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Research Article

Efficacy of Bacteriocin from *Lactobacillus* Sp. (AMET 1506) as a Biopreservative for Seafood's Under Different Storage Temperature Conditions

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Abstract

The safety of seafood is more important and the consumer awareness about quality of their food is gaining fast momentum recently. The quality of the seafood depends on many factors like microbial load, environmental problem, storage conditions and the nature of food. In this paper deals to control some common seafood borne pathogens by using lactic acid bacteria. Morphologically differed 30 *Lactobacillus* spp. strains were isolated from curd sample using MRS agar medium and they were screened against seafood pathogens by agar well diffusion assay. Potentially six strains were selected by their inhibitory activity and they were chosen for the bacteriocin production. Among the six strains, the bacteriocin of strain AMET 1506 has showed the maximum zone of inhibition against the seafood borne pathogens. Thus, strain AMET 1506 was selected for mass scale production of bacteriocin to in order to perform preservation studies. The biochemical tests are also confirmed the strain (AMET 1506) was *Lactobacillus* sp. The shrimps were collected from Rayapuram landing centre, Chennai, Tamil Nadu, India. They were divided in to two groups. One group of the shrimps were stored directly and another group of the shrimps were dipped in cold distilled water containing bacteriocin of *Lactobacillus* sp. and both treatments were stored at different temperatures like -4^0C and -20^0C for 30 days and the microbial load was assessed at different time intervals (1^{st} , 8^{th} , 16^{th} , 24^{th} and 30^{th} day). The presence of pathogenic microbial load in both treatments such as total heterotrophic bacteria, total coliforms and *Escherichia coli*, *Vibrio cholerae*, *V. parahaemolytcus*, *Salmonella* sp., and *Shigella* sp. were assessed by using most probable number technique with specific media. The results of the present study clearly indicated that, the microbial load was reduced in the treatment which is preserved with bacteriocin of *Lactobacillus* sp. (AMET 1506).

***Keywords*:** Lactic acid bacteria, bacteriocins, seafood borne pathogens, Biopreservation.

INTRODUCTION

Shrimps are the most important seafood items among the other exported marine food products in the global fishery trade (Csavas, 1991). Generally, seafood can be associated with many potential risks, especially shrimp are highly susceptible to microbiological contamination due to many factors such as, water quality, temperature, harvesting area, type of sediment, size of shrimp and storage method (Jeyasekaran *et al*., 2006). The quality of shrimp is most important because of the increasing demands for this product in markets, so that major goal for the food processing industries is to provide safe, wholesome and acceptable food to the consumer and control of microorganisms (Baggen-Ravn *et al*., 2003). In general, the preservation processes consist of a combination of mild heat stress and low concentration of chemical preservatives to control the spoilage of food and the outgrowth of food borne pathogenic bacteria.

Nevertheless, these methods have many drawbacks because; it will change the natural flavor, texture and nature of the food (Rasooli, 2007). To solve the above problem and improve the safety, controlling the microbial load by without changing the quality of food is being replaced by innovative technologies. Recently, a new method is employed in the food industry and achieving a good result by controlling the microbial load in the foods is the biopreservation technology. Biopreservation is an alternative natural technology and it is an efficient way for extending the shelf life of food, by inhibiting the pathogenic bacteria by without altering the nutritional quality of raw materials and food products by the use of beneficial or probiotic bacteria and/or their bacteriocins (Dortu and Thonart 2009; Galvez *et al*., 2010). Lactic acid bacteria (LAB) are most traditionally used in many sea food processing industries for the improvement of food flavor, texture and shelf-life (Indira *et al*. (2011) because all Lactic acid bacteria's are generally recognized as safe (GRAS) (Holzapfel *et al*., 1995). Lactic acid bacteria will produce a wide range of products from low molecular mass compounds, such as hydrogen peroxide, carbon dioxide and diacetyl, to high molecular mass compounds, such as bacteriocins (Ammor *et al*., 2006) these products will exhibit the antibacterial activity against various pathogenic microorganisms, including gram positive and gram negative bacteria (Aymerich *et al*., 2000; Maragkuodakis *et al*., 2009) so it as recognized as safe bio-preservative bacteria due to their inhibition by the production of bacteriocin like inhibitory substances (BLIS) (Matamoros *et al*., 2009; Zaheer *et al*., 2010) and some bacteriocins are commercially used as a natural bio preservatives in several food industries. In this context, this study was carried out to determine the effectiveness of *Lactobacillus* sp. (AMET 1506) and their bacteriocin in preservation of economically important and exported seafood shrimp at different temperature storage condition.

