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Screening of bacteriocin producing bacteria obtained from marine soil sediments and their antibacterial activity against diabetic foot ulcer pathogens

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Abstract

Marine bacteria are an important and relatively unexplored resource for novel microbial products. Bacteriocins were first detected by Andre Gratia in 1925. Bacteriocins function as a natural bacterial immune weapon system. Gram-positive and Gram-negative can produce many kinds of bacteriocins that allow bacteriocin-producing bacteria to have the ability to inhibit the growth of sensitive bacteria. They are ribosomally synthesized extracellularly released low molecular mass peptides, produced by different types of bacteria, Gram-positive, Gram-negative and Archaea. They can be produced spontaneously or induced by certain chemicals such as mitomycin C. In this study, several bacterial strains were isolated from the soil and screened for bacteriocin production. This newly isolated strain showed antibacterial activity against several Gram-positive and Gram-negative bacteria. Different concentrations of tryptone, yeast extract, and NaCl and physiochemical factors such as temperature, pH, and incubation period were selected as variables for maximum production of bacteriocin by using the agar well diffusion method. Totally19 bacterial colonies were isolated from three different marine environments of different places mangrove, saltpan, and coastal area of kodiyakarai area in vedaranyam, Nagapattinam district of Tamil Nadu. Probiotic properties viz., resistance to low pH and tolerance against bile salt 0.3% were tested. Six isolates had shown antibacterial activity against identified pathogens such as *Enterococcus* sp, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus pneumoniae.*

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Introduction

The Marine ecosystem covers almost 70% of the earth's surface (Vijayakumar *et al.*, 2007). Soil has a variety of microbes like fungi, actinobacteria, and eubacteria. The major constituents of soil are microbes which produce a variety of primary as well as secondary metabolites, which can play an important role in soil ecology and are beneficial for human beings. *Bacillus* spp. is one of the most common soil bacteria that produce various bioactive metabolites such as bacteriocins and antibiotics. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity, against those bacteria which are closely related to the producer strain (Katla *et al.*, 2001; Jones *et al.*, 2008). Bacteriocins are highly specific antibacterial proteins produced by strains of bacteria active mainly against some other strains of the same or related species (Gaur *et al.*, 2004). The production of antimicrobial substances is an important factor in microbial ecology. Many substances play a key role in bacterial interactions, among them bacteriocins are highly specific and efficient antagonists (Sahl, 1994). Bacteriocins are peptides and protein antibiotics which are produced by several species and have antimicrobial properties usually against other closely related species (Cladera-Olivera *et al.*, 2004). Bacteriocins produced by Gram-positive bacteria have been largely studied and also biochemically and genetically characterized (Navaratna, 1998). Bacteriocin activity is very specific and due to differences in cell wall composition, the activity spectra by Gram-positive bacteria are wider as compared to Gram-negative bacteria. Bacteriocins of Gram-positive and Gram-negative bacteria have evolved differently in terms of size and specificity (Jack, 1995). Many bacteriocins and bacteriocin-like inhibitory substances have been classified for LAB, but still, now limited classification scheme has been devised for *Bacillus* bacteriocins (Abriouel *et al.*, 2011). One of the most important species of the genus *Bacillus* is *Bacillus subtilis* which is commonly recovered from water, soil, and the environment and it can survive in extreme conditions of heat and desiccation because of the production of endospore (Alexander, 1977). This concern is becoming a challenge for researchers to investigate new antimicrobial agents produced by bacterial strains of apparent low virulence and having antibacterial activity against a wide range of clinically significant organisms (Jack, 1995). Bacteria, which are treated with antibiotics, are by far the most common cause of infectious-related deaths. It is advisable to screen antibiotic-producing bacteria as they are very easy to isolate culture and maintain and to improve their strains. Bacteria hold a prominent position as targets in screening programs due to their diversity and their proven ability to produce novel antibiotics. Keeping in mind the above-mentioned points, this study deals with the isolation of new strains of bacteria from soil and screening its antimicrobial activity against microbes. The search for new antibiotics effective against multidrug-resistant pathogenic bacteria is presently an important area of antibiotic research.

