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# Isolation of a Bacteriocin-Producing *Lactococcus lactis* and Application of Its Bacteriocin to Manage Spoilage Bacteria in High-Value Marine Fish Under Different Storage Temperatures

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**Abstract** The bacteriocins of lactic acid bacteria have considerable potential for biopreservation. The *Lactococcus lactis* strain PSY2 (GenBank account no. JF703669) isolated from the surface of marine perch *Perca flavescens* produced antibacterial activity against pathogenic and spoilage-causing Gram-positive and Gram-negative bacteria viz. *Arthrobacter* sp., *Acinetobacter* sp., *Bacillus subtilis*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and possessed broad inhibitory spectrum. The biopreservative efficacy of the bacteriocin PSY2 was evaluated using fillets of reef cod, *Epinephelus diacanthus*. The fillets (10 g) were sprayed with 2.0 ml of 1,600 AU/ml bacteriocin, wrapped and kept under different storage temperatures viz., 4, 0 and −18 °C. The biopreservative extended the shelf-life of fillets stored at 4 °C to >21 days as against <14 days observed in the untreated samples. The total count of spoilage bacteria was reduced by 2.5 logarithmic units in the treated sample during the 14th day of storage as against the control. Chemical analysis revealed a significant change ($P<0.05$) in the pH value, free fatty acid (as % oleic acid), total volatile base nitrogen and total methyl amine content in the treated samples. The overall acceptability in terms of sensory attributes was significantly higher in the bacteriocin-treated samples stored for 21 days at 4 °C while the untreated samples became unacceptable by the 14th day. The biopreservative gave no significant effect at −18 °C. Thus, the bacteriocin derived from *L. lactis* PSY2 gave increased protection against spoilage bacteria and offers an alternative for the preservation of high-value sea foods.

**Keywords** *Lactococcus lactis* PSY2 · Bacteriocin PSY2 · Biopreservative · Storage temperature

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## Introduction

The use of chemical preservatives during storage of food and their potential side effects have created consumer concerns in recent years [1]. This concern had necessitated a search for an alternative strategy for food preservation with the sole objective of extending the shelf-life of the food with least undesirable effects. Biopreservation, the strategy to extent the shelf-life of food using microorganisms and/or their metabolites, has received wide research interest and acceptance [2]. Lactic acid bacteria (LAB), enjoying the status as a generally recognized as safe organism with potential to produce the antimicrobial protein "bacteriocin," could be considered as the apt candidate for biopreservation. Though a number of bacteriocin-producing strains have been isolated from various food sources such as vegetables [3, 4], meat [5], fish [6], beverages like boza [7, 8], etc. and their bacteriocins characterized, only nisin [9] is commercialized as a biopreservative.

In the present study, a *Lactococcus lactis* PSY2 was isolated from the body surface of marine perch, *Perca flavescens.* This strain produces a broad spectrum antibacterial agent. The biopreservative efficacy of this agent was tested with fillets of the cod fish *Epinephelus diacanthus*. Since the fish is prone to spoilage with increase in storage time, the study was focused on determining the quality in 7-day intervals during storage at 4, 0 and −18 °C for 28 days.

## Materials and Methods

### Bacterial Strains

The indicator bacterial strains used in study were obtained from our laboratory stock and maintained as frozen stocks at −20 °C in the presence of 25 % glycerol. Working cultures were propagated in appropriate broth media (Table 1). Bacteria tested for bacteriocin production were isolated from the body surface of marine yellow perch fish *P. flavescens*. The samples were serially diluted in normal saline (0.85 % NaCl), and 0.1 ml aliquots were spread plated on de Mann–Rogosa–Sharpe (MRS) agar (HiMedia) and the plates were incubated for 24 h at 30 °C. After incubation, colonies were randomly selected, grown in MRS broth, Gram-stained and tested for catalase production. Gram positive and catalase negative were tested for antimicrobial activity against different indicator strains (Table 1).

