The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 1107-1113
ISSN: 1018-7081

ISOLATION OPTIMIZATION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDE PRODUCING BACTERIA FROM SOIL

N. Muhammad[1], A. Akbar[2], A. Shah[1], G. Abbas[3], M. Hussain[1] and T. A. Khan[1]

[1]Department of Microbiology, Kohat University of Science and Technology Kohat, Pakistan
[2]Department of Microbiology, Faculty of Life Science, University of Balochistan Quetta, 87300, Pakistan
[3]Department of Chemical Engineering, University of Gujrat, Gujrat, Pakistan
Corresponding Author: Niaz Muhammad (mniaz@kust.edu.pk)

ABSTRACT

In this study, soil bacteria were isolated and characterized including its antimicrobial peptide. For this, soil samples were collected from agriculture lands. Soil samples were diluted and cultured in nutrient agar plates to obtain the isolated bacterial colonies. Antimicrobial peptide producers were screened by stab overlay, agar well diffusion, cross streaking and lacuna assay methods. The isolated strains were identified through morphological, cultural and biochemical properties. Isolates with significant antimicrobial peptide producing potential, which inhibited the growth of sensitive strains in all applied assays have been identified as *pseudomonas luteola* with 98 % similarity index by API kit. Maximum antimicrobial activity of the isolated strain was observed at pH 7, 18 h incubation at 35 ˚C in brain heart infusion agar (BHI). Under optimized growth conditions, inhibitory zone was 34-36 mm, almost 20 folds higher than the initial and further increased after particle purification with 60 % ammonium sulfate precipitation. Estimated molecular size of the peptide produced by the isolates was less than 20 kda. These antimicrobial peptides lost antibacterial activity after treating with protinease K. Antimicrobial peptide obtained from producer strain was active against *Shigella*, *Salmonella*, *Staphylococcus aureus, E. coli*. Furthermore, this study suggests that clinically and industrially important peptide antibacterial compounds can be obtained from unexplored ecological niches.

Keywords: Antimicrobial peptides, Optimization, Clinical isolates, Soil bacteria

INTRODUCTION

Soil rhizosphere contains several habitats with functional microbial communities, where some microbial communities defend themselves from others by producing antimicrobial metabolites. Most of these antimicrobial metabolites are proteins in nature and have been designated as antimicrobial peptides. These antimicrobial peptides are ribosomally synthesized in bacterial cells and possess activity against other bacteria, either of the same species or across genera (Bowdish *et al*. 2005; Cotter *et al*. 2005). These antimicrobial compounds produced by bacteria are found in all major bacterial lineages (Riley and Wertz, 2002) and produced by both Gram negative and Gram positive bacteria (Savadogo *et al*. 2006). These are heterogeneous compounds with diverse biochemical and molecular properties, molecular weight, activity spectrum and mode of action (Klaenahmmer, 1998).

These antimicrobial peptides are gaining more and more attention, not only as an alternative therapeutic agent for clinical applications but also as preservatives in food industries to prevent the deterioration and spoilage of food (Akbar and Anal, 2014; Anthony *et al*., 2009). The antimicrobial peptides attracted the attention of researchers due to their broader antimicrobial spectrum, generally against their closely related bacteria. The significant progress in antimicrobial metabolite research was made by investigating the colicins. Those prototype peptidyl metabolites are produced by most species of the *Enterobacteriaceae* family and this has resulted in considerable in-depth knowledge on the genetic background, structure, synthesis and mode of action of these molecules (Messaoudi *et al*., 2013; Settanni and Corsetti, 2008).

The upcoming challenge in therapeutic agents is the resistance of pathogenic bacteria, which is increasing with time due to over-prescription of antibiotics and its inadequate use (Akbar and Anal, 2013; Talpur *et al*., 2012). This concern is a challenge for scientists to explore new antimicrobial agents from natural sources including bacterial strains with low virulence and broad antimicrobial activity against clinically significant microorganisms (Ananou *et al*., 2010). With this background, current study deals with the isolation, identification and characterization of antimicrobial peptide produced from soil and optimization of conditions for increased production of antimicrobial peptides.

