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BIOPRESERVATION OF VALUE ADDED MARINE FISHES UNDER DIFFERENT STORAGE CONDITIONS USING BACTERIOCIN FROM LACTOBACILLUS SP (AMETLAB27)

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ABSTRACT

In the present study, morphologically differed 30 strains of Lactobacillus sp. were isolated from curd sample using MRS agar medium and they were screened against seafood pathogens by agar well diffusion assay. Eight potential strains were selected based on their inhibitory activity and they were selected for bacteriocin production. Among the eight strains, the bacteriocin produced by AMETLAB27 strain has showed the maximum zone of inhibition against all the tested seafood borne pathogens that were under study. Thus, strain AMETLAB27 selected for mass scale production of bacteriocin in order to perform the preservation studies. Based on the biochemical characteristics the strain identified as Lactobacillus sp. The value added and commercially available fishes such as Lutjanus campechanus, Gerres subfasciatus and Sardina pilchardus were collected from Rayapuram landing centre, Chennai, Tamil Nadu, India and divided into two groups. One group of fishes were stored directly as control and the other group of fishes were dipped in cold distilled water containing the bacteriocin of Lactobacillus sp. (AMETLAB27) and both treatments were stored at different temperatures like –40C and –240C for 30 days and the microbial load assessed at different time intervals (1st, 8th , 16th and 24th day). The presence of pathogenic microbial load in both the treatments such as total heterotrophic bacteria (THB), Escherichia coli, Vibrio cholerae, Vibrio parahaemolytcus, Salmonella sp, Shigella sp and Listeria sp were assessed by using most probable number (MPN) technique with specific media. The results of the study indicated that, the microbial load has been reduced in the treatment which preserved with bacteriocin produced by Lactobacillus sp (AMETLAB27).

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KEY WORDS

Lactic acid bacteria; Bacteriocins; Seafood borne pathogens; Bio-preservation

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INTRODUCTION

Seafood is one of the most important constituent for maximum section of the consumers' diet worldwide and ranks the most widely consumed food after meat and poultry as staple animal protein foods. Especially fishes form a cheap source of protein. Presently seafood such as fishes and prawns are the most widely consumed foods [1]. Generally, seafood can be associated with many potential risks; especially fishes are highly susceptible to microbiological contamination due to many factors such as water quality, temperature, harvesting area, type of sediment, size and storage method [2]. Quality of seafood is the most important aspect, because of the increasing demand for its products in markets, the food processing industries fix a goal to provide safe, wholesome and acceptable food to the consumer which is devoid of harmful microorganisms [3]. The common ways of preservation are applying mild heat stress and the use of chemical preservatives in low concentration to prevent the spoilage of food by the outgrowth of food borne pathogenic bacteria. But these methods have many disadvantages as it may change the natural flavour, texture and nature of the food [4]. To overcome the above problem and to improve the safety of the food by controlling the microbial load without changing the quality of food many innovative technologies have been introduced [5]. One of the innovative technologies being followed to improve the safety of the food is Bio-preservation. Bacteriocins of Lactic acid bacteria are considered to be a safe bio-preservative, since they are assumed to be degraded by proteases in gastrointestinal tract [6]. Most of the probiotic strains produce antimicrobial substances such as lytic enzyme, hydrogen peroxide, organic acids & Bacteriocin [7]. In particular, Lactic acid bacteria will produce a wide range of products from low molecular weight compounds, such as hydrogen peroxide, carbon dioxide and diacetyl to high molecular weight compounds, such as bacteriocins [8]. Since these compounds exhibit antibacterial activity against various pathogenic bacteria including gram positive and gram negative [9], the bacteria which produce these substances are recognized as safe

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bio-preservative bacteria and also due to their inhibition by the production of bacteriocin like inhibitory substances (BLIS) [10,11]. Some bacteriocins are commercially used as natural bio-preservatives in several food industries. In this context, the study aimed to determine the effectiveness of Lactobacillus sp. and their bacteriocin in the preservation of economically important fishes such as, Northern Red Snapper (*Lutjanus campechanus*), Silver Belly Fish (*Gerres subfasciatus*) and European Pilchard (*Sardina pilchardus*) at different temperature storage conditions.