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB)

For the isolation of *Lactobacillus* spp., 1 mL of curd sample was mixed in 99 ml of sterile distilled water, and this suspension was serially diluted up to 10^{-4} in 9 mL blank, 0.1 ml of the diluted sample were taken from 10^{-3} and 10^{-4} dilutions and spreaded in MRS agar plates and incubated for 48 h at room temperature (28±2°C). After the incubation period, morphologically different colonies were selected from the MRS agar plates and each strain were individually streaked in MRS agar plates and incubated for 48 h at room temperature (28±2°C).

Antimicrobial activity of LABs

To determine the antimicrobial activity of all the isolated *Lactobacillus* sp., were tested against five different seafood borne pathogens (*E.coli*, *V. cholerae*, *V. parahaemolyticus*, *Salmonella* sp. and *Shigella* sp. which were already isolated from seafood) using agar well diffusion assay (Schillinger and Lucke 1989).

Extraction of bacteriocin

The *Lactobacillus* spp. strains which were showed the zone of inhibition (ZOI) against all the tested sea food pathogenic bacteria are subcultured individually in MRS agar plates. The strains were inoculated separately in 50 mL of MRS broth (pH 6.8) for extraction of bacteriocin, all the culture supernatants were centrifuged at 6000 rpm for 30 minutes at 4°C. The cell free supernatant were precipitated with ammonium sulphate (40% saturation) and kept for 2 h at 4°C, and later centrifuged at 10,000 rpm for 20 minutes. After centrifugation the precipitates were obtained and resuspended in 10 mL of 0.05 M potassium phosphate buffer [pH 7.0] (Arokiyamary and Sivakumar, 2012).

Determination of bacteriocin activity

Agar plates were swabbed with 100 µl of each seafood pathogenic bacteria after growing them in a broth. Once the plates were dried aseptically, 5 mm wells were bored using a sterile cork borer and about 10 µl of (extracted bacteriocin) supernatant was placed into each well. Then the plates were incubated for 24 h at 37°C. After the incubation period the antimicrobial activity was determined by measuring the diameter of the ZOI around the wells (Arokiyamary and Sivakumar, 2012). The strain which showed the maximum inhibition zone against the tested seafood borne pathogens is taken for the mass scale production of bacteriocin.

Mass scale production of bacteriocin

The bacteriocin of *Lactobacillus* sp. (AMET 1506) strain which showed the strongest antimicrobial activity against seafood borne pathogens was chosen for mass scale production of bacteriocin. The culture was inoculated in 1000 mL MRS broth (pH 6.8), after the incubation period the bacteriocin was isolated by following the aforesaid procedure.

Shrimp sample preparation and treatment application

Fresh shrimp (*Penaeus monodon*) samples were collected from Rayapuram landing centre, Chennai, Tamil Nadu, India. The shrimps were transferred by using icebox to the Laboratory within 1 hour. The shrimps were immediately manually eviscerated, headed and filleted. They were divided in to two groups. One group of the shrimps were stored directly and other group of the shrimps were dipped in cold distilled water containing LAB bacteriocin, and both treatments of shrimps were packed in polyethylene bags and stored at different temperatures at –4°C and –20°C.

Microbiological analysis

Shrimps were taken randomly from both treatments at different time intervals (1^{st} , 8^{th} , 16^{th} , 24^{th} and 30^{th} day) and homogenized using mortar and pestle, 10 g of the sample was mixed in 95 mL of sterile saline (0.85% NaCl) and this suspension was serially diluted up to 10^{-4}. For isolation of total heterotrophic bacteria (THB) spread plate method was followed using nutrient agar medium, for the isolation of total coliforms, *E. coli*, *Vibrio* spp., *Salmonella* sp. and *Shigella* sp. MPN technique was followed using EMB agar, TCBS agar and SS agar, respectively.

