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Control of Multi Drug Resistant E. Coli Strains Using Bacteriocin Produced from Lactobacillus Plantarum

KEYWORDS: antibiotic resistance, E.coli, plantaricin

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ABSTRACT *The widespread use of antibiotics has led to the development of antimicrobial resistance. The problem of multiple drug resistance is a major public health concern due to its rapid dissemination. This paper deals with the effect of bacteriocin against multidrug resistant E.coli isolated from clinical samples. Lactobacillus species were isolated from marine samples. Bacteriocin produced from potent strain was purified using DEAE-cellulose ion exchange column chromatography. The antibacterial activity of bacteriocin was determined using well diffusion. The zone of inhibition against clinical multidrug resistant E.coli was in the range of 6 to 15mm. The purified bacteriocin showed a bacteriocin activity of 12800AU/ml against L. monocytogenes. Whereas,for clinical isolates it was ranged from 1600AU/ml to 25600AU/ml. Hence it was concluded that bacteriocin may be used to combat antibiotic resistant serious human pathogens.*

Introduction

Antibiotics are the microbial product or their derivatives that can kill susceptible microorganisms or inhibit their growth. The widespread use of antibiotics has led to the development of antimicrobial resistance. Persistent indiscriminate use and rising antibiotic resistance all over the world may result in "Post antibiotic era" . The problem of multiple drug resistance is a major public health concern due to its rapid dissemination.

Lactic acid bacteria (LAB) are widely involved in food processing and spontaneous fermentation and for centuries they have been used for the production of a wide variety of fermented food such as dairy, meat and vegetable products (Stiles and Holzapfel, 1997). These microorganisms contribute, not only to flavour, aroma and texture development, but also to the improvement of food shelf-life and safety by producing several antibacterial compounds including lactic and organic acids, ethanol, hydrogen peroxide, carbon dioxide, acetoin, diacetyl, reuterin, reutericyclin, antifungal peptides and bacteriocins (Caplice and Fitzgerald, 1999 and Magnusson and Schnürer, 2001).

The term bacteriocin is applied to a wide range of bioactive compounds which are synthesized in two different ways, ribosomally from transcripts (gene encoded) or by stepwise synthesis employing either multienzyme complexes or sequential enzyme reactions (Kleinkauf and Von Dohren, 1996) and they are extracellularly released by bacteria exhibiting the antimicrobial properties, either as bactericidal or bacteriostatic level against other bacterial species.

Lactobacillus species have been used in the food industries for years, this present work deals with the effect of bacteriocin against multidrug resistant E.coli isolated from clinical samples.

MATERIALS AND METHODS

Isolation of *Lactobacillus* strains

Sediment samples collected from Parangipettai coastal waters were serially diluted using sterile distilled water. 0.1ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread plated over the surface of the MRS agar plates and incubated at 37°C for 24-48 hrs. Typical colonies were purified and stored in the same agar.

Screening *Lactobacillus* for bacteriocin production

Screening for bacteriocin production was done with closely related species as well as using indicator strains (*L. monocytogenes* 39-2 and *Staphylococcus aureus* STCC 12600) and the most potent strain was identified. The antimicrobial activity was also tested against closely related *Lactobacillus* spp. such as *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Lactobacillus delbrueckii.*

Antimicrobial activity of bacteriocin against clinical and environmental *E. coli* strains

Well diffusion method

Sterile Muller Hinton agar plates were prepared and the inoculum of test microorganisms was spread uniformly in each plate as pure culture. Wells were made and about 50µl of cell free extract of *Lactobacillus* was loaded in to individual well. The plates were observed for a clear zone of inhibition and measured after 24 hrs of incubation. Ten different *Lactobacillus* strains were tested and based on the well assay result, the most potential strain was selected and identified according to the method described by Buchanan *et al.,* 1974.

Optimization of growth of L. *plantarum*

Factors like pH, temperature, salinity, carbon and nitrogen sources were optimized for the growth of the selected strain by selecting one parameter at a time.

pH, Temperature, Salinity

Different pH (i.e.) 5, 6, 7, 8, 9 and 10, different temperature (i.e.) 25°C, 30, 35 and 40°C, different salinity such as 0.5%, 1%, 1.5%, 2.0% and 2.5% were maintained in the medium. Growth was assessed for every 6 hrs.