MATERIALS AND METHODS

Isolation of Lactobacillus sp

For the isolation of lactic acid bacteria 10ml of homemade curd sample added to 90ml of sterile distilled water and it serially diluted up to 10-6. From the dilutions such as 10-4, 10-5 and 10-6, 1ml of sample taken and spreaded over sterile deMan Ragosa and Sharpe (MRS) agar plates and incubated at room temperature for 48 hours. After the incubation period morphologically different colonies were selected and subcultured in MRS agar plates and stored for further study. The morphological nature and the biochemical characterization were studied for the bacterial isolates as colonial morphology is an important parameter for preliminary identification.

Isolation of Seafood pathogens

The sea food pathogens such as, *E. coli, Vibrio cholerae, Vibrio parahaemolyticus, Salmonella sp, Shigella sp and Listeria sp* were isolated from infected fish samples using specific media [5].

Antibacterial activity of Lactobacillus sp

To determine the antibacterial activity of all the isolated strains of *Lactobacillus* sp, they were tested against the six seafood borne pathogens (E. *coli, V.cholerae, V.parahaemolyticus, Salmonella sp, Shigella sp and Listeria* sp) using agar well diffusion assay where the pathogens were already swabbed on nutrient agar plates [12].

Extraction of bacteriocin

The strains of Lactobacillus sp. which showed the zone of inhibition (ZOI) against all the tested sea food pathogenic bacteria were subcultured individually in MRS agar plates. The strains were inoculated separately in 50 mL of MRS broth (pH 6.8). For the extraction of bacteriocin, all the culture supernatants were centrifuged at 6000 rpm for 30 minutes at 4°C. The cell free supernatant 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 precipitate obtained resuspended in 10 mL of 0.05 M potassium phosphate buffer (pH 7.0) [13].

Determination of bacteriocin activity

For the determination of the activity of the bacteriocin against the pathogens, the agar plates were swabbed with 100µl of the isolated pathogens after growing them in their respective broths. Once the plates were dried aseptically, 5 mm wells were bored using a sterile borer and about 10µl of the extracted bacteriocin poured into each well. Then the plates were incubated for 24 h at 37°C. After the incubation period the antimicrobial activity determined by measuring the diameter of the ZOI (Zone of inhibition) around the wells [13]. The strain that showed the maximum inhibition zone against the tested seafood borne pathogens inoculated in 1000 mL MRS broth (pH 6.8) for mass scale production of bacteriocin by following the aforesaid procedure.

Optimization of bacteriocin production

The factors like pH, temperature, salinity and substrate concentration which were expected to influence the production of bacteriocin by the selected strain optimized by using one parameter at a time method. To determine the growth conditions of the selected potential Lactobacillus sp strain, it inoculated in MRS medium at different pH (6.0, 6.5, 7.0, 7.5, 8.0), temperature (25°C, 30°C, 35°C, 40°C, 45°C), saline concentrations (1%, 1.5%, 2%, 2.5%, 3%), with different carbon sources at 3% (Lactose, Starch, Dextrose, Fructose, Sucrose) and nitrogen sources at 3% (Ammonium sulphate, Peptone, Beef extract, Ammonium nitrate, Sodium nitrate). The growth of Lactobacillus sp in the medium determined at every 6hrs by observing the OD at 520nm.

Determination of protein concentration and Molecular weight of the purified bacteriocin

The protein content of the bacteriocin was determined by Lowry's method using Bovine Serum Albumin as standard. The molecular weight of the bacteriocin determined by Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis

Lyophilisation of bacteriocin

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After dialysis the partially purified bacteriocin was lyophilized for 48 hours at -50˚C by using Freeze-dryer and it was stored at two different temperatures -4^{0}C and -24^{0}C in Eppendorf tubes for further use [14]. During the storage period the anti-bacterial effectiveness of lyophilized bacteriocin was determined by dissolving 0.1g of the dried sample in 1ml of distilled water. Sterile discs were dipped in the sample and placed on the nutrient agar plates where the pathogens were already swabbed. After 24 hours of incubation, the zone of inhibition around the discs measured and the activity of bacteriocin was determined.

