

Effectiveness of Bacteriocin from *Bacillus Subtilis* (KY808492) and its Application in Biopreservation

Ashwitha A¹, Thamizharasan K¹, Vithya V¹, Karthik R² and Vijaya Bharathi S^{1*}

¹Department of Biotechnology, FSH, SRM University, India

²Aquaculture Division, Guybro Chemical Pvt Ltd, Mumbai, India

Received: 02.06.2017 / Accepted: 20.06.2017 / Published: 22.06.2017

Abstract:

In this study, bacterial strains were isolated from curd and evaluated its bactericidal potential against two aquatic pathogens *Salmonella sp* and *Vibrio sp* isolated from marine fish, *Parastromateus niger* and squid, *Loligo duvauceli*. Based on the antimicrobial activity, potential strain was selected and identified as *Bacillus sp*. Further, bacteriocin was partially purified from the *Bacillus sp* by ammonium sulphate precipitation and dialysis. The molecular weight of the partially purified bacteriocin was estimated by SDS-PAGE. To understand the biopreservative ability of the purified bacteriocin, tissues of *Salmonella* and *Vibrio*-infected fish, *Parastromateus niger* and *Loligo duvauceli* were treated with bacteriocin for 30 days at different storage conditions (-4°C and -20°C) and the microbial load was counted at 5, 10, 15, 20, 25 and 30 days. Results showed that bacteriocin significantly reduced the total bacterial count at both storage temperatures in a time dependent manner. This study proved the efficiency of bactericidal and biopreservative activity of bacteriocin isolated from *Bacillus subtilis* (KY808492).

Keywords: *Bacillus sp*; Bacteriocins; Seafood pathogens; Biopreservation

*Correspondence to:

Vijaya Bharathi S, Department of Biotechnology, FSH, SRM University, India, Tel: +91-44- 27417000;
E-mail: vijayabiotech10@gmail.com

Introduction

Seafood is consumed by humans for their nutritive values which prominently include fish and shellfish. Shellfish include various species of molluscus, crustaceans etc. (WHO, 2002). Generally, seafood can be associated with m bacteria produce a wide range of antimicrobial products from low molecular weight compounds such as hydrogen peroxide and carbon dioxide, to high molecular compounds such as bacteriocins (Roy, 1997). These bacteriocins exhibit antibacterial activity against many seafood pathogenic bacteria including gram positive and negative and hence it has been used as a bio-preservative (Aymerich 2000). Some bacteriocins are synthesized commercially in industries and are used as a natural biopreservative. The present study aimed to screen the potential probiotic strain from curd with bactericidal and biopreservative potential.

Materials and Methods

Isolation of seafood pathogens

Marine fish, *Parastromateus niger* and squid, *Loligo duvauceli* was collected from local fish market in Chennai, India and the infected fishes were picked. To isolate seafood pathogens such as *Salmonella* sp and *Vibrio* sp the infected fish tissues were smashed and the extract was spread over specific media such as SS agar and TCBS agar (HiMedia) respectively and the isolated pathogens were confirmed by biochemical tests (Lee, 2008).

Screening of probiotic bacterial strain

To isolate probiotic bacteria, curd sample was serially diluted up to 10^{-6} and inoculated in the specific media MRS agar medium (HiMedia) and incubated for 48 hrs at room temperature. 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.

Antimicrobial activity of LABs

To determine the antimicrobial activity of all the isolated probiotic bacterial strains, they were tested against the seafood pathogens, *Salmonella* sp and *Vibrio* sp using agar well diffusion method (Wilson, 2016).

Partial purification of bacteriocin

The probiotic bacterial strain which showed the zone of inhibition (ZOI) against the tested sea food pathogenic bacteria was potentially selected subcultured individually in MRS agar plates. The strain was 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 was 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 was obtained and resuspended in 10 mL of 0.05 M potassium phosphate buffer.

Determination of molecular weight by SDS-PAGE

The molecular weight of the bacteriocin was determined by Sodium dodecyl sulphate polyacrylamide gel electrophoresis. 10 mL of the separating gel taken, mixed well and immediately poured between the plates till the level is about 2 cm below the top of edge of notched plate. After the gel set, the top of the separating gel washed with distilled water. 5 mL of the stacking gel prepared and immediately poured on the polymerised separating gel. The comb inserted into the gel solution carefully without trapping any air bubbles 1 cm above the separating gel. After the gel is formed the samples were loaded into the wells. The chords were connected to the power supply and the voltage set between 50-100 V and switched on. When the dye front reaches 0.5 cm above the bottom of the gel, it placed in the staining solution for 1hr and then placed in the destaining solution until the bands are clearly observed.

