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Full Length Research Paper

Enterocin from *Enterococcus faecium* isolated from mangrove environment

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Enterococcus faecium **isolated from mangrove environment produced enterocin and it showed broad inhibitory spectrum against gram positive and gram negative bacteria such as** ***Lactobacillus plantarum, Enterococcus facealis, Listeria monocytogens and Salmonella paratyphii.*** **The optimum production of bacteriocin (2400 AU/ml) from** ***E. faceium*** **was obtained when the culture conditions were maintained at pH 6.0 and 35°C. Maximum yield was 40% in ion exchange chromatography. The molecular weight of the partially purified enterocin was estimated as 5 KDa by SDS PAGE electrophoresis.**

Key words: *Enterococcus faecium,* Enterocin, antimicrobial spectrum and mangrove environment.

INTRODUCTION

Lactic acid bacteria (LAB) are important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliland, 1990). The potential use of LAB as non-toxic biopreservative agents in the industrial processing of human food and animal feeds are chiefly due to the fact that they inhibit the growth of pathogenic and degradative bacteria (Cintas et al*.,* 2000) by producing bacteriocin or bactericidal proteins during lactose fermentations (Lindgren and Dobrogosz, 1990). Bacteriocins are ribosomally synthesized peptides and proteins which may inhibit the growth or eliminate certain bacterial species, affecting the permeability of the membrane or even interfering with essential roles played by the cell, such as DNA replication and translation (Bennick et al., 1998). Based on structural, physicochemical and molecular properties, bacteriocins from LAB can be subdivided into three major classes (Klaenhammer, 1993; Nes et al*.,* 1996). Class 1 bacteriocins are lantibiotics, i.e. small, cationic, hydrophobic, and heat-stable peptides that contain unusual amino acids (e.g. the thioether amino acids lanthionine and/or 3-methyl-lanthionine) that are post-translationally formed. Class 2 bacteriocins are small, cationic, hydrophobic, heat-stable peptides that are not post-translationally modified, except for cleavage of a leader peptide from the prebacteriocin peptide. Within this class, three subclasses can be distinguished: subclass 2a or pediocin-like bacteriocins with a strong antilisterial effect, possessing the consensus sequence YGNGV in their N-terminus; subclass 2b or bacteriocins that require two polypeptide chains for full activity; and subclass 2c or bacteriocins that do not belong to the other subgroups. Class 3 bacteriocins are a group of large, hydrophilic, heat-labile proteins.

Many bacteriocins from Gram-positive bacteria have fairly broad inhibitory spectra, and these bacteriocins may therefore have an applied potential as antimicrobial agents. The *Enterococcus* is a gram positive bacteria belong to lactic acid bacteria (LAB) which are well known producers of bacteriocin (Schleifer and Kilpper-Balz, 1987; Stackebrandt and Teuber, 1988; Devriese and Pot, 1995; Hardie and Whiley, 1997). Mangrove environment is rich in nutrient, highly polluted, heavy accumulation of organic matter and highly contaminated with faecal matter. Hence the strain *Enterococcus faecium* was isolated from mangrove environment. In this paper, we characterized the bacteriocins produced by *E. faecium,* which was isolated from the mangrove environment, determined the antibacterial spectrum, the optimum condition for bacteriocin production, and estimate the molecular weight of the enterocin.

MATERIALS AND METHODS

Microorganism

Enterococcus strains, were isolated from mangrove sediment samples of Vellar estuary (Lat; 11°46' Long; 79°46'), east coast of India

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by plating them on M-Enterococcus agar (Hi Media Laboratory Pvt Ltd. Mumbai. India). One of the isolates was selected for further studies which exhibited strong inhibitory activity against indicator strains and identified by physiological, biochemical and morphological tests according to Bergey's Manual of Determinative Bacteriology.

Bacteriocin production and extraction

The isolated strain was grown in MRS broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.5) seeded with 1% inoculum of overnight culture and maintained anaerobically at 37°C for 48 h. After incubation, cells were removed from the growth medium by centrifugation (5,000×g for 15 min, 4°C). The cell-free supernatant was adjusted to pH 6.5 using 1N NaOH and it was used as crude bacteriocin. The bacteriocin activity was measured by standard well diffusion assay described by Kang and Lee (2005).

