INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-4, Oct-Dec-2012 Coden : IJABPT Copyrights@2012

ISSN : 0976-4550

Received: 28th August-2012 *Revised: 31st August -2012* *Accepted: 02nd September-2012*

Research article

MOLECULAR PROFILING AND ANTIMICROBIAL ACTIVITY OF BACTERIOCIN FROM *BACILLUS SUBTILIS*

Berlina Dhas S* and J.Vimalin Hena**

*Research Scholar, ** Assistant Professor*

*9486392127 berlinadhas@gmail.com, **9486461871, post4vimalin@yahoo.com.

PG and Research Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore-28

ABSTRACT: Development of multi drug resistant organism has been high due to improper use of antibiotics. That made the necessity to develop new drug molecules. In this study an effort was made to find a new alternative. A wild type microorganism was isolated from soil and was identified as *Bacillus* and confirmed as *Bacillus subtilis* species by 16S r RNA sequencing. The strain was identified to have the ability to produce bacteriocin by stab overlay assay. Bacteriocin was produced in nutrient broth and that was extracted by organic solvent extraction using chloroform and further purification was carried out by HPLC and the molecular weight of the bacteriocin was analysed by SDS-PAGE. Antimicrobial activity was analysed on four strains *Pseudomonas* sp, *Staphylococcus* sp, *Klebsiella* sp and *Proteus* sp. and was found to be sensitive towards the analyzed strains.

Key words: Bacteriocin, Antimicrobial activity, *Bacillus subtilis*, Indicator organisms, HPLC: High Pressure Liquid Chromatography.

INTRODUCTION

The broad use of antimicrobials in medicine, animal husbandry and agriculture is developing several antibiotic resistant organisms. This makes the necessity to develope novel antibiotics and alternative therapeutic strategies (McGahee W and Lowy FD 2000). Ribosomally synthesized antimicrobial polypeptides, bacteriocins are usually inhibitory only to strains closely related to the producing bacteria. Bacteriocin from Gram positive bacteria are often membrane-permeabilizing cationic peptides with fewer than 60 amino acid residues (Jack, et. al., 1995 and Klaenhammer, et. al., 1993). Cinical antimicrobial use of RAMPs is because, they are active against antibiotic resistant isolates, have limited natural bacterial resistance, are proven to kill bacteria in animal model etc (Pag, et. al., 2004). Bacteriocins are of different groups, small peptides that undergo extensive post-translational modification to produce the active peptides example nisin and are grouped as Class I bacteriocins (lantibiotics). Heat stable, low molecular weight, membrane active peptides are grouped as Class II bacteriocin members of class III are large heat labile proteins, and a fourth class (complex bacteriocin) has also been suggested, requiring nonprotein moieties for activity. The genus *Bacillus* encompasses a number of bacteriocinogenic species, such as *B. subtilis* which produces subtilin and subtilosin, *B.coagulans* which produces coagulin and *B.megatirium* which produces megacin. *B. thuringiensis* is widely used in agriculture for the control of many insect pathogens (Mobolaji and Okulate, 2009). Because of the safety use in humans, probiotic strain *Bacillus subtilis* is of great interest (Irina and Philippe, 2001). It is found that subtilin and subtilosin from *B. subtilis* are active against many strains of Gram positive bacteria and these are the only bacteriocins of *Bacillus* sp to be characterized at the amino acid and DNA sequence level (Claire le Marrec and Bertrand, 2000). The present study was designed to isolate and identify *B. subtilis* from soil to characterize and partially purify the bacteriocin and to analyze the antimicrobial activity.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

MATERIALS AND METHODS

Isolation of bacterial strain

Approximately 1 gram of soil sample collected was placed in 5 ml distilled water and vortexed vigorously to dissolve the particles. The soil sample was boiled for 10 minutes in water bath and allowed to cool. 10- Fold, 100-fold and 1000- fold dilution of the re-suspended soil sample were made and 0.1 ml from each sample, including the undiluted solution was plated on Nutrient agar plates. The plates were incubated overnight at 37°C (Mobolaji and Okulate, 2009). After incubation the colonies were identified by colony morphology and biochemical characterization and also compared with MTCC strain no. 144.