Determination of bacteriocin activity

Agar plates were swabbed with 100 µl of both 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. The strain which showed the maximum inhibition zone against the tested seafood borne pathogens is taken for the mass scale production of bacteriocin (Wilson et al., 2016; Quanli et al., 2012; Karthik et al., 2013).

Mass scale production of bacteriocin

The bacteriocin of potential 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.

Identification and confirmation of bacterial strain

The potential probiotic bacterial strains were subjected to biochemical examination and based on the results they were identified by using Bergey's Manual of Determinative Bacteriology (1989). Further identification was done by 16S rRNA sequencing. PCR was used to amplify the 16S ribosomal DNA and was determined by direct sequencing. Total DNA was isolated SoluteReady® genomic DNA purification kit. Agrose gel (2%) was used to isolate 16S rRNA. 16S rRNA was separated by gel electrophoresis on gel made with 2% acrylamide and bis-acrylamide.

Biopreservation activity of purified Bacteriocin

To determine the biopreservation activity of bacteriocin, infected fish tissues from *Parastromateus niger* and *Loligo*

duvauceli were collected and taken into two groups. One group of the tissue 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 for 30 days. The tissues that were stored without bacteriocin treatment served as control.

Microbiological analysis

Stored tissue samples were taken randomly from both treatments at different time intervals (5th, 10th, 15th, 20th, 25th & 30th day) and homogenised using mortar and pestle. 10 g 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) Nutrient agar medium (HiMedia) was used. *Salmonella* sp and *Vibrio* sp were enumerated by using SS agar and TCBS agar respectively. All the experiments were performed in triplicates (Karthik et al., 2013).

Results and Discussion

Modern technologies in food processing and implementation of food safety standards have reduced the loss due to food spoilage and the related food-borne illness. However, the chemical based preservatives and food additives have their own side effects which emphasize the importance of using natural preservatives. Marine fish products are a major category of frozen foods which is an important business with huge commercial values. But the spoilage caused by bacterial pathogens is a major threat in this industry which requires a better preservative. Biopreservatives are one of the natural preservatives in which non-pathogenic microorganisms and/or their metabolites are used to improve the safety and shelf life of foods (Galvez et al., 2007). In recent years, lactic acid bacteria producing bacteriocins have received more importance for their potential application as biopreservatives in food industry (Stiles, 1996). In this study, we have analysed the biopreservation ability of bacteriocin producing probiotic bacteria isolated from curd to prevent the growth of bacterial pathogen in fish tissues.

Bacillus bacterial strains were isolated from curd and it was used as source for bacteriocin. Serially diluted curd sample was plated on MRS broth and 20 morphologically different bacterial colonies were isolated and they were named as KY808480 to KY808499. All the 20 isolates were subjected to agar well-diffusion assay to screen the potential strains with antimicrobial activity against seafood pathogens *Salmonella* sp and *Vibrio* sp that are isolated from infected fish tissues. Based on their zone of inhibition, one potential strain (KY808492) which showed the maximum activity of 10 mm, 6 mm against both the tested pathogens were selected for further study (Table 1). Then, the parameters required for the maximum growth of the selected strain was optimized as suggested by (Wilson et al., 2016). The maximum growth was observed at pH 7, 25°C , 1% salinity, 3% starch as carbon source and 3% beef extract as nitrogen source after 24 hrs of incubation at 37°C . The growth range of *Bacillus* sp in the modified medium was found to be higher than the normal MRS broth (Graph 1). Therefore, the modified medium has been used to grow *Bacillus* sp for the further assays.