Bacteriocin assay

The supernatant from 24-h culture was filter sterilized by passing through a 0.22 µm pore size membrane (Millipore, Bedford, MD, USA). Aliquots (50 µl) of the sterile supernatant were placed in 4-mm-diameter wells of MRS agar plates previously seeded with the indicator bacteria. After 12–18 h of incubation, the diameters of growth inhibition zones were measured. Antimicrobial activity was expressed in arbitrary units (AU/per ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Ivanova et al., 1998) (i.e. turbidity ≤50% of the turbidity of control culture grown without *E. faecium* supernatant).

Influence of growth conditions on the production of bacteriocin

Bacteriocin production level in different incubation time, temperature, pH was performed with 100 ml of MRS broth in 500 ml of Erlenmeyer flasks were inoculated (1%, v/v) with an overnight culture and incubated at different temperatures (25, 30, 35, 40 and 45°C), pH (4.5, 5.0, 5.5, 6.0 and 6.5) and incubation time (6, 12, 18, 24, 30, 36, 42 and 48 h). Samples were collected after 48 h (expect for incubation time effect) and examined for bacteriocin production (AU/ml) as described earlier.

Purification of bacteriocin

The bacteriocin was purified by simple purification step similarly to the method described for pediocin PA-1 (Lozano et al, 1992). 400 g of ammonium sulfate (Ranbaxy, New Delhi) per liter of culture supernatant was added and allowed it to settle for 24 h at 4°C. The protein precipitate was pelleted by centrifugation at 6,000 *g* for 20 min and dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 6.0). It was applied at a flow rate of about 10 ml/min to a 7-ml S-Sepharose Fast Flow cation-exchange column equilibrated with buffer. The bacteriocin was eluted from the column with different gradient of NaCl concentration (0-1 M) and then eluted fractions were assayed for bacteriocin activity.

Molecular weight estimation

The molecular weight of bacteriocin was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed according to Laemmli, (1970) in Hoeffer MiniVE or LKB Bromma 2050 Midget electrophoresis units (Pharmacia Amersham Co). After electrophoresis the gel was stained with Comassie Brilliant Blue R-250. Ultra-low range moleculer markers (1060–26 600 KDa) with six polypeptides were used as a markers.

RESULTS

Isolation and identification of Enterocin producing strain *E. faecium*

The bacteriocin producing strain was isolated from the mangrove environment of the Vellar estuary and it was identified as *E. faecium* by the morphological, cultural, physiological and biochemical characteristics such as catalase negative, Gram-positive, facultatively anaerobic coccus with the ability to grow at 45°C and pH 9±6, and in the presence of both 40% (v/v) bile and 0±04% sodium azide. It did not produce gas from glucose; it gave a positive Voges Proskauer reaction and produced ammonia from arginine. The final pH in glucose broth was 4±2, and acid was produced from l-arabinose, but not from D-arabitol, sorbitol or gluconate.

Antimicrobial spectrum of enterocin from *E. faecium*

The susceptibilities of various Gram-positive and negative bacteria to growth inhibition by the supernatant of *E. faecium* (Table 1) and it shows inhibitory activity against *Lactobacillus plantarum, E. faecalis, Listeria monocytogenes, Listeria innocua, Salmonella typhi* and *Salmonella paratyphi.* Among these, maximum activity observed against *L. plantarum, E. faecalis* and *L. monocytogenes.*

Purification of enterocin

The first step in the purification protocol was to concentrate the activity from the growth medium by ammonium sulfate precipitation. The next steps in the purification were cation-exchange chromatography followed by hydrophobic interaction chromatography. The overall yield and activity is summarized in Table 1. An increased amount of biological activity has also been reported during purification of other bacteriocins in the pediocin family and may be due to the presence of some inhibitory compound at an earlier stage of the purification.