Carbon and nitrogen sources

The carbon sources such as maltose, starch, glucose, sucrose and cellulose and nitrogen sources such as peptone, yeast extract, beef extract and ammonium nitrate were tried. Growth was assessed for every 6 hrs.

Concentration of ideal carbon and nitrogen source

Different concentration of starch as carbon source (0.5 – 2.5%), Different concentration of peptone as nitrogen source (0.1-1.0%) were maintained in the medium and incubated. Growth was assessed for every 6 hrs up to 48hrs.

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Mass scale production of biomass in shake flask
The optimized conditions were maintained in the 500 ml shake flask medium and kept for incubation at 30°C in a shaker for 24 - 48 hrs.

Ammonium sulphate precipitation and dialysis
The collection of cell free supernatant from the mass scale culture was harvested at 36hrs. The % of ammonium sulphate was selected by activity based precipitation which was tested on small volume of aliquots. Dialysis was performed using dialysis membrane (with 12 kDa MWCO membrane- Himedia, Mumbai). The titer values were assessed for the purified bacteriocin.

Purification of bacteriocin using DEAE-cellulose ion exchange column chromatography
DEAE-cellulose was purchased from Sigma and activated as per manufacturer's instructions. The resin was packed into a C 10/20 column **(**AKTA prime, Amersham). The column was pre-equilibrated with the 20 mM Tris-Cl buffer, pH 8.5. One ml of the crude bacteriocin sample was loaded onto the pre-equlibrated column. The column was then washed with the same buffer (20 mM Tris-Cl buffer, pH 8.5) to remove the unbound proteins (indicated by zero absorbance at 280 nm). The bound protein was eluted by applying a linear gradient of 0-0.8 M NaCl in the same buffer at a flow rate of 0.5 ml/min. and monitored at 280 nm. The active fractions were pooled, assayed for bacteriocin activity and it was lyophilized in a Vertis lyophilizer for further analysis.

Detection of bacteriocin titers
The titer of the bacteriocin produced was quantified by serial dilutions of the bacteriocin, using the physiological saline solution. The indicator strain *L. monocytogenes*, as well as the clinical *E. coli* isolates were tested for the determination of bacteriocin titre. Aliquots of 0.1 ml from each dilution were spotted in plates seeded with the overnight culture and incubated at 30°C for 24-48hrs. The antimicrobial activity of bacteriocin titre was defined as the reciprocal of the highest dilution which showed the inhibitory activity and it was expressed as arbitrary unit (AU) (Barefoot and Klaenhammer, 1983).

RESULTS AND DISCUSSION

Screening of *Lactobacillus* isolate for bacteriocin production
The potential bacteriocin producing *Lactobacillus plantarum* was determined using indicator strains such as *L. monocytogenes* 39-2 and *Staphylococcus aureus* STCC 1260 with the zone of clearance of 17 and 11 mm respectively.

Antibacterial activity against multiple antibiotic resistant clinical *E. coli* isolates
The zone of clearance due to bacteirocin activity for various MAR clinical strains was given in table 1. Clinical *E. coli* strains with single antibiotic resistance to 10 antibiotic resistant strains were tested the zone of clearance was in the range of 6 to 15mm (fig 1). Manzoor et al., 2016 revealed that Lactobacillus isolates from spoiled fruits and vegetables displayed antimicrobial activity against antibiotic resistant uropathogens similar to the present study.

Optimization of growth of *L. plantarum*
In the present study the optimized growth conditions observed were pH – 6, temperature - 30ºC, salinity – 1%, starch -1%, peptone -0.5%. These conditions were maintained in the medium for the mass scale production .Maximum growth was observed within 36 hrs itself.. Urso *et al.*, 2006 observed 25°C as the optimum temperature for the highest growth and bacteriocin production by *Lactobacillus sakei*.

In this study 60% ammonium sulphate was used for partial purification whereas Hata *et al.,* 2010 recovered plantaricin produced by *Lactobacillus plantarum* A-1 by adding 40% ammonium sulfate.

Bacteriocin titre
The purified bacteriocin showed a bacteriocin activity of 12800AU/ml against *L. monocytogenes*. Whereas,for clinical isolates it was ranged from 1600AU/ml to 25600AU/ml . In this study, antimicrobial activity of bacteriocin was tested against different clinical *E. coli* isolates of varying antibiotic resistant patterns.

