COMPARATIVE STUDY OF TWO BACTERIOCINS PRODUCED BY REPRESENTATIVE INDIGENOUS SOIL BACTERIA

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ABSTRACT

The aim of this research work was to identify and characterized the bacteriocins produced by soil-associated bacteria. Bacillocin from *Bacillus brevis* Bb and pyocin from *Pseudomonas aeruginosa* Pa were found bioactive only against gram-positive bacteria tested. Maximum production of both the bacteriocins was observed at 32°C in BHI medium. Production of both the bacteriocins started in the early exponential growth phase while the maximum production was observed during the stationary phase. Bacillocin Bb remained stable during 1-9 pH while pyocin Pa remained stable at pH 1-11. Both of the bacterocins were found resistant to high temperature (100°C for 30 min), detergents (1% solutions of EDTA, Tween 20, Tween 80 and SDS) and organic solvents (1% solutions of Ethanol, Butanol, Methanol, Propanol, Chloroform, and Acetone). Activity of both was completely lost after proteinase K treatment suggesting their protein nature. Titre of bacillocin Bb was estimated to be 5280 AU/mL while the titre for pyocin Pa was calculated as 640 AU/mL. Both of the bacteriocins showed bacteriolytic mode of action against the indicator *Bacillus* strain BC31 and were found <10 KDa in their molecular mass.

Keywords: Bacteriocin, bacillocin, pyocin, bacteriolytic.

INTRODUCTION

Bacteriocins are bacterially produced peptide antibiotics with the ability to kill a range of bacteria (Cleveland *et al*. 2001). Both gram-positive and gram-negative bacteria have produced them. Bacteriocin-mediated antagonism is believed to occur in virtually any niche colonized by bacteria. They are heterogeneous compounds with variable molecular weights, biochemical properties, inhibitory spectra and mechanisms of action (McAuliffe *et al*., 2001; Sullivan *et al*., 2002). The bacteriocin could be a potential food bio-preservative. Nisin was the first ever reported bacteriocin approved by FDA and classified as Generally Recognized As Safe (Muriana and Luchansky, 1993).

Members of the genus *Bacillus* are rod-shaped, endospore forming aerobic or facultative anaerobic, gram-positive bacteria. Many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment (Claus and Berkeley, 1986). Members of the genus *Bacillus* carry tremendous importance because of their antimicrobial activity since they produce a variety of peptide antibiotics representing several different basic chemical structures (von Dohren, 1995). The production of bacteriocins or bacteriocin-like substances has been described for *B. coagulans, B. brevis, B. lichniformis, B. cereus, B. subtilis, B. amyloliquefaciens* and other *Bacillus* species (Hyronimus *et al*., 1998; Hyung *et al.*, 2001; Martirani *et al*., 2002; Risoen *et al*., 2004; Teo and Tan, 2005; Lisboa *et al.*, 2006). Gram-negative bacilli of the genus *Pseudomonas* are common inhabitants of soil, fresh water, and marine environments. *Ps. aeruginosa* receives more attention since it is also an opportunist pathogen, causing human diseases (Stover *et al.*, 2000). Generally, three types of pyocins are described. (i) R-type pyocins resemble non-flexible and contractile tails of bacteriophages. They provoke a depolarisation of the cytoplasmic membrane causing pore formation. (ii) F-type pyocins also resemble phage tails, but with a flexible and non-contractile rod-like structure. (iii) S-type pyocins resemble colicins and are protease-sensitive proteins (Briand and Baysse, 2002).

The purpose of this study was the isolation and characterization of bacteriocins from indigenous soil associated bacteria. The two bacteriocin preparations were compared with for their sensitivity to pH range, proteolytic enzymes, organic solvents, surfactants and thermostability. Mode of action and growth phase related bacteriocin synthesis was also measured. The characterization of the both bacteriocins have suggested their potential use for industrial applications.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Two bacteriocin producing strains were isolated from garden soil of Microbiology Department and were identified as *Bacillus brevis* Bb (gram-positive) and *Pseudomonas aeruginosa* Pa (gram-negative) according to their morpho-cultural and biochemical characteristics as given in table 1. Indicator cultures used in this study were obtained from Reference Culture Collection Lab of Microbiology Department University of Karachi while others were the clinical isolates. All the strains were maintained in BHI or MacConkey's agar and sub-cultured

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once a week. Bacteriocin producing strains were maintained in BHI broth (in the presence of 15% glycerol) and were kept at 4ºC.

