

ISOLATION AND PARTIAL CHARACTERIZATION OF A NEW BACTERIOCIN FROM BACILLUS PUMILUS DR2 ISOLATED FROM SEA WATER

Dasarathan Rajesh, Shivasanmugam Karthikeyan, and *Gurunathan Jayaraman

School of Bio-Sciences and Technology,
Vellore Institute of Technology University, Vellore Tamil Nadu-632014, India
*Author for Correspondence

ABSTRACT

Soil samples collected from sea water (Ennore, India) were screened for bacteria that produce antimicrobial compounds. The isolated microbial strain that exhibited highest antimicrobial property was identified by biochemical and 16S rRNA sequencing as *Bacillus pumilus*. The antimicrobial activity was suppressed in the presence of trypsin, indicating that the antimicrobial substance is proteinaceous in nature. The bacteriocin named Pumiviticin, was found to be halotolerant with a maximal activity of 42.37 IU/ml (Nisin equivalents) in the presence of 1M NaCl (pH 8.0), and was thermostable as it retained one-fourth of its activity even after incubation for 2 hours at 110°C. Pumiviticin (molecular weight 3.9 kDa) inhibit a wide range of microorganism belonging to the lactic acid bacteria such as *Lactobacillus jugurti*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactococcus lactis*, *Listeria monocytogenes*, *Leuconostoc mesenteroides*, *Bacillus subtilis*, *Micrococcus luteus* and also selected gram negative pathogenic bacteria including *Salmonella typhimurium* and *Proteus vulgaris*. The functional and stability characteristics of Pumiviticin produced by this isolate, *B. pumilus* DR2, is distinct from the *B. pumilus* strain reported in literature

Key Words: Alkali, Bacteriocin, Halotolerant, pH, Trypsin.

INTRODUCTION

Bacteriocins are polypeptides that have the ability to inhibit the growth of specific microorganisms. Several molecules of this type have been isolated from microorganisms from different habitats, ranging from fermented food products to soil samples (Jack *et al.*, 1995; Miranda *et al.*, 2008; Ouoba *et al.*, 2007; Rajesh *et al.*, 2009). The exhibited range and the stability of these protein molecules vary depending on the producer organism. Halocins are the bacteriocins produced by marine organism (Messeguer and Rodrigue-Valera, 1985; Messeguer and Rodrigue-Valera, 1986; Platas *et al.*, 2002; Torreblanca *et al.*, 1989). These molecules are of special importance as they are capable of being active even under saline conditions. In general, the mode of action of bacteriocins includes inhibition of transcription/translation, hydrolysis of DNA and RNA, formation of pores and disruption of cellular membranes (Barefoot *et al.*, 1992). Halocin H4 produced by *Haloferax mediterranei* R4 was the first halocin to be characterized and was found to be a single protein of about 28 kDa (Messeguer and Rodrigue-Valera, 1986). Halocin H6, produced by *Haloferax gibbonsii* has been shown to inhibit the Na⁺/H⁺ antiporter (Torreblanca *et al.*, 1989). Thereafter, several genera belonging to halophilic/halotolerant organisms (Das Sarma and Arora, 1997) have been reported to produce bacteriocins.

Organisms such as *B. marinus*, *B. badius*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus* and *B. mycoides* are from marine inhabitants of the Pacific Ocean and has been extensively investigated for the production of different secondary metabolites. Moreover the bacteriocins produced by *Bacillus sp.*, inclusive of *B. pumilus* have been shown (Duc *et al.*, 2004; Martirani *et al.*, 2002; Michael *et al.*, 1983; Pinchuk *et al.*, 2001) to inhibit the growth of a wide variety of gram positive and fewer gram-negative microorganisms. In the present study, we have shown that the antimicrobial compound produced by *B. pumilus* DR2 strain, isolated from the sea water samples of Ennore, South India. (Latitude 13°14'N

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longitude 80°22'E), inhibits a range of gram positive and selected gram negative bacteria. Also, the bacteriocin named pumiviticin, exhibits different characteristics compared to the bacteriocin produced by *B. pumilus* reported in literature (Aunpad and Bangchang, 2007).