Surprisingly all of them were found to be sensitive to *Lactobacillus plantarum* isolated from coastal water samples. The results revealed that the isolated *L. plantarum* possessed the ability to produce bacteriocin compound that effectively controlled multiple antibiotic resistant *E. coli*.

As the purified bacteriocin was tested against *E. coli* strains, it was clear that the inhibitory activity might be due to this protein only. The mode of activity of this bacteriocin on antibiotic resistant *E. coli* is not known. Though 300 bacteriocins were identified till date their mode of action is not fully under stood. But the model membrane studies with nisin have shown that lipid II acts as a docking station. After binding, nisin wedges itself into the cell membrane to form short lived pores which disturb the integrity of the cytoplasmic membrane and causes the efflux of ions and other cell components. At high concentrations of nisin, pore formation may occur in the absence of lipid II, provided the cell membrane contains at least 50% negatively charged phospholipids. Under these conditions, the positively charged C terminus of nisin is important for initial binding and antimicrobial activity. Mersacidine and the antibiotic vancomycin also bind to lipid II, but to a different part of the molecule (Bauer *et al.* 2005) (i.e), bacteriocins kill sensitive bacteria by forming pores on the cytoplasmic membrane or by inhibiting the synthesis of the cell wall (Diep *et al.,* 2006). Whereas, normally the cells producing the bacteriocins are immune to its antagonistic action and therefore might enjoy a competitive advantage over sensitive bacteria inhibiting the same ecological niche (Aslam *et al.,* 2011).

The present study clearly indicated that *Lactobacillus* spp., (or) their bacteriocin may be used to combat antibiotic resistant serious human pathogens.

Table 1: Values of Bacteriocin Titre and zone of inhibition

Bacteriocin (from *L. plantarum*)	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	Zone of inhibition (mm)
L. monocytogenes	+	+	+	+	+	+	+	+	-	-
E.coli 029	+	+	+	+	+	+	+	-	-	12
72	+	+	+	+	+	+	+	+	+	15
9	+	+	+	+	+	+	+	-	-	11

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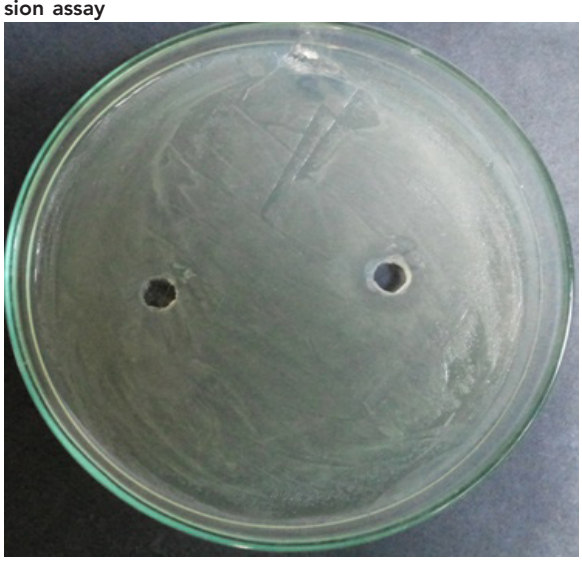
016	+	+	+	+	-	-	-	-	-	7
038	+	+	+	+	+	+	+	+	-	13
039	+	+	+	+	+	+	+	+	-	12
044	+	+	+	+	+	+	+	+	-	14
64	+	+	+	+	+	+	+	-	-	11
78	+	+	+	+	+	+	+	-	-	12
113	+	+	+	+	+	+	+	-	-	12
26	+	+	+	+	+	+	+	-	-	12
117	+	+	+	+	+	+	-	-	-	10
133	+	+	+	+	-	-	-	-	-	6
145	+	+	+	+	+	+	+	-	-	11
157	+	+	+	+	+	+	+	-	-	10
158	+	+	+	+	+	+	+	+	-	13
166	+	+	+	+	+	+	+	+	-	13
167	+	+	+	+	+	+	-	-	-	9
168	+	+	+	+	+	+	-	-	-	6
173	+	+	+	+	+	+	+	-	-	10

Fig 1: Antibacterial activity of bacteriocin –Well diffusion assay



Strain 016 (7mm)

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