Efficacy of Bacteriocin of *Enterococcus faecalis* CD1 as a Biopreservative for High Value Marine Fish Reef Cod (*Epinephelus diacanthus*) under Different Storage Conditions

A. R. Sarika*, A. P. Lipton*, M. S. Aishwarya* and R. S. Dhivya**

*Central Marine Fisheries Research Institute, Vizhinjam Research Centre of CMFRI, Vizhinjam, Kerala, India  
**Department of Zoology, Sree Devi Kumari College for Women, Kuzhithurai, Tamil Nadu, India

ABSTRACT

The increased consumer demands for food with fewer chemical preservatives created a shift toward biopreservation in recent years. The bacteriocin based strategy has gained increased attention from among the different approaches of biopreservation. In the present study, the effect of enterocin CD1, a broad spectrum bacteriocin produced by *Enterococcus faecalis* CD1 isolated from commercial curd was tested in the high value marine cod (*Epinephelus diacanthus*) fish fillets as crude bacteriocin supernatant during storage at 4°C, 0°C and -18°C and compared to that of the chemical preservative sodium benzoate. Samples were analysed for the sensory and microbiological attributes after 7, 14, 21 and 28 days of storage at the above different temperatures. Although the treatment did not completely eliminate the spoilage bacteria on the stored fish, the results showed that the fillets treated at 10% level reduced the total viable count by 2 log units when stored at 4°C for 28 days as against the control which was kept untreated. The treatment also reduced the load of spoilage bacteria at 0°C; however no significant impact could be noticed when stored at -18°C. The sensory attributes also showed variations with respect to the control for the samples stored at 4°C and 0°C. The results of the bacteriocin treatment were promising in the light of the extension of shelf-life of the high value sea foods.

Key words: *Enterococcus faecalis* CD1, enterocin CD1, biopreservation, *Epinephelus diacanthus*.

INTRODUCTION

Biopreservation approaches for food items are gaining importance and interest both for industry and consumers. In this context, the bacteriocinogenic lactic acid bacteria and/or their isolated bacteriocins are regarded as safe additives (GRAS), useful to control the growth of pathogens and spoilage microorganisms in foods and feed. The spreading of bacterial antibiotic resistance
and the demand for products with fewer chemicals again necessitated to explore new alternatives, such as bacteriocins.

The antimicrobial potential of lactic acid bacteria (LAB) is significant in the preservation, microbiological stability and development of starter cultures for fermented foods [1]. The use of bacteriocin also allows biological control of food-borne pathogens without resorting to more severe physical treatments [2]. The antimicrobial effect has been attributed to their capability for acid secretion, competitive nature for extracting nutrients, formation of hydrogen peroxide, CO₂, diacetyl or antimicrobial peptides, such as bacteriocins [3]. As suggested by Calo-Mata et al., the biocontrol of foodborne pathogens by bacteriocin producing lactic acid bacteria or by bacteriocin extract could be an attractive alternate method [4].

Many bacteriocins have been isolated for use as natural food biopreservative [5, 6]. The bacteriocin, nisin produced by Lactococcus lactis sub sp. lactis, has been studied extensively and as a result it is approved for application in the food industry. Nisin is active against Gram positive food-borne pathogenic microorganisms such as Staphylococcus aureus and Listeria monocytogenes, while are generally inactive against Gram negative bacteria due to the resistance conferred by the outer membrane [7].

The bacteriocin enterocin CD1, is produced by Enterococcus faecalis CD1 isolated from commercial curd. Compared to nisin, enterocin CD1 showed a broader antimicrobial spectrum and had higher antimicrobial activity against spoilage bacteria. The present study is conducted to study the effect of the bacteriocin enterocin CD1 on the keeping quality of the high-value marine cod fish (grouper, Epinephelus diacanthus) at different temperatures of storage.

MATERIALS AND METHODS

Bacterial culture
The bacteriocin producer Enterococcus faecalis CD1 was grown in MRS medium [8] and incubated at 35°C. The indicator organisms viz., Staphylococcus aureus, Bacillus subtilis, Bacillus pumilus, Escherichia coli, and Pseudomonas aeruginosa used for the bacteriocin assay were stored in Nutrient Agar (Hi-Media) slants and Listeria sp. in Listeria selective agar. These strains were propagated as and when required. The 24 h culture of the indicator strain was used for bacteriocin assay.

Preparation of bacteriocin extracts
Two millilitres of the log phase culture of E. faecalis CD1 was transferred to 500 ml of MRS broth. Once the pH has reached about 4.95 after 96 h of incubation at 35°C, the resulting fermentation broth was centrifuged (10,000 rpm for 30 min) and the cell-free culture broth supernatant was filter sterilized (0.45 µm pore size, Hi-Media) under vacuum and used for checking the activity.