MATERIALS AND METHODS

Sample collection and screening

Sea water samples from various spots of Ennore (Tamilnadu, India) were collected. To ensure broader sampling, fifty samples were collected from various locations (depth of one meter and a distance of one kilometre from seashore) using sterile screw cap bottles, and inoculated into nutrient and Luria Berthoni broth and was grown aerobically and anerobically (Oxoid anaerobic Gas-Pak system Hampshire, England), at 37°C for 48 hrs. Individual colonies were isolated by serial dilution method and by plating in respective medium. The purity of the culture was checked by repeated streaking. The pure strains were then screened for the production of antimicrobial compounds.

Bacterial strains and culture media

All indicator strains used in the present study were procured either from IMTECH (Chandigarh, India) or NCIM (Pune, India). These cultures were maintained in recommended medium and stored in 20 % glycerol (v/v) at -20°C. Throughout the experiments, strains were subcultured every 2 weeks on agar plates and kept at 4°C. Before use in experiments, cultures were propagated twice in the respective broth (MRS/ Nutrient) overnight.

Bacterial culture and assay for antimicrobial activity

The production of antimicrobial compounds was monitored by agar well diffusion assay (Tramer and Fowler., 1964). Accordingly, 15 ml of 1.5 % agar (w/v) was used for bottom agar. The top agar (5 ml of 1.0% w/v) was mixed with 200 µl of *L. jugurti* harvested at the early exponential growth phase (OD 600 nm of 0.2 with inoculum strength of 10⁶ log CFU/ml) and was overlaid on bottom agar. The plates were then incubated at 4°C for 3 hours, and wells of 6 mm diameter were cut using gel borer. 50 µl of test supernatant was added to respective wells and the plates were then incubated at 37°C for 24 hr and the zone of inhibition was measured. All experiments were performed with proper controls and the values given represent the average of two experiments. Inhibition values are given in terms of Nisin-equivalents. To eliminate growth inhibition caused by organic acids, the cell-free supernatant was adjusted to pH 6.5 with 1N NaOH and filtered through 0.2µm pore-size polysulphone membrane filters.

Identification and characterization of bacteria

The biochemical assay such as gram reaction, motility, catalase, citrate utilization, gas production, growth at varying temperature and NaCl, hydrolysis of casein, urea, starch, gelatin and arginine was performed. Species level confirmation was performed by analyzing the 16S rRNA sequence analysis (Bangalore Genei, Bangalore, India).

Effect of enzymes

B. pumilus DR2 was grown at 37°C for 24 hr and was centrifuged at 8000 X g for 10 min, (4°C). The pH of the cell-free supernatant (CFS) was adjusted to 6.5 with sterile 1N NaOH. Aliquots of these samples were treated with different hydrolytic enzymes (papain, chymotrypsin, trypsin, pepsin, lipase and amylase) and were incubated for 2 hr at 37°C. Incubation of the CFS with the hydrolytic enzymes (obtained from Sigma Aldrich) was done at their respective optimal pH conditions. The assay conditions used were: chymotrypsin (0.05 M Tris HCl, 0.01 M CaCl₂, pH 8.0); pepsin (0.2 M citrate, pH 6.0); trypsin (0.05 M Tris HCl pH 8.0); lipase (0.05 M Tris HCl, 0.01 M CaCl₂ pH 8.0); papain (0.05 M acetate, 0.2 M NaCl, pH 4.5). After incubation, the pH of the solution was adjusted to pH 6.5 before assaying the antimicrobial activity.

Effect of temperature and pH

The effect of pH and temperature on the activity was tested by adjusting the cell-free supernatants to specific pH in the range 2.0 to 12.0 (at increments of two pH unit) with sterile 1N NaOH or 1N HCl (incubation time 0-150 min; temperature 50-110°C). The samples were tested for antimicrobial activity by

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using the agar well diffusion assay. The inhibition values obtained at different conditions were normalized with respect to the inhibitory activity obtained at zero time periods, for pH 8.0.

