CFF	FC	CFF
MSUIS1	11	11	13	13
MSUIS2	7	7	9	9
MSUIS3	7	11	10	12
MSUIS4	-	-	-	11
MSUIS5	-	-	10	-
MSUIS6	7	9	8	11
MSUIS7	8	9	11	12
MSUIS8	10	11	11	12

*FC – Free cells CFF – Cell Free Culture Filtrate

lactic acid bacteria against pathogenic microorganisms are based on the action of bacteriocins and a combination of antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriophages or due to the presence of appropriate receptor sites in the cell wall of the susceptible organisms. (Lima *et al*., 2007). Lindgren and Dobrogosz (1990) reviewed the antagonistic activity of lactic acid bacteria against pathogens and spoilage bacteria and stated that the mechanisms involve the production of lactic and acetic acids, nutrient depletion, hydrogen peroxide production, changes in oxidation/reduction potential, and production of antibiotic-like compounds Maintaining a safe food supply has become an ever-changing endeavour as new information on pathogenic bacteria is discovered (Djadouni and Kihal, 2012). The microbiological safety of minimally processed sea foods is also of concern owing to the possible presence of several pathogenic bacteria that may harm the consumers (Ananou *et al*., 2007). Bacteriocins have been discovered in a wide range of habitats and conditions, such as fresh and cured meats and sea foods, milk and milk products, spoiled salad dressing and soybean paste. Moreover Shayesteh *et al*. (2014) has reported bacteriocin producing bacteria from marine clams. As is the case in the present investigation, many other reports have been published regarding the inhibitory activity of different LAB against viruses, pathogenic and spoilage microflora in fish, especially processed seafood such as cold-smoked seafood or packaged aquatic food

products (Leroy and De Vuyst 1999; Chen and Hoover 2003; ; Murua *et al*., 2013; Gomez-Sala *et al*., 2016). However, there are only a few reports on the purified bacteriocins produced by the same in application as biopreservative of fish. Currently, nisin and pediocin are the only bacteriocin which is commercially accepted to be used as a biopreservative and marketed as Nisaplin® and pediocin PA-1, marketed as Alta® 2341 (Yang *et al*., 2014). According to some previous studies (Arques *et al*., 2015), nisin in combination with other bacteriocins can induce a greater inhibitory potential than the use of a single bacteriocin and this observation can be used advantageously to design efficient natural food biopreservatives.

CONCLUSIONS

The antimicrobial activity of the bacteriocins produced by the LAB isolated in this research could act as a barrier to inhibit food spoilage and / or growth of pathogenic microorganisms during preservation of Tuna flesh. Further work to evaluate the nature of the substances, their stability in refrigeration conditions, and applicability in biopreservation techniques need to be carried out.

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