MATERIALS AND METHODS

Isolation of bacterial strains from soil: Soil samples were collected from irrigated soil around Kohat

University of Science and Technology (KUST) Kohat, Khyber Pukhtoon Khwa Pakistan. Samples were immediately transported to lab in sterile bags and stored at 4 ˚C before further processing. Soil samples were serially diluted (10 fold) in sterilized distilled water. An amount (100 µl) of each dilution was spread homogeneously with the help of sterilized glass spreader on Nutrient agar (Oxide, UK, Ltd) plates and incubated overnight at 37 ºC. Bacterial colonies were isolated by sub-culturing on Nutrient agar and Brain heart infusion (BHI) agar (Oxide). Fresh culture of bacterial colonies was preserved in 20 % glycerol and stored at -20 ºC for further experimental use accordingly (Yousef *et al*., 1991).

Identification of soil isolates: Different morphological and biochemical identification tests were performed for preliminary identification of bacterial isolates, which include Gram reaction, microscopy and biochemical test according to the Bergey`s manual of determinative bacteriology (Holt *et al*., 1994). The bacterial isolates identification was confirmed with the help of analytical profile index (API) 20E kit (BioMerieux, Germany), according to user's instructions manual and results were recorded after 24 and 72 h of incubation at 37 ºC. These results were used to generate numeric code for identification purpose. Data were submitted to API-20E web using API web software (Apiweb identification software) and results were interpreted.

Screening of antimicrobial peptide producers: The antibacterial activity of soil bacterial strains was determined by Lucana count Stab-overlay, Cross-streak and Agar-well diffusion assays. Lacuna assay technique was used following the method by Hardy (1987) to check the antimicrobial peptide production frequency of producer cells from the population of representative producer isolate. Fresh culture (1.0 ml) of producer soil isolate was serially diluted (tenfold). Then, 0.1 ml of each dilution was spread in sterilized Petri plate, poured over with soft melted nutrient agar and incubated at 37 ºC for overnight. Bacterial colonies have been replicated to check the most potent antimicrobial peptide producers. The bacterial growth in control plates was inactivated by inverting the plate for 20-30 min on filter paper socked with chloroform (Merck, Germany) Bacterial growth inactivated plate was overlaid with 3-5 ml of soft agar containing 100 µl of sensitive strain and incubated at 37 ºC for overnight. After incubation, the lacuna - inhibition zone was measured. Bacterial colonies with larger inhibitory zone were obtained from replica plates for further use.

Nutrient agar plates were stabbed with soil bacterial isolates obtained from Lucana assay and incubated overnight at 37 ºC. After incubation plates were exposed to chloroform (Merck) vapors to inactivate the bacterial cells. In brief, bacterial cell containing plates were inverted on larger plates containing filter paper soaked with chloroform and kept closed for 20-30 min. After inactivation of bacterial growth, cells were removed and plates overlaid with 3 ml Nutrient soft agar containing 1 % of standardized inoculums of target bacteria strains. Plates were again incubated at 37 ºC for overnight and inhibitory zone was observed around producer strains accordingly (Akbar and Anal 2014). For further identification, Producer isolates were streaked across the surface of nutrient agar plates, incubated at 37 ºC for overnight and exposed to the chloroform to inhibit the growth of streaked bacterial cells. Dead cellular mass was scraped-off from the plate surface. The selected sensitive strain was cross streaked and incubated at 37 ºC for overnight. At crossing point, growth inhibition of sensitive strain was checked.

Nutrient agar plates were overlaid with 5 ml nutrient soft agar having 100 µl of target strain culture (fresh). Wells of 5 mm diameter were bored in agar plates and equal amount of cell free supernatant (CFS) of producer strain was placed in each well. Plates were incubated at 37 ºC for overnight and inhibitory zone was measured in mm accordingly (Akbar and Anal, 2014).