Fish sample preparation and treatment

Fresh fish samples (Lutjanus campechanus, Gerres subfasciatus, Sardina pilchardus) shown in **Figure– 7, Figure– 8, Figure– 9** were collected from Rayapuram landing centre, Chennai, Tamil Nadu, India. The fishes were stored in icebox and brought to the laboratory within 1 hour. One group of the fish samples were stored directly and other group of the fish samples were dipped in cold distilled water containing bacteriocin produced by the strain, and both the treatments were packed in sterile polyethylene bags and stored at different temperatures at –4°C and –24°C. The fishes that were stored without bacteriocin treatment served as control.

Microbiological analysis

Fishes were taken randomly from both treatments at different time intervals (0th, 1st, 8th, 16th and 24th day) and homogenised using mortar and pestle. 10g of the sample was mixed in 90 ml of sterile distilled water and this suspension was serially diluted up to 10-4. For the analysis of Total Heterotrophic Bacteria (THB) pour plate method was followed by using Nutrient agar medium. For the isolation of *E.coli, Vibrio sp., Salmonella sp., Shigella sp.,* and *Listeria* sp., MPN technique was followed by using EMB agar, TCBS agar SS agar and PALCAM agar respectively.

RESULTS AND DISCUSSION

The In spite of the modern technologies, safety concepts and preservation techniques, the number of food borne illness is in rise and the safety of food is still an important public health issue to be noticed [1]. Hence bio-preservation is an emerging technique to the seafood industries where lactic acid bacteria is used as preservatives in food products and it will provide health benefits to the consumers [15].

Isolation of Lactic Acid Bacteria from curd sample

In this study morphologically differed 30 strains of Lactobacillus sp. were isolated and they were named as AMETLAB01 to A*coli*, *V. cholerae*, *V. parahaemolyticus*, *Salmonella* sp, *Shigella* sp and *Listeria* sMETLAB30. The morphological characteristics, biochemical characteristics and gram nature of the 30 bacterial colonies w *Lutjanus campechanus, Gerres subfasciatus* and *Sardina pilchardus* ere noted. To determine their antimicrobial activity, all the 30 strains were tested against 6 different seafood borne pathogens (E. *coli, V. cholerae, V. parahaemolyticus, Salmonella sp, Shigella sp and Listeria s*p) using agar well diffusion assay. Based on their zone of inhibition (ZOI), the eight strains namely AMETLAB01, AMETLAB02, AMETLAB03, AMETLAB07, AMETLAB09, AMETLAB27, AMETLAB28, AMETLAB29 which showed the maximum inhibitory activity against all the tested seafood pathogens were potentially selected for further study.

All these eight strains were taken for bacteriocin production to determine their bacteriocin activity against seafood pathogens. Among the eight strains the strains namely AMETLAB02, AMETLAB07 and AMETLAB27 that showed the maximum inhibitory activity towards all the tested pathogenic bacteria were again confirmed for the activity of their bacteriocin towards all the seafood pathogens and the strain AMETLAB27 was found to be more potential than the other strains. The phenotypic and biochemical tests were performed to identify that the strain (AMETLAB27) was Lactobacillus species [16]. The strain was found to be gram positive, non-motile, non-spore forming and rod shaped.