Determination of Molecular weight

The CFS (100ml) of *B. pumilus* DR2 was subjected to precipitation using ammonium sulfate (70%). The crude precipitate was then centrifuged for 15 min at 15,000 g (4°C). The resulting pellet was resuspended in 2ml (0.1M Phosphate buffer) and was dialyzed against same buffer using MW CO 500 Da (Spectrum, CA). The apparent molecular mass of the bacteriocin was determined using 15% (w/v) SDS-PAGE. Electrophoresis was carried with vertical electrophoresis unit using 10 x 10 mm² PAGE gel running at 15 mA. The molecular weight of the protein was determined by comparing with low range molecular weight standards (Bangalore Genei, Bangalore). After electrophoresis, the gel was removed and cut in two halves. A part of the gel with the molecular weight marker was stained with silver stain while the other part was assayed for antibacterial activity against *L. jugurti* (Bhunja et al, 1987).

RESULTS AND DISCUSSION

Isolation of bacteria and screening for antimicrobial compounds

Microbial screening was carried out for 55 samples collected from various spots of Ennore sea water. Individual colonies were screened for the production of antimicrobial substances against *L. jugurti*, *L. casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Listeria monocytogenes*. A single potent strain that inhibited the growth of all these organisms was selected for further investigation.

Strain identification and production of antimicrobial substance

The isolated strain was positive for gram reaction, motility, catalase and Voges-proskauer and grew at 2-3% NaCl. It appears as creamy colony morphology, which can grow in the temperature range of 15-55°C, and the pH range of 4.0-9.0. The strain was capable of forming spores and displayed hemolytic activity. These characteristics indicate that the organism is *B. pumilus* (Inanova et al., 1999). In addition, 16S rRNA sequence analysis indicates 99% homology to the previously published *B. pumilus* strain (Gene bank ID AB301019) (Aunpad and Bangchang, 2007).

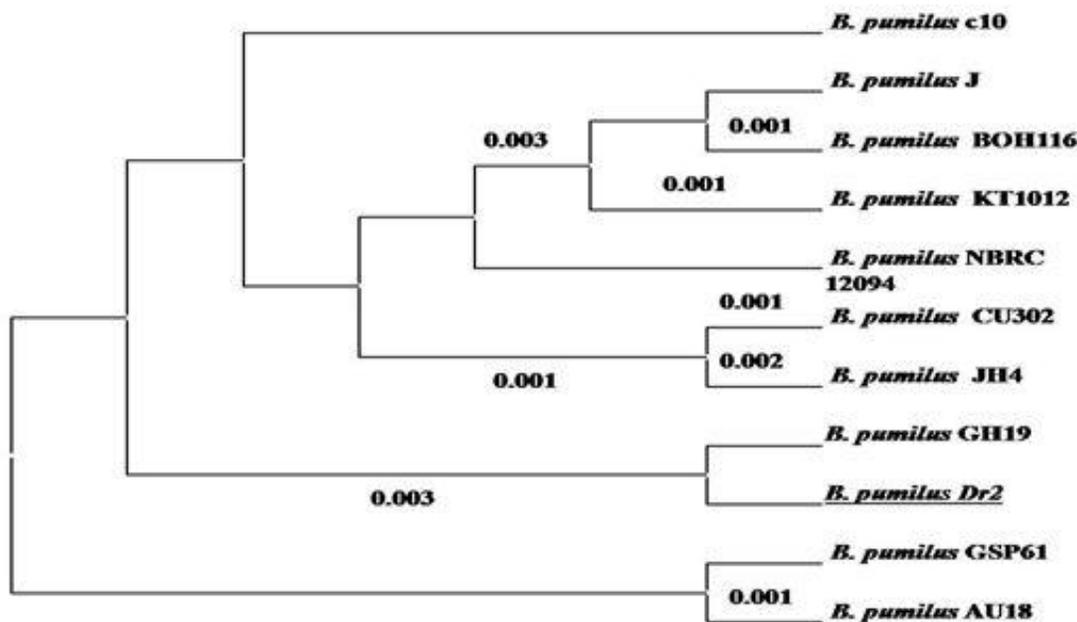


Figure 1: 16S RNA sequence correlations by neighborhood joining method showing the position of the present isolate (dotted underline). As per the sequence identity, the strain described in the present study is *B. pumilus*.