Materials and methods

Isolation and identification of diabetic foot ulcer bacterial pathogens (Baby Joseph et al., 2013)

Wound swabs were collected from diabetic foot ulcer patients from Government Hospital, Thiruvidaimaruthur, Kumbakonam, and were processed in blood agar medium and analysis done by standard microbiological methods. Genotypic identification was done based on Bergey's manual of systemic bacteriology (Fig. 1).

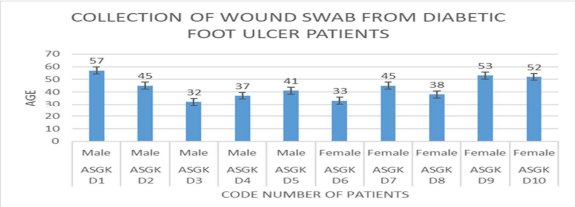

Fig. 1. Collection of wound swab from diabetic patients

Culture media

The cultural media used for testing antimicrobial activity was processed in Mueller Hinton agar. The culture media was prepared and sterilized following the manufacturer's instructions.

Detection of the strain to produce bacteriocin stab overlay assay (Pinchuk et al., 2001)

The 24 h culture of *Enterococcus* sp, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptococcus pneumoniae* was stabbed on a nutrient agar plate and incubated at 37 °C for 24 h. After incubation, the colonies on the stabbed area were scrapped and the plate was exposed to chloroform vapors for 15 min. The soft agar was prepared and a 24-hour-old indicator organism was added vortexed poured on a stabbed plate and incubated for 24 h at 37 °C. After incubation, plates were examined for the zone of inhibition around the stabbed area.

Effects of ph, temperature and incubation period on Bacteriocin stability (Baby Joseph et al., 2013)

The effects of temperature on bacteriocin stability, the partially purified bacteriocin were incubated at various temperatures (28, 32, 36, 40, and 44) for 30 minutes. After that incubation period the agar well diffusion method were carried out to analyse the activity of bacteriocin based on different temperature. The effects of pH on bacteriocin stability was determined by agar well diffusion method to adjusting the pH of the partially purified bacteriocin with diluting HCL and NaoH as 3, 5, 7, 9 and 11 and allowed for incubation for 2 hrs at 37 °C, and samples were tested for antimicrobial activity. The effects of Incubation period on stability of bacteriocin production started after 24, 48, 72, 96, and 140 hours and observed bacteriocin production. In all the three cases, untreated partially purified bacteriocin served as control. The effects of pH, Temperature and incubation time on the production of bacteriocin stability against the DFU pathogens results were tabulated.

Screening of antimicrobial activity

Anti-bacterial assay

The supernatants of each suspension were assessed for the antibacterial property against bioassay strains of bacteria viz., *Enterococcus* sp, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae* (Pathogens were isolated from the wound samples of infected patients and identified by morphological and biochemical test). To perform this test, a bioassay strain was carried out on Mueller Hinton agar and wells were made in plate agar using a sterile cork borer. 50 and 100 µl of each supernatant were added to each well and plates were incubated at 35°C for 24 h. The exhibition of a clear zone of growth inhibition was observed, measured, and quantified which was considered as antimicrobial activity.

Bacteriocin extraction and purification (Mezaini et al., 2009)

Nutrient broth was seeded with culture and incubated at 37 °C at 150 r/min for 24 h. The sample was centrifuged at 10 000 r/min for 20 min, and the supernatant was collected. The culture supernatant (100 mL) was stirred vigorously with chloroform (100 mL) and transferred in a separating funnel. The interface layer between the aqueous and organic phases, which contain bacteriocin was harvested, and the residual chloroform was eliminated by speed vacuum.