All the experiments were performed in triplicate, and results presented are the means of triplicate trial.

Table 1: Phenotypic identification of *Bacillus brevis* Bb and *Pseudomonas aeruginosa* Pa

Characteristics	*Bacillus brevis* Bb	*Pseudomonas aeruginosa* Pa
Morphological characteristics		
Gram-Reaction	Positive	Negative
Shape	Long rods	Short rods
Arrangement	Chains	Scattered
Spores	Present	Absent
Cultural Characteristics		
Nutrient Agar	Large, irregular, white, wrinkled colonies	Green-pigmented, medium size, viscid, irregular colonies
MacConkeys agar	No growth	Non-lactose fermentation, pinpoint colorless colonies
Biochemical Characteristics		
Sugar fermentation		
Glucose	+	+
Lactose	-	-
Sucrose	+	-
Mannitol	-	-
Catalase test	+	+
Oxidase test	-	+
IMViC test		
Indole	-	-
MR	-	-
VP	-	-
Citrate	-	+

Key: (-) = negative; (+) = positive; MR = methyl red; VP = Voges-Proskauer.

Bacteriocin bioassay

Bacteriocin screening was performed by agar-well diffusion method as described by Iqbal *et al.* (1999). Cell-free neutralized supernatant (CFNS) was adjusted to pH 7 with 1N NaOH to prevent the inhibitory effect of the acid. *Bacillus* strain BC31 (a soil isolate) was found the most sensitive and hence used as indicator throughout this study.

Effect of different growth media and incubation temperatures on bacteriocin production

For this purpose, both the producer strains (*Bacillus brevis* Bb and *Pseudomonas aeruginosa* Pa) were inoculated in different growth media (brain heart infusion broth, lactose broth, Luria basal broth, trypticase soy broth and nutrient broth) and incubated at different temperatures (30ºC, 32ºC, 37ºC and 40ºC) for 16-18 hrs and activity was measured by agar-well diffusion assay (Lisboa *et al.,* 2006).

Titration of Bacteriocins

The titre of bacillocin Bb and pyocin Pa was carried out by two-fold serial dilution in PBS (pH 7) and 0.1 ml of each dilution was placed into wells made on a BHI plates seeded with $1x10^6$ indicator cells (Saeed *et al.,* 2006). Next day zones of inhibition were measured. The antimicrobial activity of a bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator strain and is expressed as activity units (AU/mL).

Growth curve

Bacteriocin producing strains were inoculated into 300 ml of BHI broth and incubated at 32ºC. After every hour, 1.0 ml of samples were drawn. After centrifugation at 6,000 rpm for 15 minutes, supernatants were assayed for antibacterial assay. At the same time OD_{600} was recorded and CFU/ml were also calculated (Karmen and Bojana, 2003).

Inhibitory spectrum of bacillocin Bb and pyocin Pa

The agar well diffusion method was used to access the antimicrobial activity of crude bacteriocin preparations (CFS) of both *Bacillus brevis* Bb and *Pseudomonas aeruginosa* Pa towards several gram-positive and gram-negative bacteria (Hyung *et al.,* 2001). All strains were sub cultured in BHI agar medium, propagated in BHI broth for two hours (to get logarithmic phase) and were then inoculated into the BHI soft-agar.

Physico-chemical characterization

Cell free supernatants (CFS) of bacillocin Bb and pyocin Pa were 80% precipitated with ammonium sulfate at 4ºC, with constant stirring for 18-19 hours. Proteins were isolated by centrifugation at 6000 rpm for 45 minutes and pellets were resuspended in 50mM PBS (pH 7.0). This was designated as cell-free precipitated bacteriocin preparations. Effect of different enzymes (protease, proteinase K, pepsin, trypsin and lipase), temperatures (40ºC, 60ºC, 80ºC, 100ºC) for 30min, 121ºC (at 15 psi, 15 min), 4ºC (6 months), pH range (1-14), organic solvents (acetone, butanol, ethanol, methanol, propanol and chloroform) and detergents (tween20, tween80, sodium dodecyl sulphate and ethylene diamine tetra acetic acid) on cell-free precipitates was observed by agar well diffusion method (Saeed *et al*. 2006). The ability of the bacteriocins to pass through dialysis membrane (pore size 12,000 Da: Sigma) was also assessed to estimate their molecular weight (Ahmad and Rasool, 2003).