**Table 1** Indicator bacteria and culture medium used for their growth

| Bacterial strains | Medium[a] |
|---|---|
| *Arthrobacter* sp. | TS |
| *Acinetobacter* sp. | TS |
| *Bacillus pumilus* | TS |
| *Bacillus subtilis* | TS |
| *Escherichia coli* | TS |
| *Lactobacillus acidophilus* TS1 | MRS |
| *Listeria monocytogenes* | UVM-L |
| *Pseudomonas aeruginosa* | TS |
| *Staphylococcus aureus* | TS |

*TS* trypticase soy (HiMedia), *MRS* de Mann–Rogosa–Sharpe (HiMedia), *UVM-L* UVM-*Listeria* (Merck)[a]Media were broth in the case of propagation and agar slants for storage

## Bacteriocin Activity Detection

The bacteriocin-producing strains were screened against the indicators using well-diffusion method as described by Barefoot and Klaenhammer [10]. The diameters of the inhibition zones were measured after 24 h of incubation. One strain was chosen for the further investigation and was identified based on biochemical tests and phylogenetic characterization [11]. The bacteriocin activity of the selected strain was determined by serial two-fold dilution of the sample, and the reciprocal of the highest inhibitory dilution was used to calculate the arbitrary units (AU) per millilitre. The uninoculated media were tested for inhibitory zones as a control.

## Elimination of the Influence of Organic Acids and Hydrogen Peroxide as Inhibitory Agents

The pH of the test supernatant was adjusted to 7.0 with 10 M NaOH to rule out the effect of organic acids. The neutralized supernatant was filter sterilized with a 0.22-μm Millipore filter membrane and tested by the well-diffusion assay for persistence of the inhibition zone. To exclude the influence of hydrogen peroxide on inhibitory activity, catalase (HiMedia) was incorporated in the overlay agar as described by Barefoot and Klaenhammer [10]. To confirm the proteinaceous nature, the metabolite was treated with 1 mg/ml trypsin and incubated for 2 h and assayed.

## Determination of Biopreservative Efficacy in Fish Fillets

### *Fish Samples*

Reef cod, *E. diacanthus*, weighing 11.8 kg procured from fish landing centre located in Vizhinjam, Thiruvananthapuram, India was immediately brought to the laboratory in insulated containers and washed in potable water. The fish as a whole was dipped in chlorine water, washed with sterile water, beheaded, eviscerated, washed in sterile water and the skin was removed. Pieces were cut from the whole fish and further cut into pieces of 10 g with a surface area of 5.0×2.5×0.5 cm and surface-sterilized by exposure to UV radiation (Arklite, India) for 15 min.

## Treatment of Fish Fillets Using the Bacteriocin

The bacteriocin PSY2 produced by *L. lactis* PSY2 isolated from marine perch fish in the current study was assessed for its in situ biopreservative effect in the high-value cod fish fillets. The fish fillets (10 g) were sprayed with 2.0 ml of 1,600-AU/ml bacteriocin PSY2 dissolved in sterile distilled water, while the fillets sprayed with autoclaved distilled water (2.0 ml) served as the control. The treated samples were wrapped in aluminium foil, kept in separate boxes and stored at 4, 0 and −18 °C for 28 days and for each treatment analysed in triplicate at 0 (initial day) after 7, 14, 21 and 28 days of storage.

## Microbiological Analyses

### *Plate Counting Method*

Microbiological analyses were performed on three independent samples initially after treatment and in 7-day intervals. One gram of each treated sample was aseptically mixed

with 9 ml sterile saline (0.85 % NaCl), crushed to homogeneity and serially diluted and plated in nutrient agar (HiMedia) to determine the total viable count of bacteria in the sample. The determination of the load of psychrophilic spoilage bacteria was performed on different selective media viz., MRS (HiMedia) to determine LAB population, mannitol salt agar (Merck) for *Staphylococcus* sp., thiosulphate citrate bile salts sucrose (HiMedia) for *Vibrio* sp., MacA (Merck) for *Enterobacteriaceae* and *Pseudomonas* agar base (HiMedia) for counting pseudomonads. Bacterial counts were expressed as log10 CFU/g of fish fillet.

### Sensory Analyses

The sensory evaluation was performed by five trained panelists. The assessment was conducted for odour and appearance of fish samples using a nine-point hedonic scale [12]: 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much and 9, like extremely.