RESULTS AND DISCUSSION

In spite of the modern technologies and safety concepts there are wide range of preservation techniques are available, but the number of food borne illnesses is in rise and the safety of food is still an increasingly important public health issue (WHO, 2002). Hence, the biopreservation is an emerging technique to the seafood industries using lactic acid bacteria as preservatives in food products and it will confer health benefits to the consumers (Olaye and Idowu, 2010). In this present study morphologically differed 30 *Lacto bacillus* spp. strains were isolated and they were named as AMET 1501 to AMET 1530. To determine their antimicrobial activity, all the 30 strains were tested against 5 different seafood borne pathogens (*E. coli*, *V. cholerae*, *V. parahaemolyticus*, *Salmonella* sp. and *Shigella* sp.) using agar well diffusion assay. Based on their ZOI, the strains AMET 1503, AMET 1506, AMET 1518, AMET 1519, AMET 1526, AMET 1530 were showed the maximum inhibitory activity against all the tested seafood pathogens (Table 1). All these six strains were taken for bacteriocin production to determine their bacteriocin activity against seafood pathogens. Among the six strains the strain AMET 1506 has showed the maximum inhibitory activity towards all the tested pathogenic bacteria (Table 2). The phenotypic and biochemical tests were performed to identify the strain (AMET 1506), according to the Bergey's Manual of Systematic Bacteriology (1984) guidelines; the LAB strain (AMET 1506) is identified as *Lactobacillus* species. Previously the researchers also described that the antibacterial activity of *Lactobacillus* sp. may be due to the production of its many metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Ennahar *et al*., 2000; Lasagno *et al*., 2002 and Valenzuela *et al*., 2010). Ponce *et al*., (1998) also stated that the antimicrobial properties of lactic acid are attributed to the un dissociated lactic acid molecule and to a reduction of pH below the level at which the growth of many bacteria is inhibited. Organic acids and their salts can potentiate the activity of bacteriocins greatly, whereas acidification enhances the antibacterial activity of both organic acids and bacteriocins.

Regarding the results from the tables (3 and 4), the number of THB was higher in directly preserved shrimp sample than in bacteriocin treated and preserved shrimp sample at both temperature conditions. It confirms that, the growth of THB have been reduced by the bacteriocin of *Lactobacillus* sp. (AMET 1506). When assessing the total number of coliforms, it was suddenly reduced and there was no more presence of coliforms from 8^{th} day onwards in bacteriocin treated and preserved shrimp sample than directly preserved shrimp sample. The reduction of coliforms in bacteriocin treated shrimp treatment could be due to the acidification by inhibitory compounds formed by *Lactobacillus* sp. (AMET 1506). Murali *et al*. (1985) also reported that the complete inhibition of coliform was observed in mutton preservation with *Lactobacillus* sp. and the reduction of coliform number might ensure good biopreservation against pathogenic microorganisms (Ndaw *et al*., 2008).

Table1: Antimicrobial activity of 30 *Lactobacillus* sp. isolates against seafood borne pathogens

LAB Strains	Zone of inhibition (in mm) against Sea food pathogens				
	E. coli	*V. cholerae*	*V. parahaemolyticus*	*Salmonella* sp.	*Shigella* sp.
AMET 1501	+	++	-	+	+
AMET 1502	+	-	++	-	-
AMET 1503	+	++	++	++	++
AMET 1504	++	+	-	+	+
AMET 1505	+	+	-	+	++
AMET 1506	+++	+++	++	+++	+++
AMET 1507	+	-	-	++	+
AMET 1508	-	+	-	+	+
AMET 1509	+	-	-	+	-
AMET 1510	++	+	+	-	+
AMET 1511	+	-	+	-	-
AMET 1512	+	-	-	+	-
AMET 1513	+	-	+	-	-
AMET 1514	-	-	+	+	+
AMET 1515	++	-	+	+	-
AMET 1516	-	+	-	+	+
AMET 1517	+	+	-	-	-
AMET 1518	++	+	+	+	+
AMET 1519	++	++	++	++	++
AMET 1520	-	+	+	-	-
AMET 1521	-	-	+	+	+
AMET 1522	+	+	-	-	+
AMET 1523	+	+	-	+	-
AMET 1524	-	+	+	+	+
AMET 1525	-	+	-	+	+
AMET 1526	++	++	++	++	++
AMET 1527	+	-	-	+	+
AMET 1528	+	-	+	-	-
AMET 1529	-	+	-	+	-
AMET 1530	++	++	++	++	+

−: no inhibition zone; +: ZOI up to 4 mm; ++: ZOI up to 8 mm; +++: ZOI up to 12 mm