Influence of growth conditions on the production of bacteriocin

The bacteriocin was secreted into the growth medium, and production was started from late log phase itself and maximum was obtained in the early stationary growth phase at 18th h of the culture (Figures 1 and 2). Growth beyond the stationary phase resulted in a decrease in bacteriocin production. Incubation temperature and pH

Table 1. Antimicrobial spectrum of *Enterococcus faecium* isolated from mangrove environment

Indicator strains	Source/Strain No	Inhibitory activity (mm)
Escherichia coli	ATCC 25922	2
Lactobacillus plantarum	ATCC 8014	6
Enterococcus faecalis	*	8
Pseudomonas eruginosa	ATCC 15442	-
Listeria onocytogenes	ATCC 15313	8
Listeria innocua	ATCC 33090	7
Proteus mirabilis	*	-
Vibrio sp.	*	-
Staphylocoocus ureus	ATCC 25923	-
Salmonella typhi	ATCC 14028	2
Bacillus subtilis	*	-
Salmonella paratyphi	ATCC 9150	1
Bacillus cereus	*	3
Klebsiella pneumoniae	*	-

*Isolated from mangrove environment.

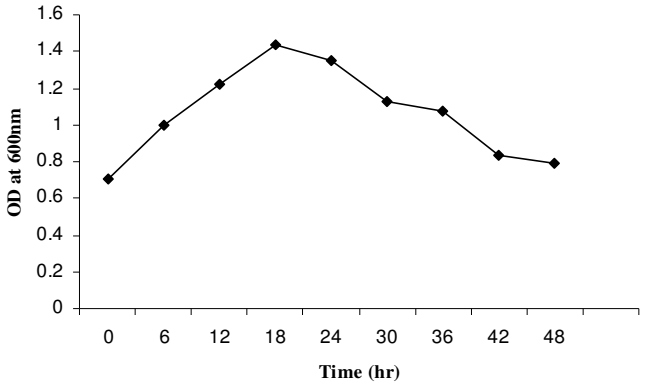

Figure 1. Growth kinetics of *Enterococcus faecium* isolated from mangrove environment.

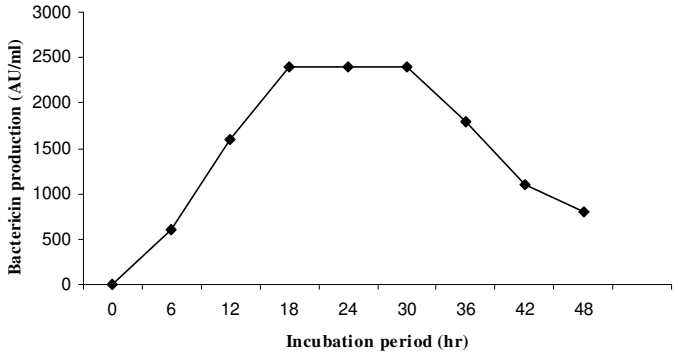

Figure 2. Bacteriocin production by *Enterococcus faecium* isolated from mangrove environment.

were played an important role in cell growth as well as bacteriocin production (Figures 3 to 6). Furthermore, the higher levels of bacteriocin production were recorded at 35°C (2,400 AU/ml), and relatively lower levels were recorded at 45°C (800 AU/ml). Regarding pH, maximum bacteriocin (2400) AU/ml level was observed at pH 6.0 and minimum bacteriocin level (800 AU/ml) was observed at pH of 4.5.

Molecular weight determination

Molecular weight of the bacteriocin protein was estimated by SDS-PAGE gel electrophoresis (Figure 7). Single protein band was observed when stained with Comassie blue and it clearly indicates the purity of the protein. The molecular weight was estimated as approximately 5 KDa.

DISCUSSION

Enterococci are natural inhabitants of the gastrointestinal tract. Although they are considered to be indicator strains for faecal pollution of water and foods, *Enterococci* are often used as additions to starter cultures for the presservation of various fermented foods, including cheese and fermented vegetable products. Indeed, the antimicrobial activity of *E. faecium* has been widely investigated and discussed (Kang and Lee, 2005). This inhibitory activity is attributable to antibacterial peptides called bacteriocins. A number of reports describing bacteriocin activity in *Enterococcus* species have appeared recently (Lopez-Lara et al., 1991; Parente and Hill, 1992; Salzano et al., 1992; Siragusa, 1992; Villani, et al., 1993). But it is the first report on *E. faecium* bacteriocin isolated from mangrove environment.