Activity spectrum of enterocin CD1 against spoilage causing bacteria
The antibacterial activity of the enterocin CD1 was determined against spoilage causing bacteria Gram positive and Gram negative bacteria viz., S. aureus, B. subtilis, B. pumilus, Listeria sp., E. coli, and P. aeruginosa. For detecting the bacteriocin activity, ten µl of the cell free filtrate was placed on an agar plate containing an overlay of indicator strains. The presence or absence of inhibitory activity against the indicator organism was confirmed after incubating the agar plate for 24 h. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the
highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU/ml) [9].

**Fish sample preparation and treatment**

Fish sample Reef cod (*Epinephelus diacanthus*), procured from fish landing center located in Vizhinjam, Thiruvananthapuram, India were 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 and eviscerated. The fish was allowed to bleed for 15 min and washed again in sterile water and the skin removed.

The fish chunks were cut from the whole Grouper of 11.8 kg. The fish flesh was cut into pieces of 10 g with surface area of 5.0 x 2.5 x 0.5cm. The fish pieces were surface sterilized by exposure to UV for 15 min. Sterility was monitored by individual sampling of pieces of the fish using the enumeration procedures described below.

**Treatment of the fish flesh using bacteriocins as biopreservatives**

To study the effect of bacteriocin in controlling the spoilage causing bacteria, the fish flesh (10 g) were glazed with 0.1, 1.0 and 10.0% v/v of 1000 AU/ml enterocin CD1. The autoclaved distilled water served as the control. The effectiveness of the bacteriocin was compared with that of the conventional chemical preservative, sodium benzoate (0.01g/10g). The treated fish samples were wrapped with aluminium foil and kept in separate boxes and stored at 4°C, 0°C and -18°C for 28 days. Samples were taken every 7 day interval and the attributes of sensory and microbial load were evaluated.

**Sensory evaluation**

The sensory evaluation was performed by 5 trained panelists. The assessment was conducted for the odour and appearance of fish flesh samples using a 9-points hedonic scale [10] viz., 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; 9, like extremely.

**Microbiological analyses**

To sample the fish flesh, 1.0 g sample was cut using sterile knives and homogenized using sterile mortar and pestle. Serial decimal dilutions were made and then plated in triplicate on nutrient agar and incubated at 37°C for 24 h. The initial count and the count at the 7, 14, 21 and 28th day of the storage of the treated fish samples were determined and expressed as CFU/g.

**Statistical analysis**

The obtained results were expressed as mean±SE.

**RESULTS AND DISCUSSION**

**Activity spectrum of Enterocin CD1**

The inhibitory activity pattern of enterocin CD1 against spoilage causing bacteria is given in Table 1. The pathogenic Gram positive bacteria *B. subtilis, B. pumilus* and *Listeria* sp. and the Gram negative *P. aeruginosa* and *E. coli* were inhibited by the bacteriocin produced by *E. faecalis* CD1. *Staphylococcus aureus* was highly inhibited with an activity of 1000 AU/ml. Enterocin CD1 showed inhibitory activity against the tested Gram positive and Gram negative pathogenic bacteria and hence could be considered to possess a broad inhibitory spectrum. The activity of the bacteriocin against Gram negative bacteria is unusual phenomenon and defies the observations of Klaenhammer [3] that bacteriocins are proteins which show inhibitory activity.
against closely related organisms. The present finding was supported by the observations made earlier by Mandal et al. [11].

Table 1. Antimicrobial spectrum of the bacteriocin produced by *E. faecalis* CD1

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Well-diffusion assay (AU/ml) *</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus pumilus</em></td>
<td>600</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>800</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1000</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>400</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>600</td>
</tr>
<tr>
<td><em>Listeria sp.</em></td>
<td>800</td>
</tr>
</tbody>
</table>

*AU/ml - Bacteriocin activity (AU – Activity Unit)