Target strain inoculums preparation: Tenfold dilution of overnight incubated culture of target strain was prepared in sterilized distilled water and the pH was neutralized by sodium phosphate buffer. Absorbance of each dilution was made at 600 nm (spectrophotometer) against blank buffer and viable cell number was determined. Ten micro litter of each dilution was inoculated in pre-poured nutrient agar plates. Plates were allowed to dry and incubated at 37 ºC for overnight. Colony forming unite per ml (CFU) of each dilution was calculated and plotted in graph against absorbance to standardize the inoculums size. Different volumes of overnight grown indicator cells were seeded in 5.0 ml of soft agar and spread over pre-poured nutrient agar plates. Wells of same diameter were bored and equal volumes of producer' s supernatant from BHI broth were poured in each well and incubated for 24 h at 37 ºC. After incubation inhibitory zones was measured in mm diameter accordingly (Iqbal *et al*., 1999).

Optimization of conditions for antimicrobial peptide production: Conditions such as temperature, nitrogen source, incubation time and pH were optimized for the maximum production of peptide antibiotic. For temperature optimization, conical flasks containing BHI broth with 1 % inoculums culture were maintained at different temperatures (30, 35 and 40 ºC) for overnight. Antibacterial activity was measured by agar well diffusion assay. Energy sources were optimized for maximum production of peptide antibiotic by culturing producer strain in different media (Luria broth, BHI broth and Nutrient broth). All cultures were incubated at 37 °C

and CFS was checked for antibacterial activity against target strain by agar well diffusion assay.

Impact of incubation time for maximum production of antimicrobial peptide was optimized by 1 % culture of producer strain grown in conical flasks containing 50 ml BHI broth maintained at 37 ºC. Aliquots were collected at desired time intervals (each 2 h for 24 h). The cell free supernatant was obtained by centrifugation at 4000 g. Antimicrobial activity of CFS obtained from each aliquot was checked against indicator strain by agar well diffusion assay. To check the effect of pH on production of antimicrobial peptide, conical flasks were maintained at different pH values (4, 5, 6, 7, 8 and 9) containing 50 ml BHI broth with 1 % culture of producer strain. Flasks were incubated at 37 ºC for overnight. After incubation CFS was harvested by centrifugation at 4000 g for 1 h. Antimicrobial activity of CFS was checked against target strain by agar well diffusion assay.

Production and partial purification of antimicrobial peptides: Producer soil isolates were inoculated in100 ml BHI broth (1 %) and incubated at 35 ºC for 16 h (optimized). After incubation, it was centrifuged at 4000 g for 1 h. The CFS was harvested and filtered through 0.45 µm pore size filter paper in order to remove the remaining cells and contaminants. Hydrogen ion concentration (pH) of CFS was neutralized by 10 % hydrochloric acid (HCl) and one molar sodium hydroxide (NaOH). Cell free supernatant was referred as antimicrobial peptide (Muriana and klaenhammer, 1991) and antimicrobial activity was measured by agar well diffusion assay.

Cell free supernatant was precipitated by adding ammonium sulfate slowly with constant stirring at 4 °C till the level of 60 % concentration to attain the optimum precipitation of the protein. After saturation of ammonium sulfate, it was incubated at 4 ºC for overnight. Ammonium sulfate precipitates were separated by centrifugation at 4000 g for 60 minutes at 4 ºC. Supernatant and precipitates were collected separately in sterilized tubes. The pellet was solublized in 100 ml of 50 mM sodium phosphate buffer (pH 7.0) and designated as crude antimicrobial peptides. Antimicrobial activity of supernatant and precipitates was checked by agar well diffusion method and inhibitory zone was measured in mm.