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The phylogenetic tree (Figure 1) constructed by neighborhood joining method indicates that the isolate reported in this study was a strain in *B. pumilus* cluster. The 16S rRNA nucleotide sequence of the present *B. pumilus* DR2 strain has been deposited in GenBank (ID FJ743437). *B. pumilus* DR2 exhibited a doubling time of 30 min in the LB medium and the growth reached the stationary phase at 20 h as indicated by optical density at 600 nm (Figure 2). The production of the antimicrobial substance was also monitored during the growth. Significant antimicrobial activity was observed only after the stationary phase (greater than 20 h of growth) was reached indicating that the antimicrobial compound(s) are the product of secondary metabolism.

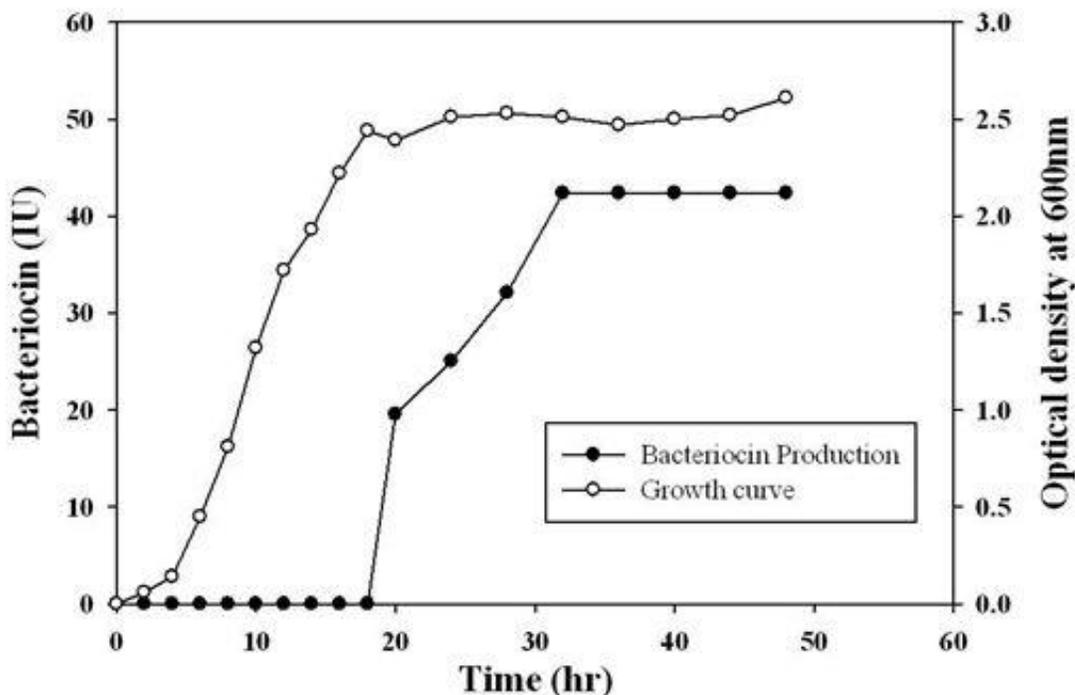


Figure 2: Correlation between cell growth and Pumiviticin production. Production of Pumiviticin starts during the onset of the stationary phase indicating that this is a product of secondary metabolism.

Nature of the antimicrobial compound

In order to find out the nature of the antimicrobial compound the CFS was incubated with different hydrolytic enzymes. When the culture supernatant was incubated with trypsin, the antimicrobial activity was substantially reduced indicating that the bacteriolytic component in the culture supernatant is proteinaceous in nature. Moreover the bacteriocin retained its activity even after incubation with either amylase or lipase, confirming that the non-proteinaceous part, if present, has no effect on the structure and activity (Table 1).

It has been reported that in a few bacteriocins the non-proteinaceous substances, in particular, the carbohydrate is essential for activity (Klaenhammer, 1993). In fact such post translational modified proteins have been indicated to play significant role in host cell recognition events. As the antimicrobial substance is proteinaceous in nature, SDS-PAGE (Figure 3) was performed to determine the molecular weight of the compound. The molecular weight of the bacteriocin, Pumiviticin, was found to be 3.9 kDa which is larger than the size of bacteriocin produced by *B. pumilus* strain reported earlier (Aunpad and Bangchang, 2007).