Table 2: Screening of *Lactobacillus* sp. bacteriocin against seafood borne pathogens

LAB Strains	Zone of inhibition (in mm) against Sea food pathogens				
	E. coli	*V. cholerae*	*V. parahaemolyticus*	*Salmonella sp.*	*Shigella sp.*
AMET 1503	+	++	++	+	+
AMET 1506	+++	+++	+++	+++	+++
AMET 1518	+	++	++	++	++
AMET 1519	++	+	+	+	+
AMET 1526	+	++	+	+	++
AMET 1530	+	+	++	++	++

−: no inhibition zone; +: ZOI up to 4 mm; ++: ZOI up to 8 mm; +++: ZOI up to 12 mm

Table 3: Microbial load on directly preserved shrimp samples

Seafood pathogens	0 hr	Microbial load at –4°C (in Days)					Microbial load at –20°C (in Days)				
		1	8	16	24	30	1	8	16	24	30
Total Heterotrophic Bacteria (CFU/g)	5.2×10^5	2600	190	11	-	-	210	6	-	-	-
Total coliforms MPN/100 mL	140	21	17		-	-	17	-	-	-	-
E. coli MPN/100 mL	90	14	12	-	-	-	14	-	-	-	-
V. cholerae MPN/100 mL	70	11	9	-	-	-	13	-	-	-	-
V. parahaemolyticus MPN/100 mL	70	9	7	-	-		12	-	-	-	-
Salmonella sp. MPN/100 mL	33	12	-	--	-	-	7	-	-	-	-
Shigella sp. MPN/100 mL	34	11	-	-	-	-	9	-	-	-	-

Table 4: Microbial load on *Lactobacillus* sp. (AMET 1506) bacteriocin impregnated preserved shrimp samples

Seafood pathogens	Microbial load at –4°C (in Days)					Microbial load at –20°C (in Days)				
	1	8	16	24	30	1	8	16	24	30
Total heterotrophic bacteria (CFU/g)	110	-	-	-	-	220	-	-	-	-
Total coliforms MPN/100 mL	6	-	-	-	-	14	-	-	-	-
E. coli MPN/100 mL	7	-	-	-	-	11	-	-	-	-
V. cholera MPN/100 mL	9	-	-	-	-	11	-	-	-	-
V. parahaemolyticus MPN/100 mL	2	-	-	-		9	-	-	-	-
Salmonella sp. MPN/100 mL	2	-	--	-	-	6	-	-	-	-
Shigella sp. MPN/100 mL	4	-		-	-	6	-	-	-	-

Yusuf and Varadaraj (1999) also reported that the bacteriocin produced by *Lactobacillus* sp. was inhibited the growth of *E. coli* in their study, the similar results also observed in this study also very similar to their results. Moreover, *E. coli* load was totally reduced in bacteriocin treated shrimp sample comparing to the directly preserved shrimp treatment and the growth of *E. coli*, have been reduced in bacteriocin treated shrimp treatment due to the effectiveness of the bacteriocin from *Lactobacillus* sp. (AMET 1506). The presence of *V. cholerae* and *V. parahaemolyticus* was reduced in bacteriocin treated shrimp sample from 8th day onwards at both temperature treatments. Chae *et al*. (2009) and Indira *et al*. (2011) also reported that the growth of *V. cholerae* and *V. parahaemolyticus* was inhibited in fish pickle and other products by *Lactobacillus* sp. In addition, Indira *et al*. (2011) reported that their *Lactobacillus* sp. strains were showed the inhibitory activity against *Salmonella* sp. and *Shigella* sp. food pathogens in fish pickle products, whereas Lozano *et al*. (2002) reported that, the strains of *Lactobacillus* sp. has not inhibited the *salmonella* sp. in meat products. In our study, there was no more presence of *Salmonella* sp. and *Shigella* sp. from 8th day onwards in both treatments at both temperature conditions. From the results, the work highlighted the presence of microbial load between the two treatments, and the results from both treatments were clearly indicated that the directly preserved shrimps have more microbial load than the *Lactobacillus* sp. (AMET 1506) bacteriocin impregnated preserved shrimps. Hence, this study clearly demonstrated the effectiveness of *Lactobacillus* sp. (AMET 1506) and their bacteriocins against seafood borne pathogens and their efficiency in biopreservation.

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