Results and discussion

The current study of 19 morphologically distinct colonies was selected and screening of bacteria by determination of Bacteriocin production. The organisms were identified as *Aeromonas* sp, *Bacillus cereus, Bacillus subtilis, Bacillus* sp, *Clostridium butyricum, Eschericia coli, Edwardsiella tarda, Flavobacterium odoratum, Klebsiella pneumoniae, Proteus vulgaris, Proteus* sp, *Pseudomonas aerogenosa, Pseudomonas facilis, Pseudomonas* sp, *Serratia fonticola, Staphylococcus aureus, Staphylococcus* sp and *Streptococcus pneumonia* (Table 1).

The identified bacterial strains allowed for bacteriocin production by stab overlay assay with indicator Pathogenic organism. It exhibited antibacterial activity producing a clear zone of inhibition around the indicator strain (Fig. 2).

The Sensitivity to temperature, pH and enzymes to determine the effect of temperature on bacteriocin activity, aliquots of partially purified bacteriocin were incubated at various temperatures (40, 60, 80 and 100 °C) for 30 min.

Table 1. Morphological characteristics of isolated bacteria

Name of the strains	Growth	Shape	Surface	Margin	Color	Elevation	Consistency	Opacity
ASGK D1	Rapid	Punctiform	Smooth	Even	White	Convex	Viscous	Transperent
ASGK D2	Slow	Punctiform	Rough	Irregular	White	Umbonate	Adhesive	Opaque
ASGK D3	Slow	Punctiform	Smooth	Wavy	White	Flat	Buttery	Opaque
ASGK D4	Slow	Circular	Smooth shiny	Entire	Pale yellow	Pulvinate	Buttery	Opaque
ASGK D5	Slow	Irregular	Smooth	Even	White	Convex	Adhesive	Translucent
ASGK D6	Slow	Punctiform	Smooth	Wavy	White	Flat	Buttery	Opaque
ASGK D7	Slow	Circular	Smooth	Entire	Creamy white	Convex	Buttery	Opaque
ASGK D8	Rapid	Punctiform	Smooth	Even	White	Convex	Viscous	Transperent
ASGK D9	Slow	Irregular	Smooth	Even	White	Convex	Adhesive	Translucent
ASGK D10	Slow	Punctiform	Smooth	Wavy	White	Flat	Buttery	Opaque

Table 2. Effect of pH, temperature and incubation time on the production of bacteriocin against the DFU pathogens

Factors	Treatment	Zone of inhibition diameter (mm)					
		Enterococcus sp.	*E. coli*	*K. pneumoniae*	*S. aureus*	*S. pneumoniae*	*P. aeruginosa*
pH	3	0	0	0	28.5±0.02	13.2±0.05	0
	5	0	0	14.5±0.02	33.5±0.02	27.3±0.05	28.1±0.05
	7	0	12.8±0.04	34.4±0.05	34.6±0.03	28.9±0.05	32.5±0.02
	9	28.1±0.05	28.5±0.02	34.2±0.06	38.4±0.05	38.3±0.05	3.55±0.01
	11	32.6±0.05	36.6±0.03	41.1±0.04	45.2±0.05	28.5±0.02	29.4±0.00
Temperature (°C)	28	12.9±0.02	12.3±0.07	10.1±0.87	12.1±0.23	8.98±0.11	7.41±0.08
	32	13.6±0.12	12.9±0.85	13.4±0.36	13.2±0.57	13.1±0.64	11.6±1.23
	36	15.3±0.23	13.8±0.66	14.3±0.92	13.9±0.28	15.5±0.88	13.4±0.69
	40	18.2±0.32	15.3±0.58	14.6±0.71	16.2±0.05	16.2±0.32	13.6±0.27
	44	19.9c0.18	17.8±0.87	15.2±0.55	17.5±0.68	20.3±0.85	16.4±0.71
Incubation time (hrs)	24	12.4±0.12	12.9±0.52	-	-	-	-
	48	12.6±0.34	13.1±0.14	10.5±0.87	9.54±0.35	9.21±0.85	7.14±0.34
	72	14.4±0.25	13.4±0.56	14.1±0.25	10.3±0.11	9.48±0.63	8.23±0.27
	96	14.7±1.23	13.6±0.15	15.2±0.32	14.5±0.23	9.99±0.00	13.9±0.35
	140	16.2±0.87	14.1±0.13	16.5±0.84	14.8±0.55	10.2±0.14	13.1±0.82