Many Enterocin and other members of the pediocin-like

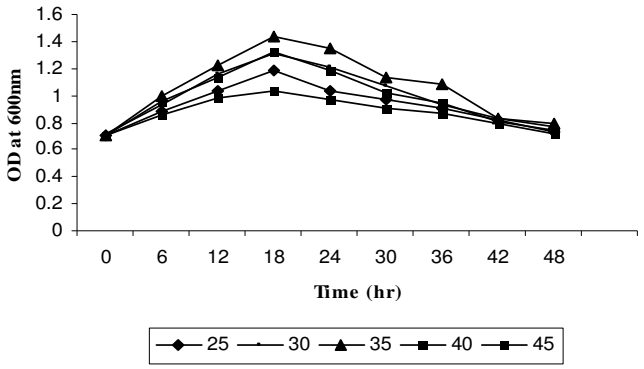

Figure 3. Cell growth of *Enterococcus faecium* isolated from mangrove environment at different temperatures.

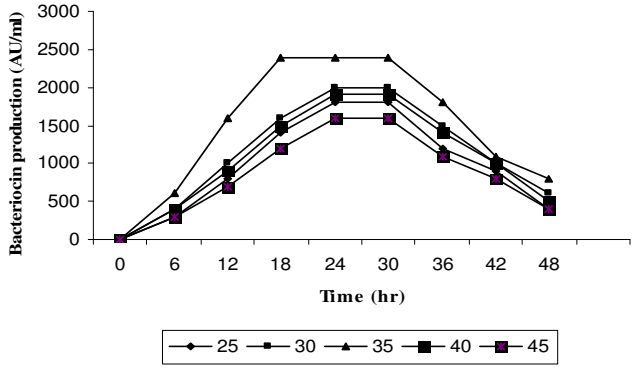

Figure 4. Enterocin production by *Enterococcus faecium* isolated from mangrove environment at different temperatures.

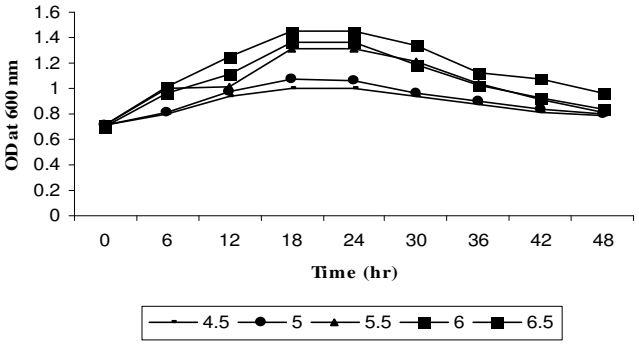

Figure 5. Cell growth of *Enterococcus faecium* isolated from mangrove environment at different pH.

family of bacteriocins exhibit a broad inhibitory spectrum that includes most of the gram-positive bacteria (Aymerich et al*.,* 1996; Booth et al., 1996; Casaus et al., 1997; Cintas et al., 1997). Our bacteriocin also strongly inhibited *L. monocytogenes* strains *L. plantarum, E. faecalis, L. innocua, S. typhi* and *S. paratyphi.*

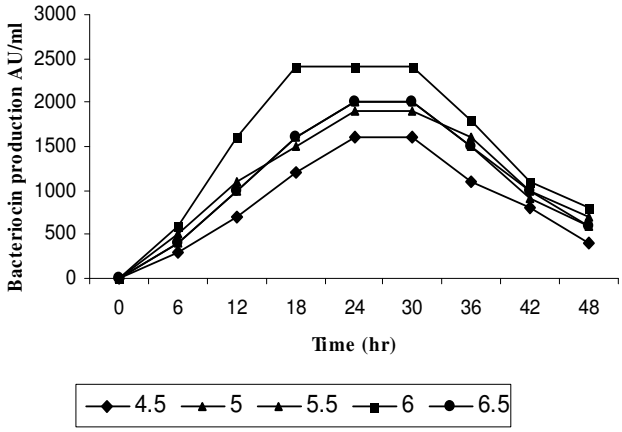

Figure 6. Enterocin production by *Enterococcus faecium* isolated from mangrove environment at different pH.