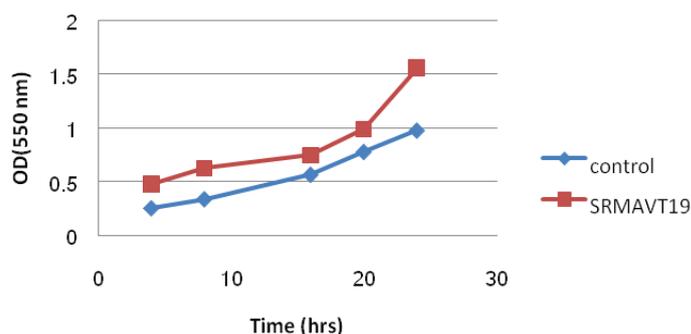
The screened potential probiotic strain KY808492 was identified as *Bacillus subtilis* by 16SrRNA sequencing (Figures 1-7). According to previous reports, *Bacillus* sp was considered as one of the important species with significant broad range of antimicrobial action against bacteria and fungus. Also many strains of *Bacillus* sp have been reported with potential bacteriocin producing ability. But the prospect of using bacteriocin produced by *Bacillus* sp as biopreservative to preserve frozen fish tissues have not been studied. Therefore, this study aimed at analyzing the biopreservative ability of the bacteriocin purified from the selected *Bacillus* sp strain. To confirm the bioactivity of the partially purified bacteriocin, well-diffusion assay was performed against both the bacterial pathogens. Interestingly, higher antimicrobial activity of 12 mm and 18 mm was observed in purified bacteriocin than the crude extract (Table 2). This specifies the bioactivity of the purified bacteriocin against both the fish pathogens, *Salmonella* sp and *Vibrio* sp.

Molecular weight of both the crude extract and the partially purified bacteriocin has been determined by SDS-PAGE Electrophoresis which corresponds to about 17 KDa and 20 KDa (Figure 1). The one prominent band of purified bacteriocin in the SDS-PAGE was cut and the antimicrobial activity was determined against the isolated seafood pathogens using agar diffusion method. After incubation of 24 hrs, the clear zones were observed around the bands and it was measured up to 25 mm and 20mm (Figure 2) which confirms the inhibitory activity of bacteriocin against the seafood pathogens and the viability of bacteriocins.

The biopreservation ability of bacteriocin produced by *Bacillus* sp in lowering the bacterial load of frozen tissues of *Parastromateus niger* and *Loligo duvauceli* with *Salmonella* sp and *Vibrio* sp infections was observed. Infected tissues were treated with bacteriocin and stored at two different temperatures (-4°C and -20°C) for 30 days and the total heterotrophic bacterial

Table 1: Zone of inhibition of probiotic strains against seafood pathogens.

Strain Name	<i>Salmonella</i> sp	<i>Vibrio</i> sp
KY808480	-	5 mm
KY808485	-	8 mm
KY808490	5 mm	-
KY808492	10 mm	6 mm



Graph 1: Growth kinetics of *Bacillus subtilis* (KY808492) in the optimized MRS broth.

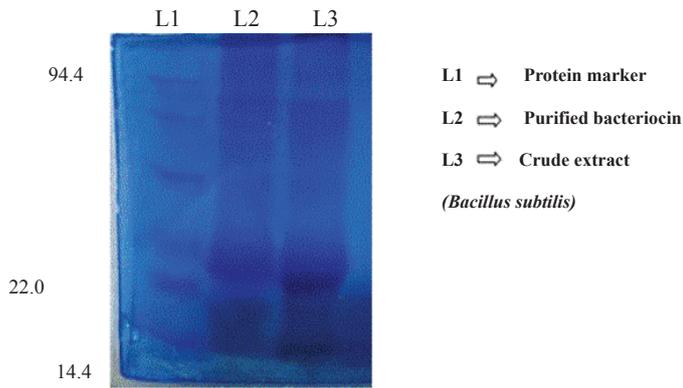


Figure 1: Molecular weight determination- SDS-PAGE.

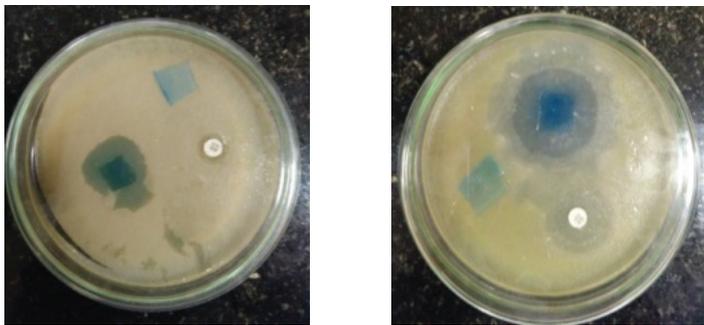


Figure 2: Antimicrobial activity of purified protein bands against *Vibrio sp* and *Salmonella sp*.