### Physical–Chemical Analyses

The pH measurement was carried out using a Cyberscan model 500 pH meter (Euteon Instruments). The samples (1 g) were homogenized using mortar and pestle with 9 ml of distilled water, and the homogenate was subjected to pH determination. Measurement of free fatty acids: Free fatty acid (FFA) content was estimated by following the titration method of Ke et al. [13]. Total volatile base nitrogen (TVB-N) and total methyl amine (TMA) were determined by the Conway's method as described by Conway [14].

All the chemical analyses were conducted on samples derived from a pool of three fish for each storage time and carried out in triplicate. Reagents were of analytical grade.

### Statistical Analysis

The data were expressed as mean±standard deviation and analysed using analysis of variance using Excel 2007.

## Results and Discussion

### Isolation of Bacteriocin-Producing LAB and Its Spectrum of Activity

Five strains of LAB isolated from the body surface of marine perch fish exhibited inhibitory activity against at least a few of the indicator strains tested (Table 2). One strain with broad spectrum of activity against the tested indicator strains was characterized based on 16S rRNA sequencing, as *L. lactis* PSY2 (GenBank account no. JF703669). The antibacterial agent, bacteriocin PSY2, produced by *L. lactis* PSY2 was selected for determining the efficacy as a biopreservative in high-value cod fish.

### Nature of the Inhibitory Substance Produced by *L. lactis* PSY2

The substance produced by *L. lactis* PSY2 was neither hydrogen peroxide nor organic acid. The inhibitory activity was not affected by catalase and was retained in neutralized supernatant fluid. The inhibitory substance had bactericidal or bacteriostatic mode of action as evidenced by the clear zone of inhibition against the indicator strains (Table 2). The

**Table 2** Inhibitory spectrum (zone of inhibition in millimetres) of the five LAB strains isolated from body surface of marine perch fish

| Isolates | Zone of inhibition against Indicator bacteria | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Arthrobacter* sp. | *Acinetobacter* sp. | *Bacillus pumilus* | *Bacillus subtilis* | *Escherichia coli* | *Lactobacillus acidophilus* TS1 | *Listeria monocytogenes* | *Pseudomonas aeruginosa* | *Staphylococcus aureus* |
| PSY1 | 12 | – | 10 | 13 | – | 8 | 9 | – | 13 |
| PSY2 | 10 | 14 | 12 | 15 | 12 | 21 | 14 | 13 | 16 |
| PSY3 | 10 | 12 | – | 13 | 12 | 13 | 8 | – | 8 |
| PSR1 | – | – | 8 | 10 | – | – | – | 13 | 12 |
| PSR2 | – | 8 | 12 | 11 | 9 | – | 10 | 8 | – |

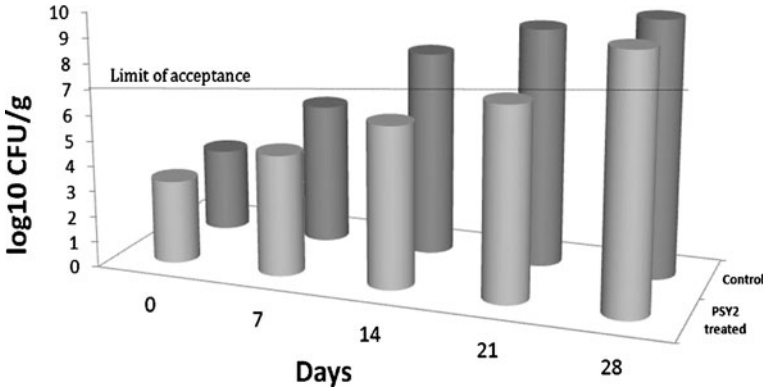


**Fig. 1** Total viable count in reef cod fillets treated with bacteriocin PSY2 stored at 4 °C for 28 days

inhibitory substance showed broad spectrum of activity, and the proteinaceous nature was confirmed by its loss in activity when treated with trypsin.