Characterization of antimicrobial peptides: Crude antimicrobial peptides were diffused through dialysis membrane (pore size < 20 kda molecular weight cut-off membrane) to estimate the molecular weight of antimicrobial peptides. One ml of crude ammonium sulfate precipitates was covered in dialysis membrane and placed in a beaker containing sterilized distilled water. Whole setup was placed on stirrer for overnight at room temperature. The antimicrobial activity of dialyzed antimicrobial peptide was checked by agar well diffusion assay as described in previous study (Ahmed and Rasool 2003). Sensitive target strain was grown in BHI broth in two different flasks containing 30 ml in each flask with equal inoculums size. One flask was designated as test and another for control and incubated at 37 ˚C and samples were collected at 1, 2, 3 and 4 h, 2 ml of crude antimicrobial peptide was added to test flask at log phase and samples harvested with same pattern. The absorbance was measured at 600 nm.

To confirm the peptide or protein nature of antimicrobial peptides, crude antimicrobial peptides were treated with proteinase K enzyme and activity was checked by agar well diffusion assay. Proteinase K enzyme was dissolved in tris HCL buffer of pH 8 (Sigma, USA). Enzyme was used at final concentration of 1 mg mL^{-1} as per Muriana and klaenhammer, (1991). The proteinase K treated peptides were placed in wells and incubated overnight at 37 ˚C. Inhibitory zone against sensitive strain was checked and compared with control. Antimicrobial spectrum of ammonium sulfate precipitates of crude protein obtained from NK 127 was assayed against clinical isolates (*Shigella* spp, *Salmonella* spp, *Staphylococcus aureus, Escherichia coli and Streptococcus* spp). Fresh cultures (100 µl) of target strains was added to 3 ml of soft agar (1 %) separately and overlaid on pre-poured nutrient agar plates. After solidification of overlaid agar, wells were bored by sterilized borer and 150 µl of ammonium sulphate precipitates were transferred by micro-pipette in respective wells. Incubated at 4 ºC for 6 h so that proper diffusion could be made and plates were incubated at 37 °C for 24 h. After incubation, inhibitory zone was measured and interpreted.

The maximum inhibitory zone of the antimicrobial peptides, against target strains, the producer strain was grown in 100 ml BHI broth containing 1 % fresh starter culture of producer strain and incubated at 37 ºC in shaking incubator. Aliquots were collected at desired time intervals (after each 6 h for 50 h) and processed for optical density (OD) at 600 nm and CFS by centrifugation at 4000 g at 4 ºC. The CFS was separated and antibacterial activity was measured by agar well diffusion method and a graph was constructed between OD and inhibitory zone.

RESULTS AND DISCUSSION

Isolation of bacterial strains from soil and identification: Ten different bacterial species were isolated from seven different soil samples, out of which only one bacterial strain was found producing antibacterial peptides. The single isolated bacterial strain with code ID NK127 was identified on the basis of morphology, growth and biochemical characteristics using API 20 E kit (Table 1). The producer strain was G-

ve rod in shape and was confirmed as *Pseudomonas luteola* with 98 % similarity index shown by API web. The antimicrobial peptide producing potential of producer strain was determined by lacuna count, stab overlay and cross streak methods. In all screening techniques, producer strain significantly inhibited the sensitive strain. The strain NK127 with good inhibitory zone against sensitive strain was antimicrobial peptide producer as also reported in a previous study (Muriana and Klaenhammer1991; Bizaani *et al*. 2005). Similarly Heu *et al*. (2001) reported several soil bacteria including *Corynebacterium, Erwinia, Pseudomonas, Xnthomonas* and *Agrobacterium* with production of antimicrobial peptide.