Figure 4: Sugar fermentation test of *Bacillus subtilis* (KY808492).

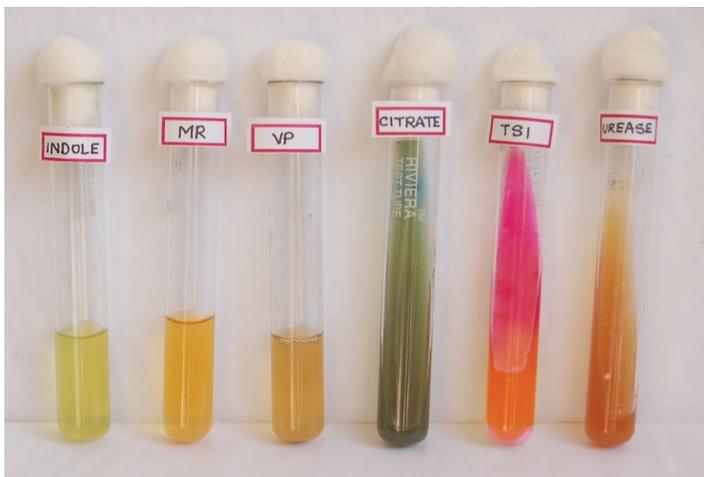


Figure 3: Biochemical tests of *Bacillus subtilis* (KY808492).

load and the counts of *Salmonella sp* and *Vibrio sp* loads were enumerated. Observations showed that after 6 days of treatment, the Total Heterotrophic Bacteria (THB) load were found reduced in the samples preserved with bacteriocin at both the temperatures than the tissues preserved without treatment. Similarly, the loads of *Salmonella sp* and *Vibrio sp* were also reduced in bacteriocin-treated tissues. After 15 days of treatment, a drastic reduction in cell count was observed. After 20 days of treatment, no colony was observed whereas no colonies were found even after 30 days of storage (Tables 3-6; Graphs 2-9). But in earlier researches it was reported that the strains of Lactic acid bacteria have not

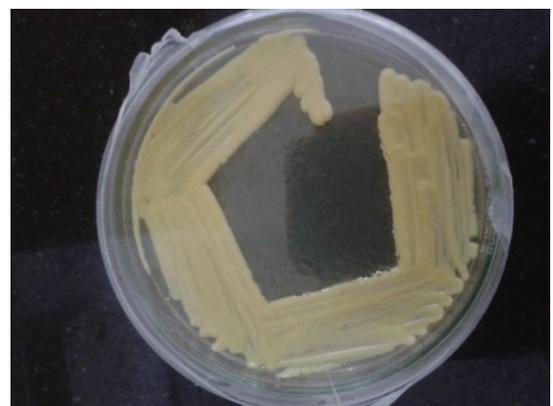


Figure 5: Pure culture of *Bacillus subtilis* (KY808492).



Figure 6: Microscopic view of *Bacillus subtilis* (KY808492).

been inhibited the *Salmonella sp* in meat products (Lozano et.al, 2002). Overall results confirmed that bacteriocin purified from *Bacillus sp* had efficiently reduced the total bacterial count which suggested their ability to use as biopreservative agent. Also, it should be noted that bacteriocin gets easily digested by the proteolytic enzymes present in the human gastrointestinal tract,

thereby confirming that it does not cause health hazards to human body. Other bacterial species bacteriocins also have the potential to inhibit seafood pathogens and hence it can also be used as a natural biopreservative (Lasango et al., 2002; Ray et al., 2001; Wilson et al., 2016).