## Biopreservative Efficacy of Bacteriocin PSY2 in Cod Fish Fillets

### Microbiological Analyses

#### *Effect of Bacteriocin PSY2 in Reducing the Total Bacterial Load*

The results of plate counts highlighted a relevant increase of microbial groups detected during storage at 4 °C (Fig. 1) both in bacteriocin PSY2 treated and untreated sets compared to the experimental sets stored at 0 and −18 °C. The comparison of the data (Fig. 2) on the total viable count revealed that PSY2-treated fish samples remained within the maximum limit of acceptability which is $10^7$ counts/g as recommended by the International Commission of Microbiological Standards for Foods [15], till the 21st day as against the untreated sets which became unacceptable before the 14th day of storage. At day 14 at 4 °C, the total viable count was noted as 6.3±0.03 and 8.0±0.10 log10 CFU/g for the PSY2 treated and untreated fish fillets, respectively. Though the bacteriocin treatment reduced the total load throughout the period of storage for samples stored at 0 and −18 °C compared to the control, both remained within the acceptability limits till 28 days of storage.

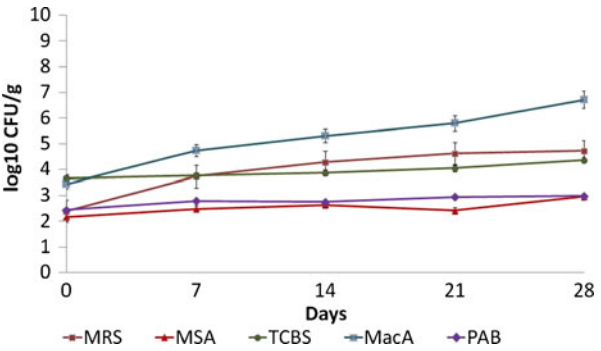


**Fig. 2** Microbiological analysis of reef cod fillets treated with bacteriocin PSY2 stored at 4 °C for 28 days

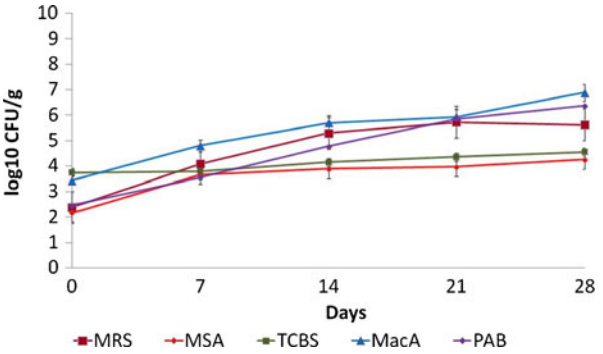


**Fig. 3** Microbiological analysis of untreated reef cod fillets stored at 4 °C for 28 days

*Effect of Bacteriocin PSY2 in Managing Spoilage Bacteria*

The results of microbial counts, obtained from samples at 0, 7, 14, 21 and 28 days of storage (4 °C), are reported in Figs. 2 and 3. All the microbial groups increased during storage. For example, the *Enterobacteriaceae* reached its highest count of >6.0 log10 CFU/g at the 28th day of storage in both samples. Counts of lactic acid bacteria were slightly lower (4.72±0.23 log10 CFU/g) in the PSY2-treated fish samples when compared to the untreated sets (5.86± 0.18 log10 CFU/g) when evaluated at day 28. The maximum inhibitory effect of bacteriocin PSY2 was observed against *Staphylococcus* sp. and *Pseudomonadaceae* which were reduced by 1.8 and 3.37 logarithmic units in the PSY2 treated sets compared to the control. The influence of storage temperature was further evident from the observations made with samples stored at 0 and −18 °C, in which the load of spoilage bacteria remained stable and controlled throughout the period of storage. According to Oramadike et al. [16], the storage of fish at the higher temperature of 4 °C facilitates the proliferation of psychrophilic bacteria. The role of bacteriocin PSY2 in managing the spoilage bacteria hence could be clearly evidenced from the observations noted with the fish samples stored at 4 °C.

Sensory Analyses

The average scores given by the panelists to the sensory attributes in terms of odour and appearance examined on each sampling day is given in Table 3. The fresh odour which was prominent on day 0 became increasingly weaker with the advancement in storage period. The sensory scores declined with the storage as was revealed from the decrease in the score