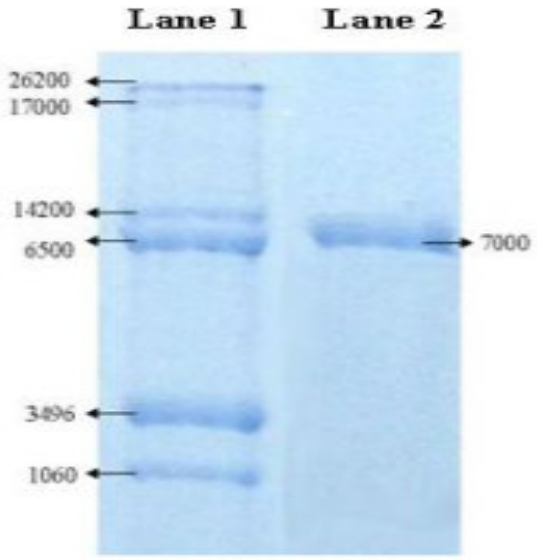

Figure 7. Molecular weight of enterocin produced by *Enterococcus faecium* isolated from mangrove environment determined by SDS-PAGE electrophoresis. Lane 1, molecular weight markers; and lane 2, bacteriocin.

Even though Gram-negative bacteria are usually considered to be resistant to the many of bacteriocins from *Enterococcus* strains, we observed some Gram-negative strains were sensitive to our bacteriocin. Some reports have stated to support sour findings that certain lactic acid bacteriocins, especially the class 2 bacteriocin pediocin, can inhibit a limited number of Gram-negative bacteria including *Shigella* sp*, Salmonella* sp (Laukova et al*.,* 1998) and *Pseudomonas*, *Shigella flexneri* (Kwon et al., 2002).

Bacteriocin production can be influenced by pH, temperature, incubation time, and other environmental factors (Biswas et al., 1991; Eijsink et al., 1996; Mortvedt-

Abildgaard et al., 1995). According to the results obtained in this study, and those reported in the literature (Daeschel et al., 1990; Jimenez-Diaz et al., 1993; Todorov et al., 2000), the optimal production of *E. faecium* bacteriocins occurs during the early logarithmic growth phase, usually at a pH above 4.5. The decrease in antimicrobial activity observed after a longer incubation time could be due to the degradation of the bacteriocin by proteolytic enzymes present in the medium, or else by the low pH (Torri Tarelli et al., 1994). By growing the cells at a constant pH it was possible to obtain good bacteriocin production in liquid media. The bacteriocin production was highly dependent on the pH of the growth medium; the optimum pH was 6.5, and there was very low production at pH below 6. Similar results have been reported for other bacteriocins (Daeschel et al., 1990; Jimenez-Diaz et al., 1993; Todorov et al., 2000), the optimal pH for enterocin production ranged between pH 5.7 - 6.0. Maximal growth occurred at pH 6.2 - 7.0 (Herranz et al., 2001). Optimal levels of growth as well as bacteriocin production by *E. faecium* were obtained at a pH of 6.0, at temperatures ranging from 30 to 35°C.

The majority enterocins have been characterized so far class 2a, with molecular weight under 10 KDa (Eijsink et al., 2002). Enterocin 32 produced by *E. faecium* has a molecular weight of 5 KDa and enterocin P has 4.5 KDa (Ennahar et al., 2000). In the present study, the molecular weight of enterocin fell under this category which was 5 KDa. However, in some cases very low molecular weight proteins were reported; enterocin O12, produced by *Enterococcus galinarum* presented a molecular size of 3.4 KDa (Jennes et al., 2000) and enterocin ON-157, produced by *E. faecium* strain NIAI 157, showed one of the lowest molecular weight characterized as 2.5 KDa (Ohmomo et al., 2000).

The present study described the production of enterocin from a mangrove stain, *E. faecium*, partial purification, influence of growth condition on bacteriocin production and molecular weight determination. To our best knowledge, it is a first study on bacteriocin producing strain isolated from mangrove environment. Also the capacity to inhibit both gram positive as well as negative strains (*L. monocytogenes* and *S. typhi*, important pathogens associated with food poisoning) indicates that *E. faecium* can be used in food preservation strategies.

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