Table 2: Antibacterial activity of bacillocin Bb (from *B. brevis* Bb) and pyocin Pa (from *Ps. aeruginosa* Pa) against gram-positive and gram-negative bacteria

Test organism	Inhibition zone (mm)	
	Bacillocin Bb	Pyocin Pa
Gram-positive bacteria		
Bacillus subtilis	0	0
Bacillus strain BC31	28	34
Staphylococcus aureus	15	32
Staphylococcus epidermidis	0	30
Streptococcus pneumoniae	0	0
Streptococcus pyogenes	0	18
Enterococcus faecalis	0	42
Enterococcus faecium	0	22
Micrococcus luteus	19	22
Corynebacterium diphtheriae	26	24
Corynebacterium xerosis	18	19
Corynebacterium hoffmanni	19	21
Gram-negative bacteria		
Escherichia coli WT	0	0
Salmonella typhi	0	0
Salmonella paratyphi A	0	0
Salmonella paratyphi B	0	0
Shigella dysenteriae	0	0
Shigella flexneriae	0	0
Pseudomonas aeruginosa	0	0
Proteus vulgaris	0	0
Proteus mirablis	0	0
Klebsiella pneumoniae	0	0
Vibrio cholerae	0	0

Key: 0 = no zone of inhibition observed.

Mode of action of inhibition

This was done to investigate the mechanism of action of both the bacteriocins. For this, 0.1 ml of the exponential growth phase cells of indicator strain (BC31) was added in the cell-free precipitated bacteriocin preparations (final concentration 200 AU/ml). Sterile BHI medium was added to the control tubes. Changes in the turbidity of the cultures were recorded at an O.D. of 600 nm after every hour and the number of colony-forming units (CFU) was also determined by plating the samples on BHI agar plates (Cladera-Olivera and Caron, 2003).

RESULTS AND DISCUSSION

Activity spectrum

Inhibitory spectrum of both the bacteriocin preparations was determined by agar-well diffusion assay and shown in table 2, fig. 1. Antimicrobial activity of both the bacteriocins was directed against most of the gram-positive bacteria tested. Bacillocin Bb was active against *S. aureus, M. luteus, C. diphtheriae, C. xerosis* and *C. hoffmanni* while pyocin Pa was active against *S. aureus, S. epidermidis, S. pyogenes, E. faecalis, M. luteus, C. diphtheria, C. xerosis* and *C. hoffmanni*.. Our results are in close agreement with (Cherif *et al.,* 2001 and Martirani *et al*., 2002) who also reported the activity of thuricin 7, a bacteriocin produced by *Bacillus thuringiensis* and bacillocin 490 by a thermophilic strain of *Bacillus Licheniformis* only against gram-positive bacteria. However, (Hyung *et al.,* 2001) reported the broad-spectrum antimicrobial activity of brevicin, produced by *Bacillus brevis* active against *B. anthracis* and *S. dysenteriae*. Iwalokun *et al*. (2006) also reported the broad-spectrum antimicrobial activity of pyocins from *Pseudomonas* spp.

Titration of bacillocin Bb and pyocin Pa

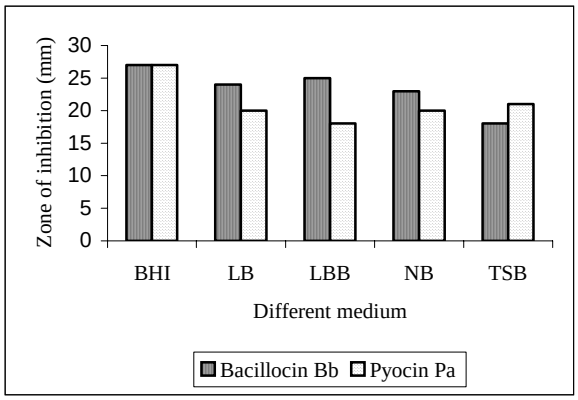
The titre(s) of bacillocin Bb and pyocin Pa were calculated as 5280 and 640 AU/ml respectively. Critical dilution method is the most commonly used procedure to quantify bacteriocin activity on solid media (Hardy, 1987).