Identification and confirmation of bacterial strain

Further identification was done by 16S rRNA sequencing. PCR was used to amplify the 16S ribosomal DNA was determined by direct sequencing. Total DNA was isolated by using SoluteReady® Genomic DNA purification kit. Agarose gel (2%) were used to isolate 16SrRNA. 16SrRNA was separated by gel electrophoresis on gel made with 2% acrylamide; bis-acrylamide. The confirmed strain was then screened for bacteriocin production.

Detection of the strain producing bacteriocin

Stab overlay assay (Paik, et. al., 2000)

The 24 hrs culture of *Bacillus subtilis* was stabbed on Nutrient agar plates and incubated at 37°C for 24 hrs. After incubation the colonies on the stabbed area was scrapped and the plate was exposed to chloroform vapors for 15 minutes. Then soft agar was prepared and 24 hrs old indicator organism (*Micrococcus leuteus*) was added and vortexed and was poured on stabbed plate and incubated for 24 hrs at 37°C. After incubation plates were examined for zone of inhibition around the stabbed area (Irina V and Philippe B, 2001).

Bacteriocin extraction and purification

Nutrient broth was seeded with *Bacillus subtilis* and incubated at 37°C and 150 rpm for 24 hours. The sample was centrifuged at 10,000 rpm for 20 min and the supernatant was collected. The culture supernatant (100 ml) was stirred vigorously with chloroform (100ml) and transferred in a separating funnel, the interface layer between the aqueous and organic phase, which contain bacteriocin was harvested, and the residual chloroform was eliminated by speed vacuum (Abdelkader M and Nour Eddine C, 2009).

Purification by HPLC

The purification of the bacteriocin was carried out by using HPLC in South Indian Textile Research Association (SITRA) Coimbatore. This analytical technique has been shown to be extremely valuable for the analysis of these peptide antibiotics, since peptide antibiotics are generally resistant to different organic solvents used as mobile phase and the high pressure employed through the chromatographic process. Here the chromatographic process was carried with C_{18} column. The mobile phase was water/trifluroacetic acid as eluent A and acetonitril/ trifluroacetic acid as eluent B. The flow rate was 1 ml/min the peaks were measured by UV- photo diode array detector.

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) for 30 min. the residual bacteriocin activity was determined by agar well diffusion method.

Effect of pH on bacteriocin were determined by adjusting the pH of the partially purified bacteriocin with dilute HCL and NaOH as 3, 4, 9 and 10 and incubated for 2 hrs at 37°C and all samples were then readjusted to neutral pH and then tested for antimicrobial activity. For enzyme stability, partially purified bacteriocin was treated for 2 hrs with enzyme lysozyme at a final concentration of 1 mg/ml and bacteriocin activity was analyzed by agar well diffusion method against indicator organism. In all the three cases untreated partially purified bacteriocin served as control (Atta, et. al., 2009).

Molecular weight determination by SDS-PAGE

The molecular weight of the isolated bacteriocin was identified by performing SDS-PAGE using 12% gel. Following electrophoresis which was conducted at 50 V for 40 min, the gel was stained with coomassic brilliant blue.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

Antimicrobial activity of partially purified bacteriocin

Inhibitory activity of partially purified bacteriocin was analyzed by agar well diffusion method for *Pseudomonas* sp, *Staphylococcus* sp, *Klebsiella* sp and *Proteus* sp.

RESULTS AND DISCUSSION

Isolation of bacterial strain:

On dilution of the soil sample the results obtained are tabulated (Table. 1).

Confirmation of *Bacillus subtilis*:

Bacillus subtilis was isolated from soil and the strain was identified by morphological biochemical and species level identification which was done by 16S r RNA sequencing. The sequence length was found to be 36, GCCCAACTAAATGATGGCAACTAAAATCAAGGGT. The phylogenetic tree analysis results showed that the isolate was 99% related to *Bacillus subtilis* BSF01.

Screening of the isolate for bacteriocin production

The ability of the isolate for bacteriocin production was analyzed by stab overlay assay (Paik, et. al., 1997) using *Micrococcus leuteus* as an indicator organism. It exhibited antibacterial activity producing a clear zone of inhibition around the indicator strain. *Bacillus subtilis* was outstanding in the genus *Bacillus* with regards to its potential to produce so many different antibiotics (Bushra J and Fariha H, 2007). (Fig. 2).