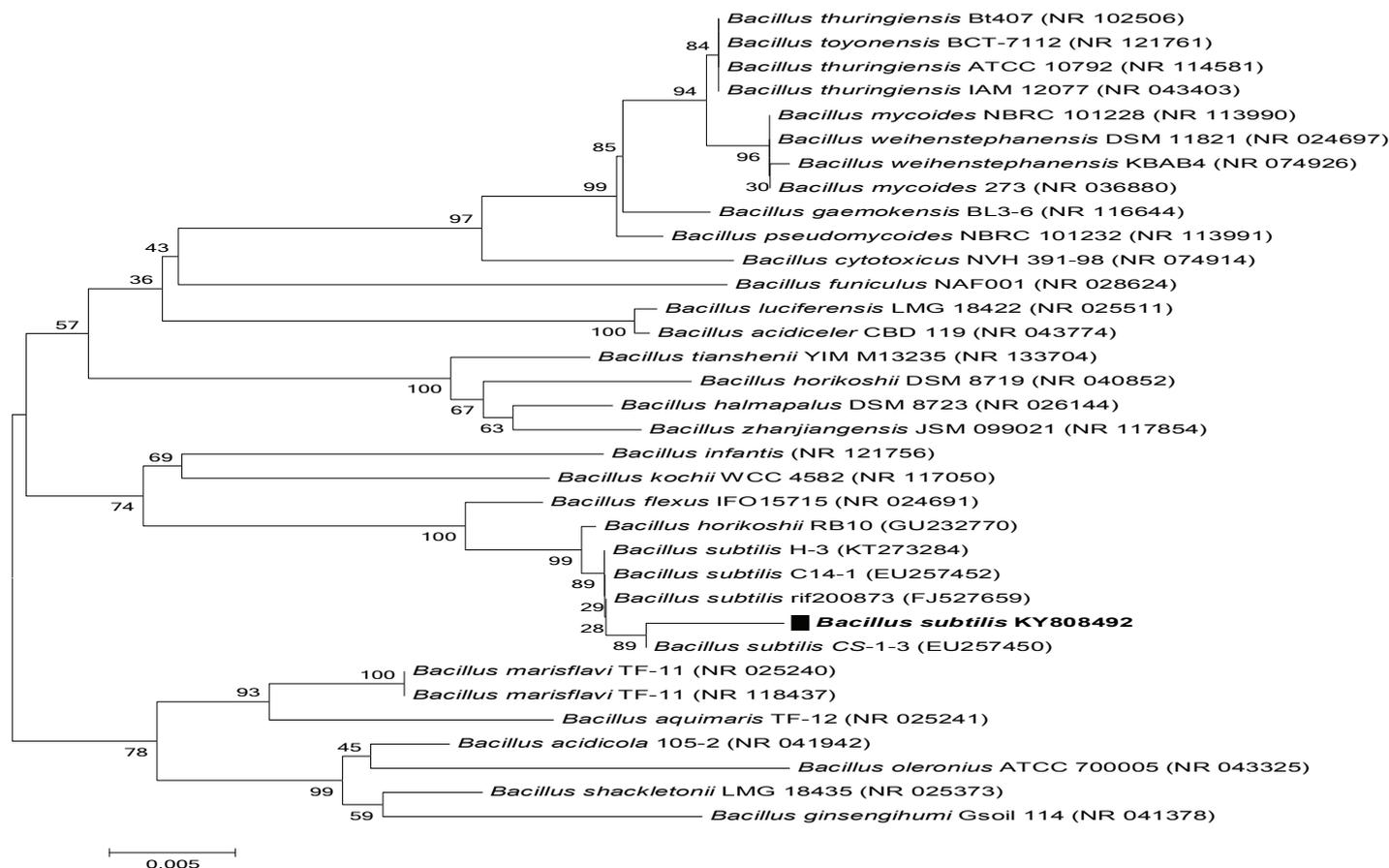


Figure 7: Phylogenetic tree of *Bacillus subtilis* (KY808492).

Table 2: Zone of inhibition of purified bacteriocin against seafood pathogens.

Seafood pathogens	Zone of inhibition of purified bacteriocin (mm)
<i>Salmonella sp</i>	12
<i>Vibrio sp</i>	18

Table 3: Microbial load in Pomfret (*Parastromateus niger*) preserved directly at -4°C and -20°C.

Seafood pathogens	5 th day	Microbial load at -4°C (in Days)					Microbial load at -20°C (in Days)				
		10	15	20	25	30	10	15	20	25	30
THB (CFU/g)	259	172	157	141	90	-	120	80	68	53	26
<i>Vibrio sp.</i> MPN/100 ml	89	63	41	29	18	-	80	40	31	22	15
<i>Salmonella sp.</i> MPN/100 ml	73	52	37	23	10	-	50	34	25	17	10

Table 4: Microbial load in Pomfret (*Parastromateus niger*) preserved with bacteriocin at -4°C and -20°C.