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Table 1: Effect of hydrolytic enzymes on the bactericidal activity of CFS of *B. pumilus*. *L. jugurti* has been used as the indicator organism.

Hydrolytic enzymes	Inhibition
Pepsin	-
Papain	-
Chymotrypsin	-
Trypsin	+
Amylase	-
Lipase	-

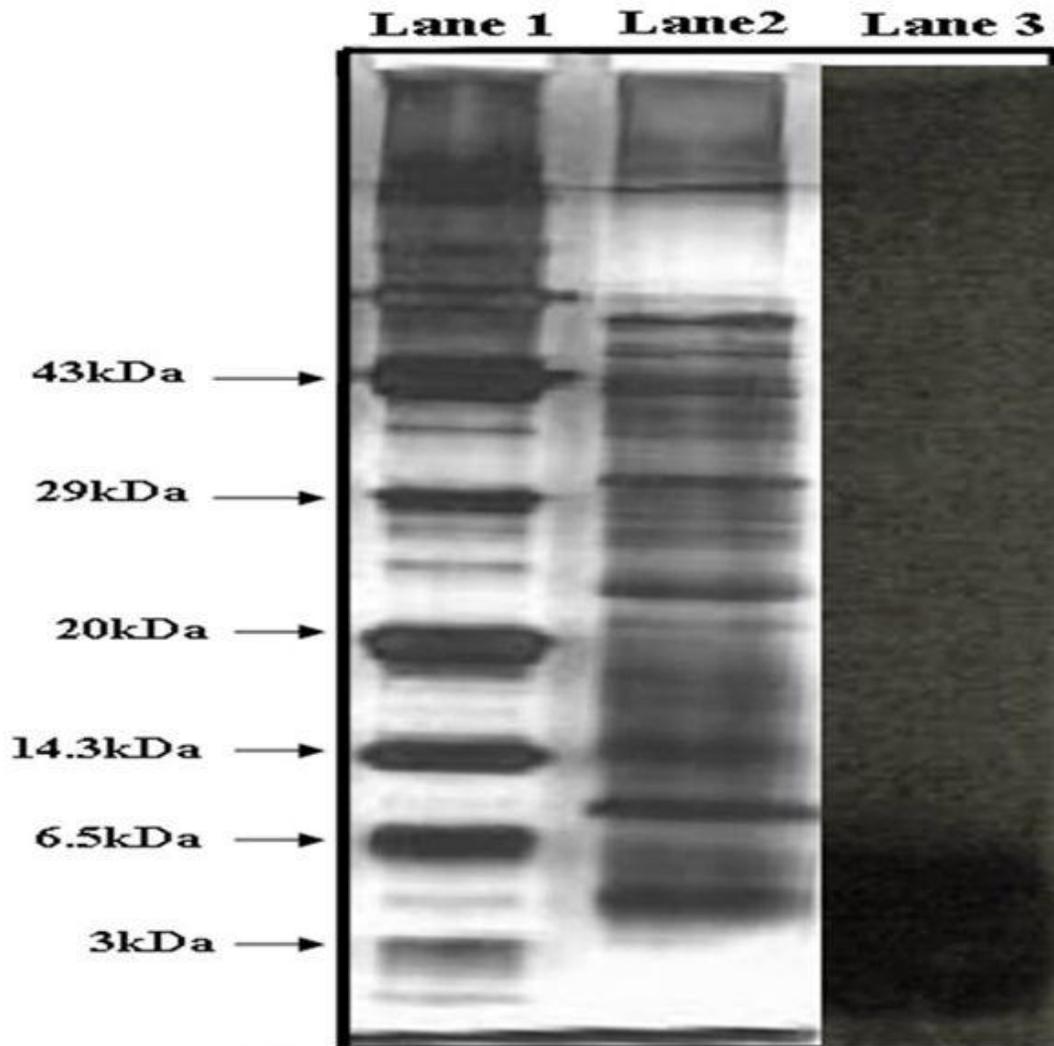


Figure 3: SDS-PAGE of the cell free supernatant. Lane 1: Molecular weight marker (low range) (43kDa-Ovalbumin, 29kDa-Carbonic anhydrase, 20kDa-Trypsin soyabean inhibitor, 14.3- lysozyme, 6.5kDa- Aprotinin, 3.0kDa –Insulin (α and β chains: 2300-3400 daltons), **Lane 2:** sample after dialyzed with 500Da membrane, **Lane 3:** Pumiviticin showing zone of clearance. *The arrow mark indicates the location of the Pumiviticin.*