Optimization of the growth medium

In the present study the results has proved the possibility of using this strain as a bio-preservative. So, the potential Lactobacillus sp strain was taken for optimization using one parameter at a time method. The optimal growth conditions for bacteriocin production were found to be at pH 7, 25ºC, 1% salinity, starch-3% as carbon source, 3% beef extract as nitrogen source with 24hrs incubation. **Figure– 1, Figure– 2, Figure– 3, Figure– 4** and **Figure– 5** shows the representation of the optimal growth conditions (carbon source, nitrogen source, pH, temperature and salt concentrations). The strain AMETLAB27 was then grown in this optimised medium and the pure culture was preserved for future study. **Figure– 6** shows the pure culture of AMETLAB27.

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Determination of protein concentration, molecular weight and lyophilisation

The potential strain of Lactobacillus sp was mass cultured in the optimized medium and the thus produced bacteriocin was partially purified by ammonium sulphate precipitation and dialysis. The total protein in the purified bacteriocin was determined as 0.43mg/ml by Lowry's method and two prominent bands were found in the SDS gel which corresponds to the molecular weight of 39KDa and 10KDa by SDS-PAGE analysis. After dialysis the partially purified bacteriocin was lyophilized for 48 hours at -50^{0}C by using Freeze-dryer and it was stored at two different temperatures -4^{0}C and -24^{0}C and its antibacterial effectiveness was found to be higher when stored at -24^{0}C.

Microbiological analysis

While preserving the value added and commercially available marine fishes (*Lutjanus campechanus*, *Gerres subfasciatus* and *Sardina pilchardus*) using the bacteriocin of Lactobacillus sp under two different temperature conditions, the microbial load in the fish samples preserved with bacteriocin was found to have reduced comparatively than the ones stored as control (preserved without bacteriocin). When the number of THB load during the preservation period was observed, it was found higher in number in the directly preserved fish sample (control) than in the samples treated with bacteriocin at both temperature conditions. This confirms that the growth of THB in the fishes has been eliminated by the bacteriocin of Lactobacillus sp (AMETLAB27) and also the bacteriocin from this particular strain can be used as a Bio-preservative in food processing industries.

Earlier it was cited that the antibacterial activity of Lactic acid bacteria was due to the production of metabolites like organic acids with low pH, hydrogen peroxide and bacteriocins [17-19]. The activity of the bacteriocins is greatly influenced by the organic acids and their salts. While reviewing another paper it was understood that these antimicrobial properties may be owing to the fact that the undissociated lactic acid molecules have a pH below the level at which the growth of many bacteria can be eliminated [20]. Other bacteriocins of Lactobacilli have been described to be potential against closely related species of mesophilic *Lactobacillus* and are considered as potential natural food preservatives [21].

E. coli load was totally reduced in bacteriocin treated fish samples comparing to the directly preserved fishes and the reduction in the growth of *E. coli* is due to the effectiveness of the bacteriocin from *Lactobacillus* sp. (AMETLAB27).

The presence of V. cholerae and V. parahaemolyticus was reduced in fish samples treated with bacteriocin from 8th day onwards in both the temperature conditions. The growth of Salmonella sp was also reduced completely from the 16th day onwards in the fish samples. But it was reported that the strains *of Lactobacillus* sp. has not inhibited the Salmonella sp. in meat products [22]. In our study, we incurred that there were no occurrence of Shigella sp and Listeria sp from 8th day onwards in the samples treated with bacteriocin at both temperature conditions.

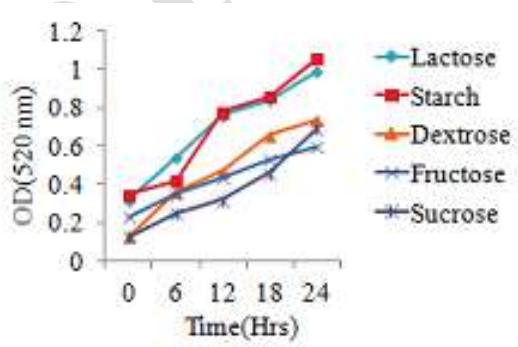

Fig: 1. Effect of carbon source on the growth of Lactobacillus sp (AMETLAB27)