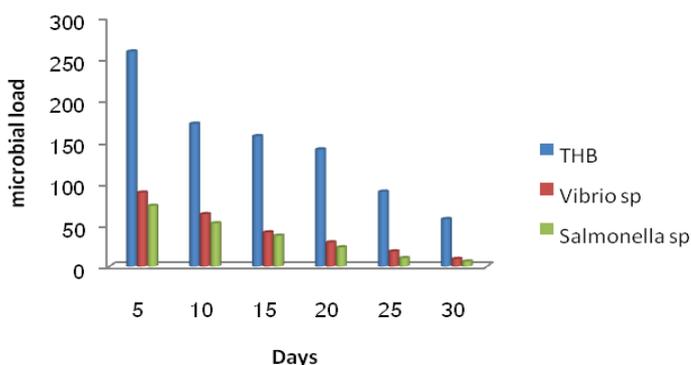
Seafood pathogens	5 th day	Microbial load at -4°C (in Days)					Microbial load at -20°C (in Days)				
		10	15	20	25	30	10	15	20	25	30
THB (CFU/g)	110	75	64	50	-	-	100	70	52	-	-
<i>Vibrio sp</i> MPN/100 ml	62	43	25	4	-	-	60	34	20	-	-
<i>Salmonella sp.</i> MPN/100 ml	57	44	26	5	-	-	30	27	16	-	-

Table 5: Microbial load in Squid (*Loligo duvauceli*) preserved directly at -4°C and -20°C.

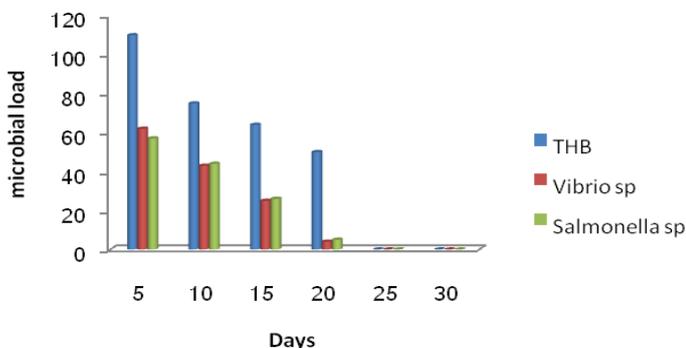
Seafood pathogens	5 th day	Microbial load at -4°C (in Days)					Microbial load at -20°C (in Days)				
		10	15	20	25	30	10	15	20	25	30
THB (CFU/g)	265	183	152	131	50	32	130	90	73	54	32
<i>Vibrio sp.</i> MPN/100 ml	101	83	52	32	21	14	70	60	48	31	20
<i>Salmonella sp.</i> MPN/100 ml	77	58	39	24	19	12	34	30	21	15	6

Table 6: Microbial load in Squid (*Loligo duvauceli*) preserved with bacteriocin at -4°C and -20°C.

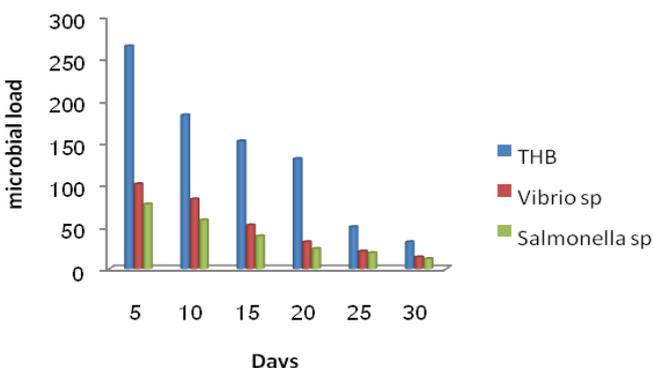
Seafood pathogens	5 th day	Microbial load at -4°C (in Days)					Microbial load at -20°C (in Days)				
		10	15	20	25	30	10	15	20	25	30
THB(CFU/g)	80	65	47	23	-	-	70	61	52	-	-
<i>Vibrio sp.</i> MPN/100 ml	69	47	24	13	-	-	63	50	35	-	-
<i>Salmonella sp.</i> MPN/100 ml	53	39	17	8	-	-	30	26	13	-	-



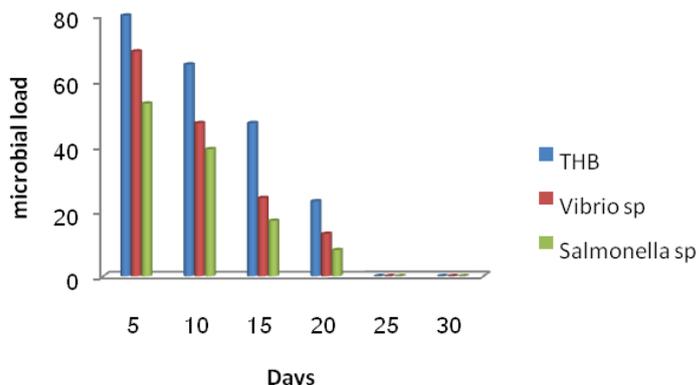
Graph 2: Microbial load in Pomfret (*Parastromateus niger*) preserved directly at -4°C.