The maximum bacteriocin production and the fermentation was carried out at different time intervals ranging from 06 to 120 hours. Optimum temperature for maximum bacteriocin production was achieved by incubating the culture medium at different temperatures from 20˚C to 60˚C for 24 hours. The effect of incubation time, temperature and pH on bacteriocin production It was observed that bacteriocin production started after 06 hours of incubation but maximum specific production rate was achieved after 24 hours and as time increases production decreases and after 72 hours no production was observed (Ansari *et al.*, 2012).

The effects of pH, Temperature and Incubation period on Bacteriocin stability were analysed by agar well diffusion method. The DFU pathogens of indicator organisms were *Enterococcus* sp, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* was swab on the agar plate and zone of inhibition was measured. The four DFU pathogens were inhibited by different pH, Temperature and incubation period of partially purified bacteriocin stability shown different size of zone formation, that each different zone was measured and values were tabulated (Table 2).

The partially purified bacteriocin showed a wide range of activity towards various temperatures, pH, enzymes, and chemicals. The partially purified bacteriocin showed antimicrobial activity towards all the bacterial isolates where a high level of activity was found against *Klebsiella pneumoniae*. The screening for bacteriocin production was done by stab overlay assay. *B. subtilis* was outstanding in the genus *Bacillus* with regard to its potential to produce so many different antibiotics (Hammami *et al.*, 2012).

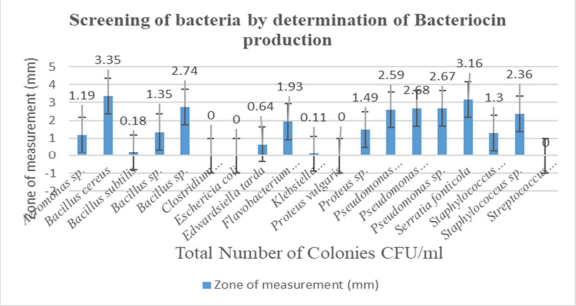

Fig. 2. Screening of bacteria by determination of bacteriocin production

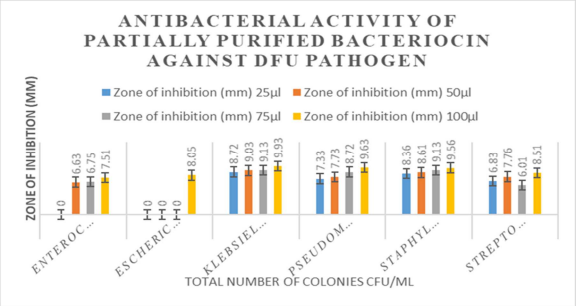

Fig. 3. Antibacterial activity of partially purified bacteriocin against DFU pathogen

To determine the antimicrobial activity of partially purified bacteriocin, the agar well diffusion method was adopted and the results revealed activity in all six isolates. A high level of activity was seen in the case of *Klebsiella pneumoniae* different concentration like 25, 50, 75 and 100 µl was 8.72±0.05, 9.03±0.08, 9.13±0.12 and 9.93±0.08 mm zone of inhibition represented (Fig. 3).

The maximum antibacterial activity was noted in *Klebsiella pneumoniae* (9.93±0.08mm). The minimum antibacterial activity observed *Enterococcus* sp (6.63±0.12mm) represented respectively.

Antimicrobial activity was assayed in duplicate using a standard paper disc assay (Mearns-Spragg *et al.*, 2012). The zone of inhibition was observed after incubating at 27°C for 36 h.

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