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Stability of Pumiviticin

The stability of Pumiviticin was investigated after incubating the CFS in different chemical (pH and NaCl), and physical (temperature) conditions. The inhibitory activity was checked over a broad pH range of 2-12 (Figure 4). The bacteriocin was found to be active at pH 6.0, 8.0 and 10.0. No inhibition was observed under highly acidic conditions. Maximal inhibitory activity was observed at pH 8.0. Incidentally, Pumilicin 4 (produced by *B. pumilus*) also exhibited inhibitory activity in the pH range of 6.0-9.0 (no report available for pH 10.0) with a maximal activity at pH 8.0.

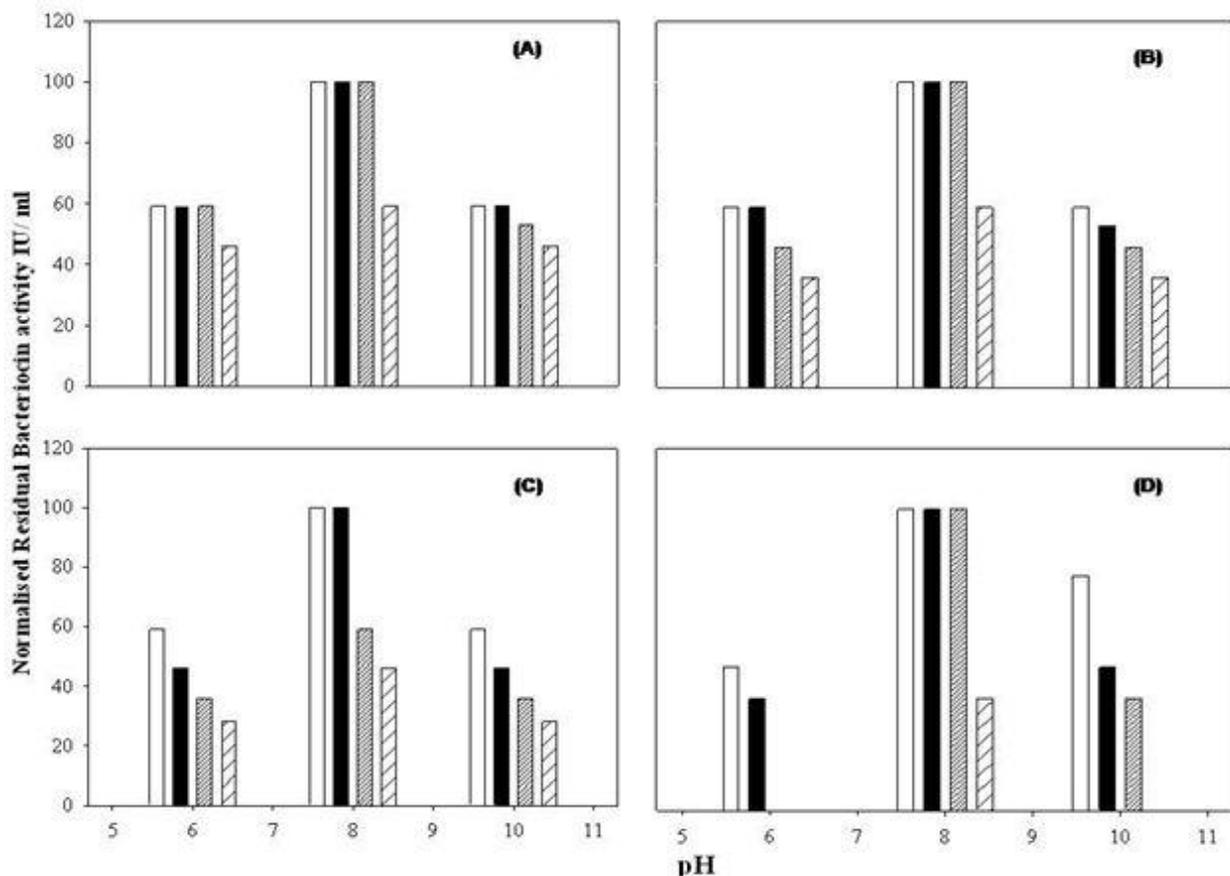


Figure 4: Stability of the bacteriocin as a function of pH and temperature [A] 50°C, [B] 70°C, [C] 90°C and [D] 110°C at different incubation periods. The time period, given for each pH value is in the order of 0 (unfilled), 60 (filled), 90 (fine hatched), and 120 minutes (coarse hatched).

The thermal stability of Pumiviticin was investigated in the temperature range of 50°-110°C (Figure 4). It could be noted that at high temperature 110°C (pH 8.0), Pumiviticin is capable of retaining the initial activity for 90 min and decreases to 37% at 120 min. Residual activity of 90% and 65 % was reported at pH 6.0 and pH 4.0 respectively for Pumilicin 4 (Jack *et al.*, 1995). However, Pumiviticin displayed 60% activity (with respect to pH 8.0) at pH 6.0 and no inhibitory effect for the sample incubated at pH 4.0. As expected, the rate of decrease in the inhibitory activity at a specified pH increases with increase in temperature (data not shown). These results confirm that pumiviticin is highly stable molecule. It should be pointed out that *L. Plantarum*, *Salmonella typhimurium* is not inhibited by pumilicin reported on bacteriocin from *B. pumilus* strains (Aunpad and Bangchang, 2007) and the bacteriocin is inactivated by chymotrypsin. *B. thuringiensis* (Cherif *et al.