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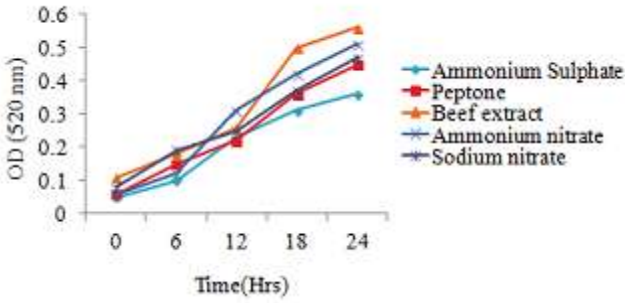

Fig: 2: Effect of nitrogen source on the growth of Lactobacillus sp (AMETLAB27)

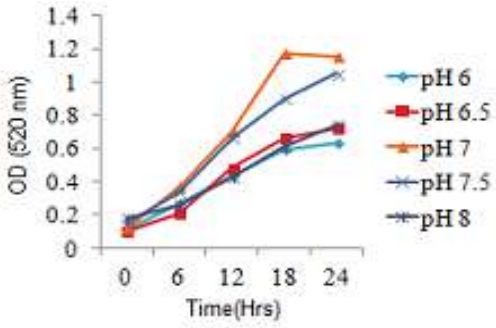

Fig: 3. Effect of pH on the growth of Lactobacillus sp (AMETLAB27)

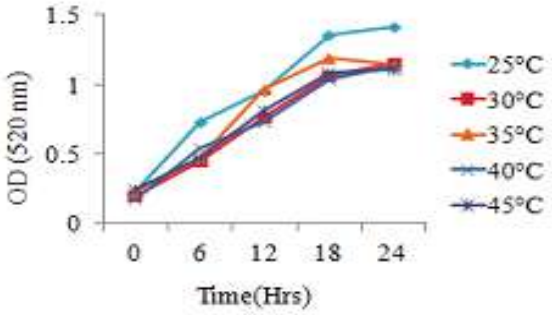

Fig: 4. Effect of temperature on the growth of Lactobacillus sp (AMETLAB27)

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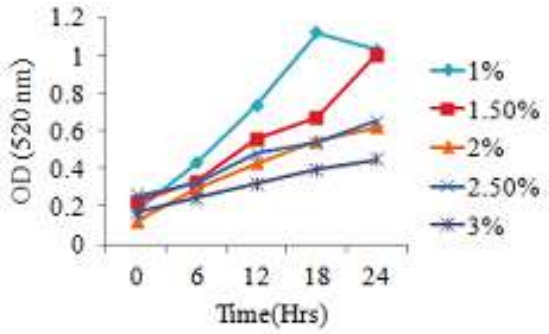

Fig: 5. Effect of various salt concentrations on the growth of Lactobacillus sp (AMETLAB27)

Fig: 6. Pure culture of Lactobacillus sp (AMETLAB27)

Fig: 7. Lutjanus campechanus Fig: 8. Gerres subfasciatus Fig: 9. Sardina pilchardus...

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CONCLUSION

From the results, the work emphasised the elimination of microbial load from the fish samples treated with bacteriocin produced from Lactobacillus sp (AMETLAB27) preserved at -40C and -240C. This study revealed that the strain *of Lactobacillus* sp (AMETLAB27) is a potential strain and their bacteriocins were efficient against seafood borne pathogens and the usefulness of them as a bio-preservative as it has antimicrobial effects on some clinically important food borne pathogens. The bacteriocins produced by gram positive bacteria especially lactic acid bacteria display a proper broad inhibitory spectrum with food preservative and therapeutic potentials. This confirmed the possibility of using the strain as a bio-preservative in fish processing industries. This exposes that the bacteriocin produced by *Lactobacillus* sp (AMETLAB27) can be applied as a defensive culture for the enhancement of the microbial safety of fermented foods and reduction in food contamination that caused various disorders and illness to the human beings in the near future.

CONFLICT OF INTEREST

There is no any form of conflict of interest

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