**Table 3** Changes in sensory attributes (hedonic scores in terms of odour and appearance) of the fish fillets treated with bacteriocin PSY2 stored at 4, 0 and −18 °C for 28 days

| Days | 4 °C | | 0 °C | | −18 °C | |
|---|---|---|---|---|---|---|
| | PSY2 treated | Control | PSY2 treated | Control | PSY2 treated | Control |
| 7 | 7.3±0.47 | 6.0±0.00 | 9.0±0.00 | 8.6±0.47 | 9.0±0.00 | 9.0±0.00 |
| 14 | 5.6±0.58 | 4.3±0.47 | 8.6±0.47 | 8.3±0.58 | 9.0±0.00 | 8.6±0.58 |
| 21 | 4.3±0.58 | 3.0±0.00 | 8.0±0.00 | 7.0±0.00 | 8.6±0.58 | 8.6±0.58 |
| 28 | 3.3±0.47 | 2.0±0.47 | 7.3±0.47 | 6.0±0.00 | 8.6±0.58 | 8.3±0.58 |

**Table 4** Changes in pH in fish fillets treated with bacteriocin PSY2 stored at 4, 0 and −18 °C for 28 days

| Days | 4 °C | | 0 °C | | −18 °C | |
|---|---|---|---|---|---|---|
| | PSY2 treated | Control | PSY2 treated | Control | PSY2 treated | Control |
| 0 | 6.81±0.01 | 6.82±0.01 | 6.81±0.01 | 6.79±0.01 | 6.79±0.01 | 6.78±0.01 |
| 7 | 6.84±0.01 | 6.87±0.01 | 6.76±0.01 | 6.72±0.01 | 6.75±0.01 | 6.75±0.01 |
| 14 | 6.87±0.01 | 7.01±0.01 | 6.75±0.01 | 6.70±0.01 | 6.75±0.01 | 6.75±0.01 |
| 21 | 6.98±0.01 | 7.25±0.01 | 6.73±0.01 | 6.68±0.01 | 6.73±0.01 | 6.71±0.01 |
| 28 | 7.21±0.01 | 7.91±0.01 | 6.70±0.01 | 6.65±0.01 | 6.71±0.01 | 6.69±0.01 |

from the seventh day of storage at 4 °C. The fish fillets treated with bacteriocin PSY2 had 5.6±0.47 score at the 14th day of storage while the control had 4.3±0.47 score. The limit of acceptability quality limit of 4.0 as per the protocol of Renitta et al. [17] was reached at the 14th day of storage in case of the untreated sample.

## Physical–Chemical Analyses

The pH of the fish fillets used in the study was in the range of 6.8 in the beginning, which increased significantly ($P>0.05$) to 7.21±0.01 for the bacteriocin treated and 7.91±0.01 for the untreated samples respectively after 28 days of storage at 4 °C (Table 4). A significant increase in the pH values with the increase of the storage time has also been recognized in brined chub mackerel [18], marinated anchovies [19] and marinated sardine [20] during their storage at refrigerated temperature. As reported by Shenderyurk and Bykowski [21], during the storage of fish, heterofermentative lactic acid bacteria grow and degrade amino acids with the formation of carbon dioxide and other decarboxylation products which raise the pH. The results of the present study indicated only a slight increase in pH along with the increase in the time of storage in treated sets as against the control. Contrary to this, the pH showed a decreasing trend for the fish samples stored at 0 °C (Table 4).

*Measurement of Free Fatty Acids* The free fatty acid content followed an increasing trend with the increase in storage periods (Table 5). In most fish, rancidity is noticeable when the FFA (calculated as oleic acid) ranged between 0.5 and 1.5 % [22]. In this respect, the FFA observed with the cod fish fillets even at day 28 (0.362±0.01) was inadequate to produce

**Table 5** Changes in free fatty acid (% oleic acid) content in fish fillets treated with bacteriocin PSY2 stored at 4, 0 and −18 °C for 28 days

| Days | 4 °C | | 0 °C | | −18 °C | |
|---|---|---|---|---|---|---|
| | PSY2 treated | Control | PSY2 treated | Control | PSY2 treated | Control |
| 0 | 0.159±0.01 | 0.159±0.01 | 0.159±0.01 | 0.159±0.01 | 0.159±0.01 | 0.159±0.01 |
| 7 | 0.235±0.01 | 0.244±0.01 | 0.186±0.01 | 0.185±0.00 | 0.181±0.00 | 0.170±0.01 |
| 14 | 0.269±0.01 | 0.284±0.02 | 0.197±0.01 | 0.201±0.01 | 0.187±0.00 | 0.185±0.00 |
| 21 | 0.311±0.01 | 0.345±0.01 | 0.206±0.01 | 0.237±0.01 | 0.195±0.01 | 0.197±0.00 |
| 28 | 0.362±0.01 | 0.380±0.02 | 0.242±0.01 | 0.264±0.01 | 0.201±0.01 | 0.208±0.01 |