Effect of different media and incubation temperatures on bacteriocin production

Maximal bacteriocin production was observed by supplementing the culture medium with growth stimulating organic factors such as sugars, vitamins and nitrogen sources and by regulating pH or by selecting the best-adapted culture medium (Vignolo *et al.,* 1995). Different culture media including: brain heart infusion broth (BHI), lactose broth (LB), Luria basal broth (LBB), trypticase soy broth (TSB) and nutrient broth (NB) were used to observe their effect on the bacillocin Bb and pyocin Pa production. Maximum production of the bacteriocins was observed when *B. brevis* Bb and *Ps. aeruginosa* Pa were grown in BHI broth at 32ºC for 16-18 hrs (Graph 1a and b). It appears that enhanced biosynthesis of both the peptides takes place in the enriched medium. Earlier, Lisboa *et al.* (2006) also reported that maximum production of bacteriocin by *B. amyloliquefaciens* was achieved in BHI medium at 30ºC and 37ºC. While, Iwalokun *et al*. (2006) reported the high yield of pyocins at 35ºC-37ºC.

Growth cycle

Maximum bacteriocin yield in a culture may be achieved at different growth phases. Graph 2a and b illustrate the growth curves of *B. brevis* Bb and *Ps. aeruginosa* Pa. The production (of both) bacteriocins started during early exponential growth phase and continued till late stationary phase. Maximum production of bacillocin Bb and pyocin Pa was achieved after nine and eleven hours of incubation respectively. Similar findings were reported by Bizani and Brandelli (2002) who reported the production of bacteriocin from *Bacillus* strain 8A started at early exponential phase and continued till stationary



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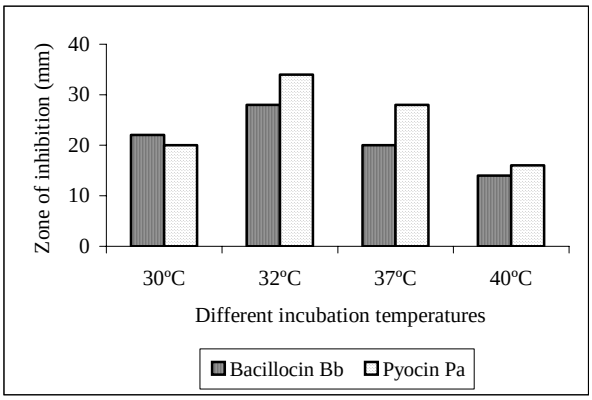

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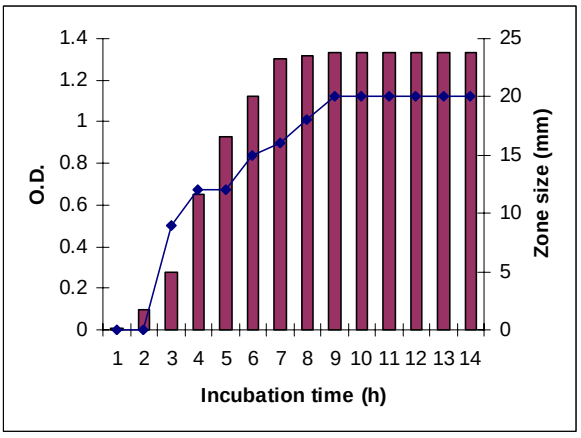
Graph 1a and b: Effect of growth media (BHI; Brain heart infusion, LB; lactose broth, LBB; Luria basal broth, NB; nutrient broth and TSB; trypticase soy broth) on bateriocin production (1a), and incubation temperatures (30ºC, 32º, 37ºC and 40ºC) for 16-18 hrs (1b)



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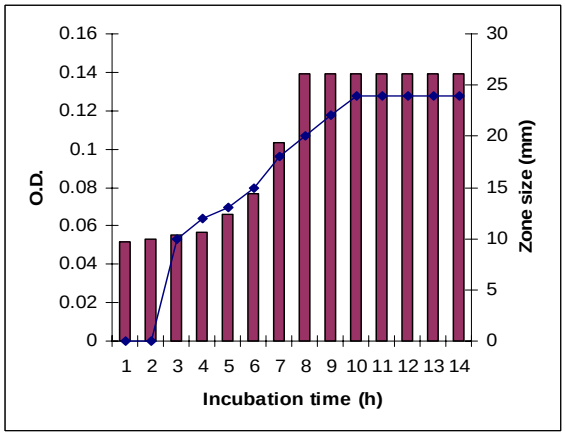

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Graph 2a and b: Growth curve of (a) *B. brevis* Bb and (b) *Ps. aeruginosa* Pa and production of bacillocin Bb (a) and pyocin Pa (b) production. Samples were taken at indicated time intervals O.D. was measured and bacteriocin production measured by zone size (mm) of growth inhibition.

growth phase. However, in a bacteriocin produced by *Bacillus cereus* called cerein (Naclerio *et al.,* 1993) and tochicin produced by *Bacillus thuringiensis* subsp *tochigiensis* (Paik *et al.,* 1997) maximum bacteriocin activity was detected at the early exponential phase with its (bioactivity) decline during the stationary phase.