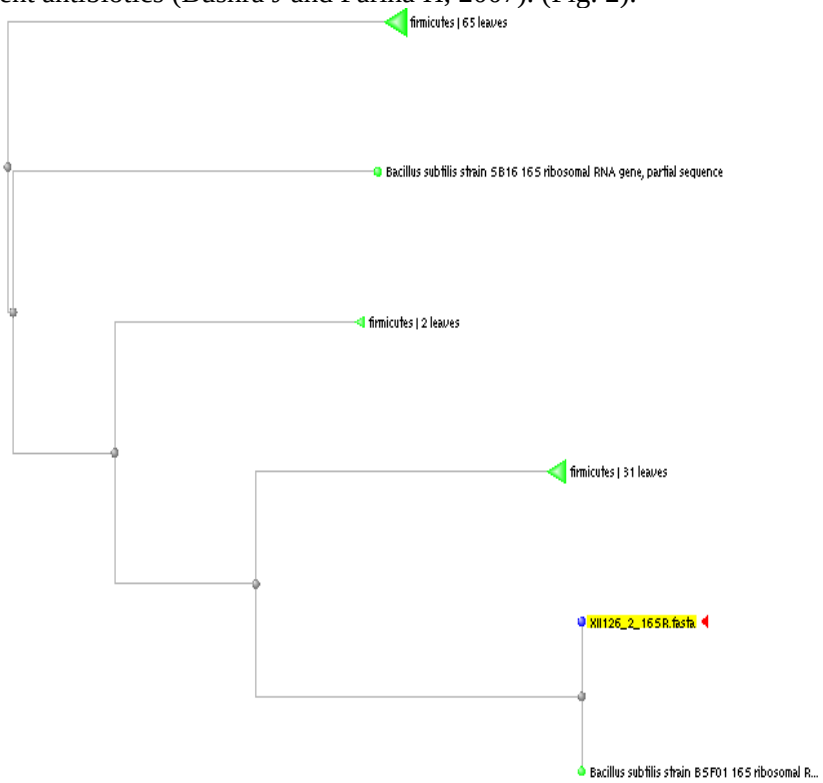

Fig. I Phylogentic Tree of *Bacillus subtilis*

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

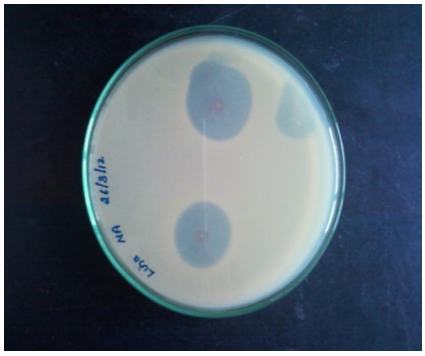

Fig. 2 Stab overlay Test of *B. subtilis*

Bacteriocin production and purification

Bacteriocin production was carried out in nutrient broth at 37°C and incubated for 24 hours. The sample was centrifuged and the supernatant was collected and mixed with chloroform for the extraction of bacteriocin in an separating funnel and mixed well for 30 min. the intermediate layer was collected and further purification was done by HPLC (Abdelkader M 2009). The peaks revealed that the extracts are bacteriocin. The peaks obtained had a height of 139619 and 19797 with Rf values of 2.082 and 112.486 respectively.

Table I. No: of colonies obtained on 1gram of soil

Dilutions	No: of colonies, CFU/ml
10-fold	TNTC
100-fold	269
1000-fold	179

Table 2. Effect of temperature, pH and enzymes on bacteriocin activity

Treatment	Activity
Temperature	
40°C	+
60°C	+
80°C	+
100°C	+
pH	
3.0	+
4.0	+
9.0	+
10.0	+
Enzymes	
Lysozyme	+

Table 3. Inhibitory activity of partially purified bacteriocin

Organisms	Zone size in mm
Pseudomonas sp	17
Staphylococcus sp	13
Klebsiella sp	21
Proteus sp	11

Effect of various temperature, pH and enzymes

The effect of various temperatures on partially purified bacteriocin was investigated. And the results are shown in Table II, bacteriocin activity was not affected in all temperature ranging from 40°C- 100°C. pH stability was observed in both acidic and basic pH were high level of activity was seen in acidic pH. On treatment of partially purified bacteriocin with lysozyme it showed reduced level of bacteriocin activity.

Molecular weight determination

The molecular weight of the isolated bacteriocin was analyzed by 12% SDS-PAGE with and protein marker. The band revealed the molecular weight of the isolated bacteriocin got on comparison with the standard protein marker to be 6.4 kDa.