Optimization of antimicrobial peptide production: Incubation time, temperature, pH of media and nutrients were optimized to get the maximum production of antimicrobial peptides. Maximum antimicrobial activity of producer strain against sensitive strain was obtained at 35 ºC with 33 mm inhibitory zone followed by 40 °C with inhibitory zone of 22 mm. The minimum antibacterial activity of CFS was observed at 30 ºC, indicating that the producer strain can produce highest amount of antibacterial peptides at 35 ˚C. The lesser inhibitory zone was noted at lower and higher temperature than 35 ˚C (Fig. 1a). These results are in agreement with the studies of Kim *et al*. (2006). Temperature affects the production of antimicrobial peptide and inactivates it to a lesser extent. In previous studies, *Enterococcus faecium* RZS C 5 produced better antimicrobial peptide between 25 °C to 35 °C than at 20 °C (Leroy and De Vuyst, 2002). The nitrogen rich media (BHI) enhanced the growth and antimicrobial activity of producer strain compared to simple nutrient media. The inhibitory zone of CFS obtained from BHI was greater (25 mm) than the nutrient broth (15 mm), indicating the nutrients have significant effect on production of antimicrobial peptide peptides. The BHI is a source rich in nitrogen and carbon as against nutrient broth as discussed by Kang and Lee *et al*., (2004) and Zendo *et al*. (2005).

Table 1. Results of API-20E kit.

TEST	Active ingredient	Reaction/ enzymes	Producer
ONGP	2-nitrophenyl-BD-galactopyranoside	b-galactosidase (ortho nitrophenyl-bd-galactopyranosidase)	+ve
ADH	L-arginine	Arginine dihydrolase	+ve
LDC	L-lysine	Lysine decarboxylase	-ve
ODC	L-ornithine	Ornithine decarboxylase	-ve
CIT	Trisodium citrate	Citrate utilization	-ve
H2S	Sodium thiosulphate	H2S production	-ve
URE	Urea	Urease	-ve
TDA	L-tryptophane	Tryptophan deaminase	-ve
IND	L-tryptophane	Indole production	-ve
VP	Sodium pyruvate	Acetoin production (Voges proskauer)	-ve
GEL	Gelatin	Gelatinase	+ve
GLU	D-glucose	Fermentation/oxidation (glucose)	+ve
MAN	D-mannitol	Fermentation/oxidation (mannitol)	-ve
INO	Inositol	Fermentation/oxidation (Inositol)	-ve
SOR	D-sorbitol	Fermentation/oxidation (Sorbitol)	-ve
RHA	L-rhamnose	Fermentation/oxidation (Rhamnose)	-ve
SAC	D-sucrose	Fermentation/oxidation (Saccharose)	-ve
MEL	D-melibiose	Fermentation/oxidation (Melibiose)	+ve
AMY	Amygdain	Fermentation/oxidation (Amygdalin)	-ve
ARA	L-arabinose	Fermentation/oxidation (Arabinose)	+ve
OX	Oxidase	Cytochrome oxidase	+ve

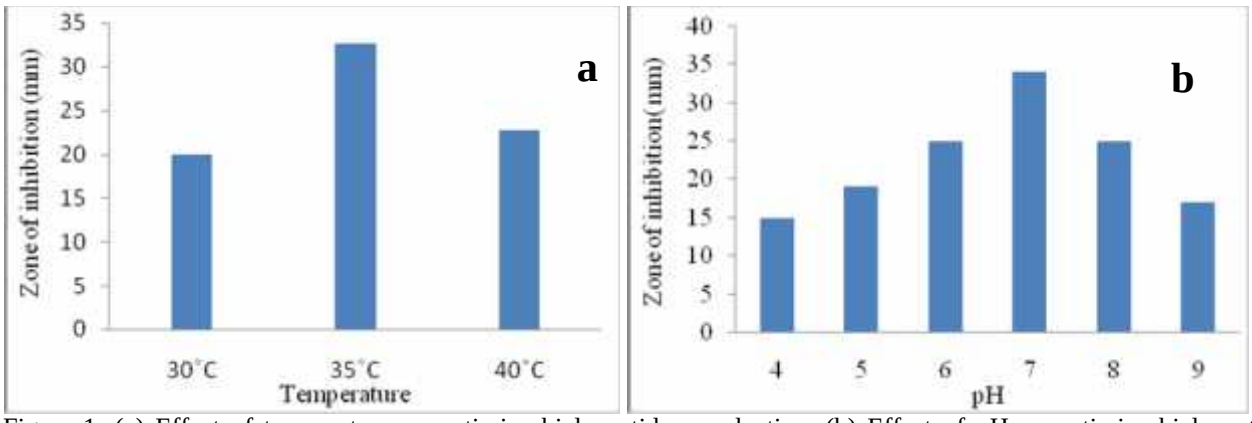

Figure 1: (a) Effect of temperature on antimicrobial peptides production. (b) Effect of pH on antimicrobial peptides production.