*, 2003) and *B. licheniformis* (Martirani *et al.*, 2002) exhibited optimal activity at neutral and moderately basic pH conditioned and retained 46% activity after incubation

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at 100°C for an hr. Entomycin 9 (from *B. thuringiensis*) had 72% of the activity upon incubation at 121°C for 20 min (Cherif *et al.*, 2003).

Effect of salt

Salt tolerance of the *B. pumilus* was studied in terms of organism growth (measured at OD 600 nm) and bacteriocin production (as a measure of activity) in the salt concentration range of 0-2 M (Figure 5). There was a steady increase in biomass production as observed by the optical density at 600 nm up to 1.0 M NaCl. Bacterial growth in the presence of 1.0 M NaCl is about 6 times greater than in the absence of salt (Figure 5). *B. pumilus* DR2 strain isolated in the present study is also capable of growing under high saline conditions reflecting its halotolerant behavior. In addition, bacteriocin production is not substantially reduced at lower salt concentrations (0-1 M). At higher salt concentration, the bacteriocin activity is proportional to the biomass production. This indicates that the bacteriocin produced is capable of withstanding higher salt concentrations. Halocin H1 from *Haloferax mediterranei* M2a has been shown to require 1.5 M of NaCl concentration to maintain its activity and therefore has an advantage to be used as a preservative in the packed foods (Platas *et al.*, 2002). Similar characteristics have been reported for other halocins (Messeguer and Rodrigue-Valera, 1986; Torreblanca *et al.*, 1989) produced by different halophilic organisms isolated from different sources.