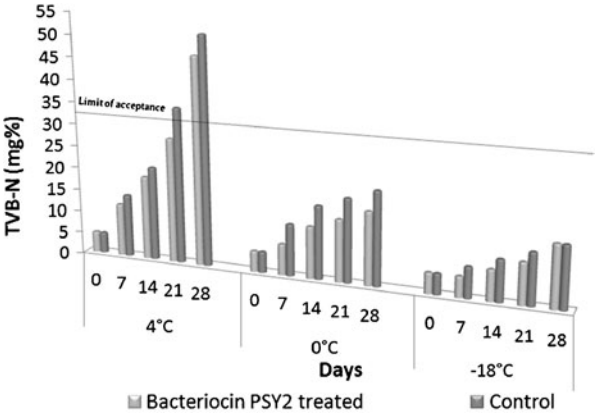


**Fig. 4** TVB-N (milligrams percent) content in the fish flesh treated with bacteriocin PSY2 at different storage temperatures

rancidity in the stored fish fillets. However, in all cases, oxidation of the fat was kept under control in the presence of bacteriocin PSY2.

*TVB-N and TMA Analyses* The results on the changes in the TVB-N and TMA content of the bacteriocin-treated and untreated fish fillets held at 4, 0 and −18 °C are shown in Figs. 4 and 5. The content of TVB-N and TMA were 4.67 and 0 mg/100 g, respectively, in fresh fish samples. The levels of TVB-N and TMA showed an increasing trend with increase in storage period irrespective of the incubation temperature. Connell [23] suggested a TVB-N level of 30–35 mg/100 g and TMA level of 10–15 mg/100 g as the maximum limit of acceptability. Accordingly, it could be estimated from the present study that the untreated fish fillets stored at 4 °C had became unacceptable by the 21st day, while the bacteriocin-treated samples remained within the acceptability limits (Figs. 4 and 5). Similar to the present observation, the biopreservation studies carried out by Brillet et al. [24] using *Carnobacterium divergens* V41 reduced the TVB-N and TMA contents of the cold-smoked Salmon. The increase in the levels of TVB-N and TMA could be attributed to the initial autolytic degradation of nucleotides and free amino acids, followed by a combination of microbiological and autolytic activities and complete microbial reduction of trimethylamine *N*-oxide to TMA [25].

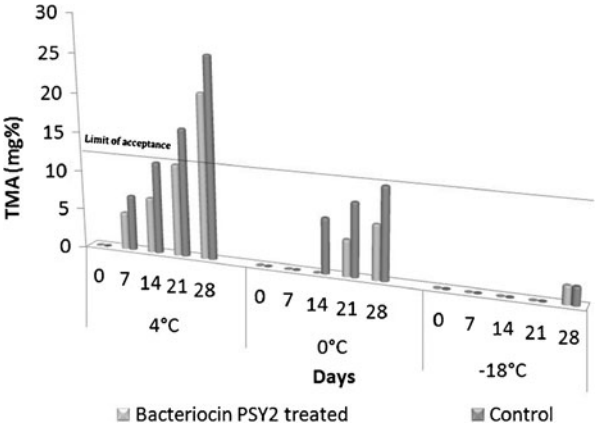


**Fig. 5** TMA (milligrams percent) content in the fish flesh treated with bacteriocin PSY2 at different storage temperatures

## Conclusions

*L. lactis* PSY2 isolated from a marine fish produces bacteriocin with antibacterial activity against a broad spectrum of pathogenic and spoilage-causing bacteria. The in situ application of this antibacterial agent as a biopreservative in high-value cod fish fillets extended its shelf-life to >21 days at the normal refrigeration temperature (4 °C), when compared to that at 0 and −18 °C. It could be concluded that the use of bacteriocin PSY2 as a biopreservative gives increased protection, a longer product shelf-life and offers a better alternative for preserving the high-value sea foods at refrigeration temperatures.

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