Microbiological analyses of the stored fish sample

The load of bacteria in fish flesh showed variations with the increase in storage period when stored at 4°C (Fig. 1). The initial total viable count (TVC) for the control set of sample was 3.24±0.11 log_{10} CFU/g, which increased with the increase in storage time and reached 11.72±0.02 log_{10} CFU/g after 28 days of storage. This could be corroborated with the findings made in the case of treatment with different concentrations of enterocin CD1. The bacterial load was noted to be 10.55±0.15, 10.01±0.05 and 9.82±0.11 log_{10} CFU/g respectively with 0.1, 1.0 and 10 % v/v of sample containing enterocin CD1. The observations made with the fish samples stored at 0°C were also consistent with the above in that the bacterial load tend to decrease with the addition of enterocin CD1 as the biopreservative in which 10% produced the maximum inhibitory effect (Fig. 2). Results of plate counts highlighted a relevant increase of microbial groups detected during storage at 4°C. The total viable count increased above 8 log_{10} CFU/g in all samples, were in agreement with previous findings [12]. This increase in bacterial load could be attributed to the congenial conditions prevailing for their growth at a higher temperature of storage as suggested by [13]. To the observations of Randazzo et al. [14], the survival of natural contaminant organisms in the fish is the result of high final load detected throughout the period of storage. It has been observed (Jemenson effect) the higher the number of most abundant organisms (spoilage organisms), the lower is the pathogen concentration [15]. However, as per the results of the present study, this observation loses significance since both the control and enterocin CD1 treated fish samples had become unacceptabel at the 28th day of storage due to the bacterial load beyond the limit [16] of acceptability. It could be estimated that the enterocin CD1 could extent the shelf-life of the fish flesh by controlling the proliferation of spoilage causing bacteria. Since the fish are prone to contamination [4], increase in storage temperature to 4°C could lead to a drastic increase in the bacterial load which could lead to deterioration of the fish leading to unacceptability. It could also be noted from the above observations that the reduction in the load of the bacteria by the bacteriocin was analogous with the inhibition by the chemical preservative sodium benzoate as is evidenced from Fig. 1 & 2. This finding could be considered noteworthy as there is a likelihood that enterocin CD1 being a biopreservative and further a compound with GRAS status, could replace or at least rationalize the use of the harmful chemical preservatives in the near future.

The findings made in the case of fish samples stored at 4 and 0°C was not repeated in the case of those stored at -18°C. As is evident from Fig. 3, no significant difference could be noticed with respect to the control and enterocin CD1 treated samples. The decreased bacterial proliferation at this temperature could be justified by the fact that such a lower temperature would be detrimental to their survival even in the food system [17].
Fig. 1. Changes in the total bacterial count (CFU/g) of the fish flesh treated with different concentration of Enterocin CD1 stored at 4°C

Fig. 2. Changes in the total bacterial count (CFU/g) of the fish flesh treated with different concentration of Enterocin CD1 stored at 0°C

Fig. 3. Changes in the total bacterial count (CFU/g) of the fish flesh treated with different concentration of Enterocin CD1 stored at -18°C
Sensory analyses
The observations made for odour and appearance were taken into account for determining the sensory attributes of the stored fish sample. Sensory analysis indicated that the fish samples at the initial day had the best sensory attributes and scored 9.0. The sensory scores declined with the storage (Fig. 4) as is revealed from the decrease in the score from the 7th day of storage at 4°C. Although, as the storage time increases the level of acceptability of the sensory attributes decreases in all the treated and untreated fish samples. There was significant difference in sensory attributes treated with enterocin CD1 and control at 21 and 28 days of storage. The fish sample treated with CD1 (10%) had 5.6±0.4 score at day 14 of storage at 4°C while the control had 4.3±0.4 score at the same time of storage. The overall scores were beyond the acceptable limit (4.0) at the end of the storage period (28 days) and were detected as 3.3±0.4 and 2.0±0.4 for enterocin CD1 treated and control respectively at 4°C. Freshness is the most important attribute when evaluating the quality of fish. The loss of freshness changes the sensory parameters, which have a direct effect on the product acceptance from the part of the consumer [17]. A deterioration in the muscle appearance occurred even at the 7th day of storage at 4°C which became unacceptable by the 21st day in the case of control. The sensory attributes with the CD1 treated fish reached the level of “dislike slightly” by the day 21 and became unacceptable by the 28th day. Several authors have attributed colour loss in muscle during storage to the oxidation of proteins which had groups, such as haemoglobin and myoglobin [18]. The results on the fish samples stored at 0°C were encouraging since the enterocin CD1 treated samples maintained its quality even at the end of storage period (28th day), though a slight reduction in quality was noticed with the control which remained within the acceptable limits (Fig. 4). No apparent variation in sensory attributes could be detected with the samples stored at -18°C.

Fig.4. Hedonic scores on two different days for each treatment
Hedonic scores represent a range from 1 (dislike extremely) to 9 (like extremely) of the mean scores ± standard deviation
CONCLUSION

The results of this experiment indicated that enterocin CD1, at 10% concentration could extend the shelf life of cod fish flesh stored in refrigeration conditions. Hence, it could be concluded that enterocin CD1 may offer novel, safe alternative for the preservation of high value sea foods. Further purification of the protein is required to enable the commercial application of this natural antimicrobial compound.

REFERENCES