Physicochemical characterization of bacillocin Bb and pyocin Pa

Ammonium sulphate precipitation (80%) of both the bacteriocin preparations was done in order to concentrate the preparations. Both the precipitated bacteriocin preparations were subjected to different physico-chemical treatments (table 3). Their bioactivity was completely lost after treatment with proteinase K thereby suggesting their proteinaceous nature. Paik *et al.* (1997) also reported the loss of bioactivity of tochicin produced by *B. thuringiensis* after proteinase K treatment. Treatment of bacillocin Bb and pyocin Pa with other enzymes such as trypsin, pepsin and lipase had no detrimental effect on their bioactivity. Resistance to the lipolytic enzyme points out towards the non-complex nature of our bacteriocins, suggesting that no lipid moiety is involved in their activity. Bacteriocin preparations were also subjected to different heat treatments (including the autoclaving for 15 min) and were found thermostable. A bacteriocin produced by *B. licheniformis* 490 could not withhold the autoclaving temperature (Olivera *et al.,* 2004). The bioactivity of bacillocin Bb was pH dependent and was found stable over a wide range of pH (1-9). Pyocin Pa was found stable at pH (1-11) range. However, its activity decreased to some extent at extreme pH 1, 2 and pH 10, 11. Both the bacteriocins (bacillocin Bb and pyocin Pa) retained 80-90% of the bioactivity after treatment with

surfactants and organic solvents. Earlier, Teo and Tan (2005) also reported that bacteriocin from *Bacillus subtilis* was resistant to organic solvents and surfactants. Molecular mass of both the bacteriocins was estimated to be <12KDa (fig. 2). A bacillocin 490 of 2KDa molecular mass was reported by Martirani *et al*. (2002).

Table 3: Effect of different physico-chemical treatments on bacillocin Bb and pyocin Pa

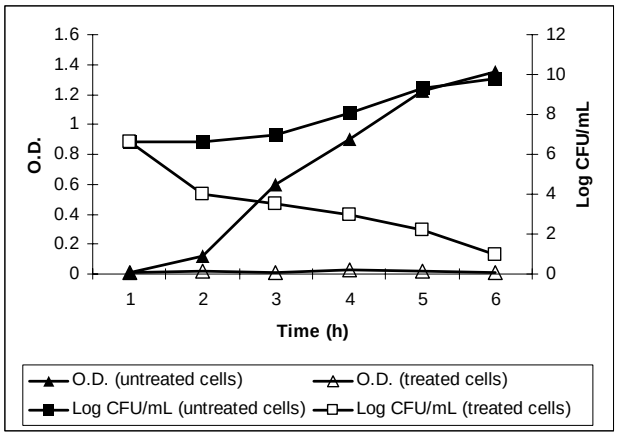
Treatments	Effect of treatment on activity	
	Bacillocin Bb	Pyocin Pa
Control (untreated)	R	R
Enzymes (1mg/ml)		
Protease	R	R
Proteinase K	S	S
Trypsin	R	S
Pepsin	R	S
lipase	R	R
Protease	R	R
Proteinase K	S	S
Trypsin	R	S
Pepsin	R	S
lipase	R	R
Temperatures		
40ºC (30 min)	R	R
60ºC (30 min)	R	R
80ºC (30 min)	R	R
100ºC 30 min)	R	R
121ºC (15psi, 15min)	R	R
4ºC (for 6 months)	R	R
pH		
1-9	R	R
10-11	S	R
Organic solvents (1%)		
Acetone	R	R
Butanol	R	R
Chloroform	R	R
Ethanol	R	R
Methanol	R	R
Propanol	R	R
Surfactants (1%)		
Tween 20	R	R
Tween 80	R	R
SDS	S	S
EDTA	R	R

Key: S= sensitive, no zone of inhibition, R= resistant, zone of inhibition comparable to untreated control.