The findings of the present study lead us to conclude that the bacteriocin producing Bacillus strains could easily be isolated from soil. The bacteriocin activity of the isolate has been made clear with stab overlay assay. Production of bacteriocin was done in nutrient broth and extraction of bacteriocin was well achieved by using chloroform as the organic solvent. Since bacteriocin and the strain *Bacillus subtilis* are found to be safe for use these antimicrobial peptides can be used as an antimicrobial in both humans and animals.

Antimicrobial activity of partially purified bacteriocin

To determine the antimicrobial activity of partially purified bacteriocin agar well diffusion method was adopted and the results reveled activity to all the four organisms and high level of activity was seen in case of *Klebsiella* sp (Table 3).

CONCLUSION

Unique combination of various properties of the partially purified bacteriocin from *Bacillus subtilis* like high thermo stability, wider pH tolerance proteinaceous nature and its antimicrobial activity against pathogenic organisms makes it as an attractive drug molecule.

ACKNOWLEDGMENT

We thank Synergy Scientific Services Chennai and South Indian Textile Research Association (SITRA) Coimbatore.

REFERENCES

Abdelkader Mezaini, Nour-Eddine Chihib, Abdelkader Dilmi Bouras, Naima Nedjar- Arroume and Jean Pierre Hornez (2009). Antibacterial activity of some lactic acid bacteria isolated from an Algerian dairy product. Journal of environmental and public health.

Bushra Jamil, Fariha Hasan, Hameed A,and Safia Ahmed (2007). Isolation of Bacillus subtilis MH-4 from soil and its potential of polypeptide antibiotic production. Pak, J. Pharm. Sci 20: 26-31.

C. Klein, C. Kaletta, N. Schnell and K. Entian (1992). Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbial 58:132-142.

Claire Le Marrec, Bertrand Hyronimus, Philippe Bressollier, Bernard Verneuil and Maria C. Urdaci (2012) Biochemical and genetic characterization of coagulin, a new antillisterial bacteriocin in the pediocin family of bacteriocins, produced by Bacillus coagulans. Applied and environmental microbiology 66: 5213-5220.

H.D. Paik, N.K. Lee, K.H. Lee (2000). Identification and partial characterization of cerein BS229, a bacterioocin produced by Bacillus cereus BS229. Journal of Microbiology and Biotechnology 10, 195-200.

H.D. Paik, S.S. Bae, S.H. Park, J.G. Pan (1997). Identification and partial characterization of tochicin, a bacteriocin produced by Bacillus thuringiensis subsp tochigiensis J. Ind. Microb. B. 19: 294-298.

H.M. Atta, B.M. Refaat and A.A. El-Waseif (2009). Application of Biotechnology for production, purification and characterization of peptide antibiotic produced by probiotic Lactobacillus plantarum, NRRL B-227. Global journal of Biotecnology and Biochemistry 4(2): 115-125.

Coden : IJABPT Copyrights@2012 ISSN : 0976-4550

I.G. Osipova, I.B. Sorokulova, N.V. Tereshkina and L.V. Grigor(1998). Safety of bacteria of the genus Bacillus, dorming the base of some probiotics. Microbiol.Epidemiol.immunobiol 6:68-70.

M. Brain, E. Mark and Mary Ann Jabra (2010). Antimicrobial Peptides: primeval molecules or future drug. PLoS Pathog 6 (10).

Mobolaji A, Okulate (2009). Antimicrobial activity of bioactive compounds produced by Bacillus species. Microbial diversity course.

R.W. Jack, J.R. Tagg and B. Ray (1995). Bacteriocin of Gram positive bacteria. Microbial. Rev. 59:171-200

T.R. Klaenhammer (1993). Genetics of bacteriocin by lactic acid bacteria. Microbial. Rev 12:39-86.

U. Pag (2004). In vitro activity and mode of action of diastereometric antimicrobial peptides against bacterial clinical isolates. JAC 53:230-239.

V. Irina, Pinchuk, B. Philippe, V. Bernard, F. Bernaard, Francis Megaraud and C. Maria (2001). In vitro anti-helicobacter pylori activity of the probiotic strain Bacillus subtilis 3 is due to secretion of antibiotics. Antimicrobial agents and chemotherapy 45: 3156-3161.

W. McGahee, F.D. Lowy (2000). Staphylococcal infection in the intensive care unit. Semin respire infect15: 308-313.