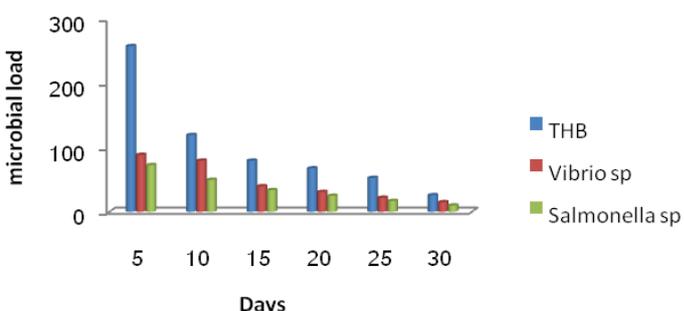
Graph 3: Microbial load in Pomfret (*Parastromateus niger*) preserved with bacteriocin at -4°C.



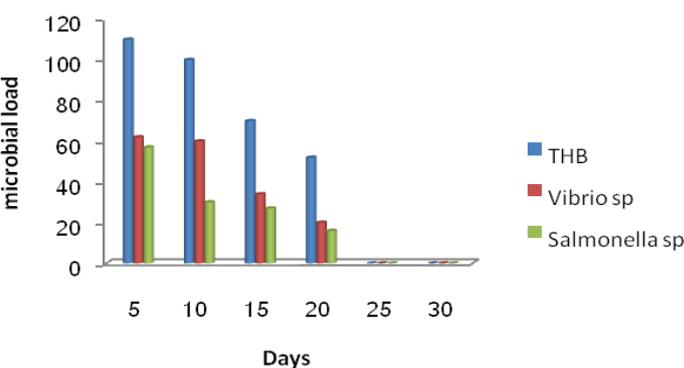
Graph 4: Microbial load in Pomfret (*Parastromateus niger*) preserved directly at -20°C.



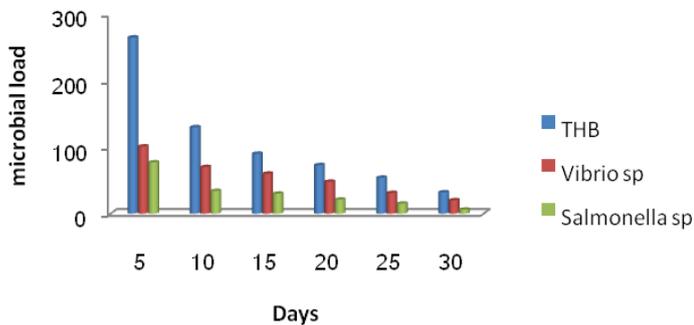
Graph 5: Microbial load in Pomfret (*Parastromateus niger*) preserved with bacteriocin at -20°C.



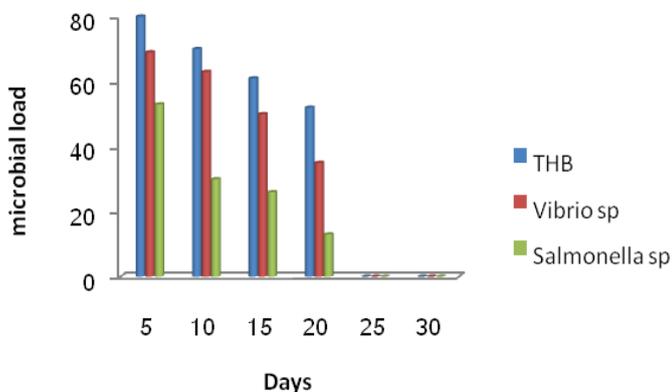
Graph 6: Microbial load in Squid (*Loligo duvauceli*) preserved directly at -4°C.



Graph 7: Microbial load in Squid (*Loligo duvauceli*) preserved with bacteriocin at -4°C.