Mode of action

The mode of action of the two bacteriocins (bacillocin Bb and pyocin Pa) was studied by measuring the viability or loses of the growth of sensitive cells along with the measurement of optical density (OD_{600}) and CFU/mL. The present study has suggested that both the bacteriocins are bacteriolytic for the sensitive cells since the viability as well as the optical density of the sensitive cells (*Bacillus* strain BC31) dramatically decreased during the course of inhibition (Graph 3a and b). Martirani *et al*. (2002) had reported bactericidal effect of bacillocin 490 (produced by *B. Licheniformis*) while, pyocin from *Ps. aeruginosa* also exhibited bactericidal mode of action against the indicator strain (Morse *et al*., 1980; Heo *et al*., 2005).

a



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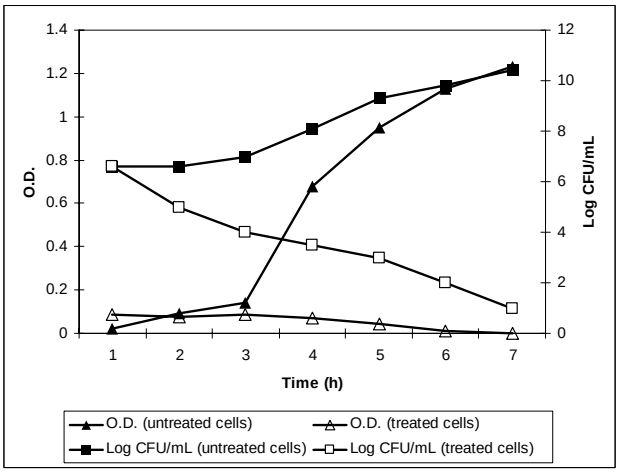

Graph 3a and b: Bacteriolytic effect of (a) bacillocin Bb and (b) pyocin Pa on sensitive cells.

CONCLUSION

We report here the identification and characterization of bacillocin Bb and pyocin Pa (from *Bacillus brevis* Bb and *Pseudomonas aeruginosa* Pa) respectively. On the basis of above-mentioned results, both the bacteriocins showed a potential to be used in medical and food industry. Stability under extreme pH values has an advantage of their application in food as biopreservatives. Furthermore, heat resistance is an advantage since bacteriocins may

remain active in foods after cooking and give protection against undesirable bacteria.

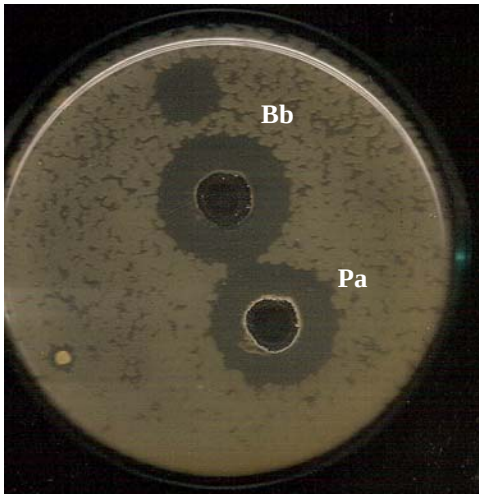

Fig. 1: Agar-well diffusion methods showing bacteriocin activity by bacillocin Bb and pyocin Pa.
Key: Bb = Bacillocin Bb, Pa = Pyocin Pa

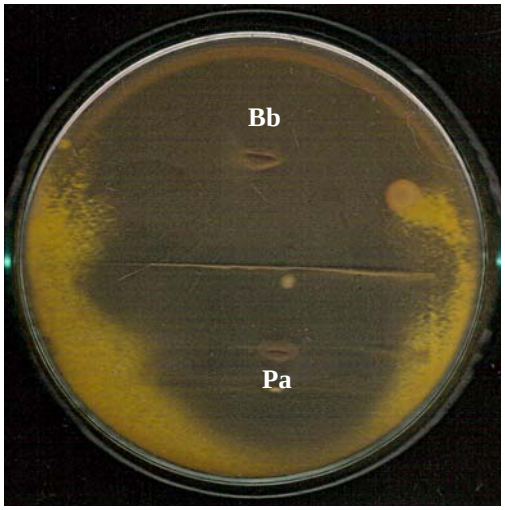

Fig. 2: Crude molecular weight estimation of bacillocin Bb and pyocin Pa by agar-overlay method over the surface of dialysis membrane pore size 12,000 Da. Zone of inhibition showed that both the bacteriocins have molecular weight <12,000 Da.
Key: Bb = Bacillocin Bb, Pa = Pyocin Pa

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