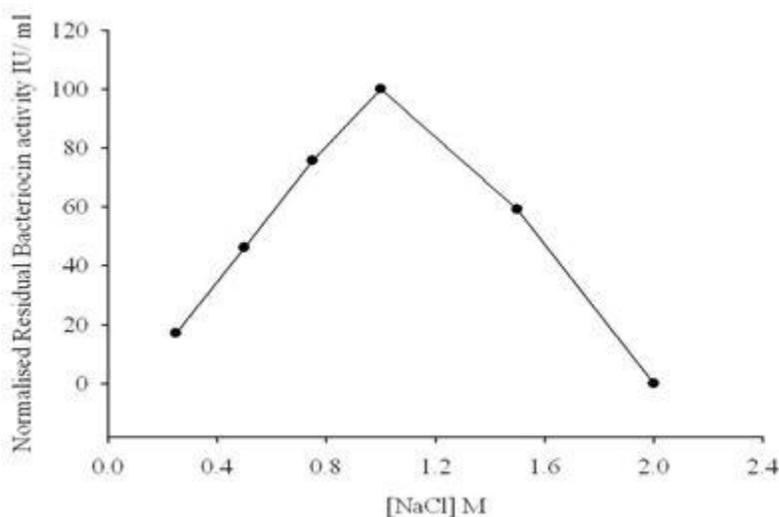


Figure 5: Relation between cell growth (-■-) and Pumiviticin (-●-) production in the presence of different concentrations of NaCl.

Inhibitory activity against pathogens

Inhibitory effects of the bacteriocins in the culture supernatant was investigated for selected gram positive and gram negative strains using the agar well diffusion assay. Based on the zone of clearance the inhibitory activity was classified as weak (< 2 mm), moderate (2-6 mm) and strong (> 6 mm). The bacteriocin produced by *B. pumilus* DR2 was found to inhibit selected gram negative strains used. The bacteriocin was able to inhibit the activity of *Salmonella typhimurium* (2 mm) and *Proteus vulgaris* (4 mm) but not *E. coli* (Table 2). Growth of *Salmonella typhimurium* that causes gastroenteritis (salmonellosis) is also inhibited, through weakly, by pumiviticin. Bacteriocin capable of inhibiting the growth of gram negative strains is very obscure. Pumulicin 4 reported recently (Aunpad and Bangchang, 2007) exhibited bacteriocidal effects towards a wide range of gram- positive strains but did not inhibit the gram negative strains used in the study. The results of this study indicate that this strain could be further developed to produce bacteriocins capable of inhibiting the growth of these gram negative strains more effectively. Pumiviticin also inhibited the growth of a wide range of gram positive species, with very strong inhibition of *Micrococcus luteus*. *M. luteus*, an obligate aerobe, is a normal flora of the mammalian

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Table 2: Bactericidal activity of CFS observed with various indicator organisms.

Indicator organisms	Inhibition
Gram positive	
<i>Lactobacillus jugurti</i> (2366)*	++
<i>Lactobacillus casei</i> (2737) \$	++
<i>Lactobacillus acidophilus</i> (2285)*	++
<i>Lactobacillus helveticus</i> (2126)*	++
<i>Lactobacillus fermentum</i> (2165)*	++
<i>Lactobacillus brevis</i> (2090)*	++
<i>Lactobacillus plantarum</i> (2085)*	++
<i>Lactobacillus bulgaricus</i> (2056) \$	++
<i>Lactococcus lactis</i> (2370) \$	++
<i>Listeria monocytogenes</i> (2091)*	++
<i>Leuconostoc mesenteroides</i> (2073) \$	++
<i>Staphylococcus aureus</i> (2891)\$	-
<i>Bacillus subtilis</i> (2189) \$	++
<i>Micrococcus luteus</i> (2871) \$	+++
Gram negative	
<i>Escherichia coli</i> (2804)\$	-
<i>Salmonella typhimurium</i> (2501)\$	+
<i>Proteus vulgaris</i> (2813) \$	++

*- Institute of microbial culture collection center, Chandigarh, India

\$-National culture collection for Industrial microorganism, Pune, India

Results (-), not inhibited; (+), (++) , (+++) were <2, 2-6, >6mm inhibition respectively.

strain and colonizes the human mouth, mucosae, oropharynx and upper respiratory tract. This organism is also being considered as nosocomial pathogen in immunocompromised patients. Thus the functional analysis also indicates that the Pumiviticin is different from Pumilicin. One of the strains of *B. pumilus* strain has been used as a commercial probiotic, Biosubtyl NT (Green *et al.*, 1999) and this opens up the possibility of using this novel bacterial strain *B. pumilus* DR2 as a probiotic in addition to the application in agro based industries and in pharmaceuticals industries.

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