Graph 8: Microbial load in Squid (*Loligo duvauceli*) preserved directly at -20°C.



Graph 9: Microbial load in Squid (*Loligo duvauceli*) preserved with bacteriocin at -20°C.

The results of the study, suggested that the isolated bacterial strain, *Bacillus subtilis* (KY808492) will play a dual role such as anti-microbial and probiotic which bring health benefits to the host and this study also proved the efficiency of bactericidal and bio-preservative activity of bacteriocin which could provide increased protection, a longer product shelf-life and acts as a better alternative for preserving the high-value sea foods.

Acknowledgment

We would like to thank the facility provided by the Department of Biotechnology, FSH, SRM University to carry out this research and the support extended by Dr. Karthik Ramachandran (Technical Product Manager [Aquaculture and Fisheries] at Guybro chemical Pvt. Ltd.), Mr. Faizal. N. M (Associate Research scholar, SRM University) and the technical support provided by Sankara Nethralaya Research centre, Nungambakkam, Chennai.

References

WHO (2002) Food safety strategic planning meeting, WHO headquarters, Geneva, Switzerland. pp: 20-22.

Jeyasekaran, G., Ganesan, P., Anandaraj, R., Jeya Shakilam R., Sukumar, D. (2006) Quantitative and qualitative study on the bacteriological quality of Indian white shrimp (*penaeus*

indicus) stored in dry ice. International J Food Microbiol **23**, 526-533.

- Rassoli, I. (2007) Food preservation: A biopreservative approach. Food Global Science Books. pp: 111-136.
- Gillor, O., Etzion, A., Riley, M.A. (2008) The dual role of bacteriocins as anti- and probiotics. Appl Microbiol Biotechnol **81**, 591-606.
- Jeevaratnam, K., Jamuna, M., Bawa, A.S. (2004) Biological preservation of foods-bacteriocins of lactic acid bacteria. Indian J Biotechnol **4**, 446-454.
- Roy, P.H. (1997) Dissemination antibiotic resistance. Medical Sci **13**, 927-933.
- Aymerich, T.M.G., Artigas, J.M., Hugas, M. (2000) Effect of sausage ingredients and additives on the production of enterocins A and B by *Enterococcus faecium* CTC492. Optimization of In-vitro production and anti-listerial effect in dry fermented sausages. J Microbiol **88**, 686-694.
- Lee, R.J., Rangdale, R.E. (2008) Centre for Environment, Fisheries and Aquaculture Science, UK, L Croci, Istituto Superiore di Sanità, Italy, D Hervio-Heath and S Lozach, Ifremer, France. Bacterial pathogens in seafood. Archimer.
- Herald Wilson, A., Anjana, B., Jerlin Jovita, S., Karthik, R., Muthezhilan, R., et al. (2016) Biopreservation of value added marine fishes under different storage conditions using bacteriocin from *LactoBacillus* sp (AMETLAB27). The IIOAB J **7**, 1-14.
- Quanli Liu, Guang Gao, Haijin Xu, Mingqiang Qiao (2012) Identification of bacteriocin subtilosin A and loss of *purl* results in its high-level production in *Bacillus amyloliquefaciens*. Res Microbiol **163**, 470-478.
- Karthik, R., Gobalakrishnan, S.C.B., Hussain, A.J., Muthezhilan, R. (2013) Efficacy of bacteriocin from *LactoBacillus* sp. (AMET 1506) as a biopreservative for Seafood's under different storage temperature conditions. J Modern Biotechnol **2**, 59-65.
- Galvez, A., Abriouel, H., Lopez, R L., Omar, N.B. (2007) Bacteriocin-based strategies for food biopreservation. Int J Food Microbiol **120**, 51-70.
- Stiles, M.E. (1996) Biopreservation by lactic acid bacteria. Springer publication **70**.
- Lasango, M., Beoletto, V., Sesma, F., Raya, R., Font De Valdez, G., et al. (2002) Selection of bacteriocin producer strains of lactic acid bacteria from dairy environment. Microbiologia **25**, 37-44.
- Ray, W., Santivarigkna, C., Jumriangrit, P., Sonomoto K., Panyim, S. (2001) Isolation of nisin- producing *Lactococcus lactis* WN C20 strain from a ham, a traditional Thai fermented sausage. Int J Food Microbiol **81**, 137-145.