The effect of pH on production of antimicrobial peptide showed the largest inhibitory zone against sensitive strains at neutral pH (7.0) (Fig. 1 B), while lesser inhibitory zone was observed at acidic and alkaline pH, indicating that neutral pH is more suitable for antimicrobial peptide production. Meanwhile there was no effect of pH on bacterial mass production as measured through OD at different pH values in BHI broth. Microorganisms produce maximum antimicrobial peptides at their physiological pH as mentioned by Parente and Ricciardi (1994) during enterocin production from enteric microorganisms and pediocin (PD-1) (Nel *et al*., 2001). The possible explanation of this phenomenon is the favorable pH for the growth of microorganism that facilitated the antimicrobial peptide production. The maximum production time observed in this study was 18 h after that a decline in inhibitory zone was observed (Fig. 3).

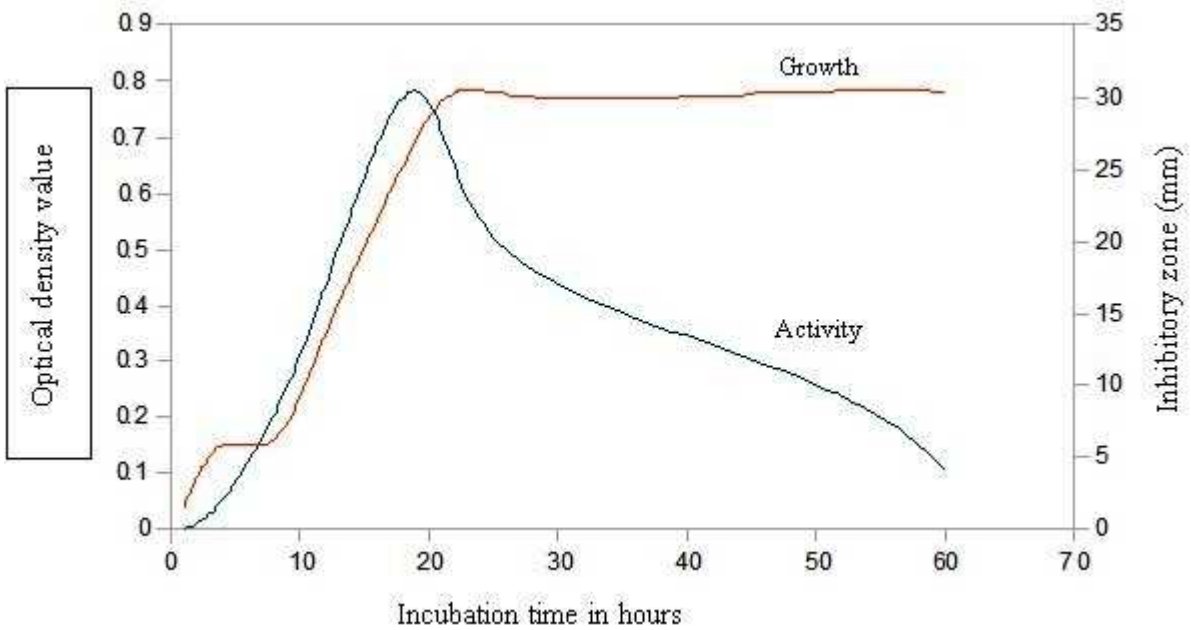

Figure 3: Inhibitory zone and growth curve optical density of NK127 bacterial strain

Furthermore antimicrobial peptides were precipitated up to 60 % and ammonium sulfate and antibacterial activity of precipitates were checked by agar well diffusion and found to be 20 fold higher than earlier (Fig. 2. a,b)

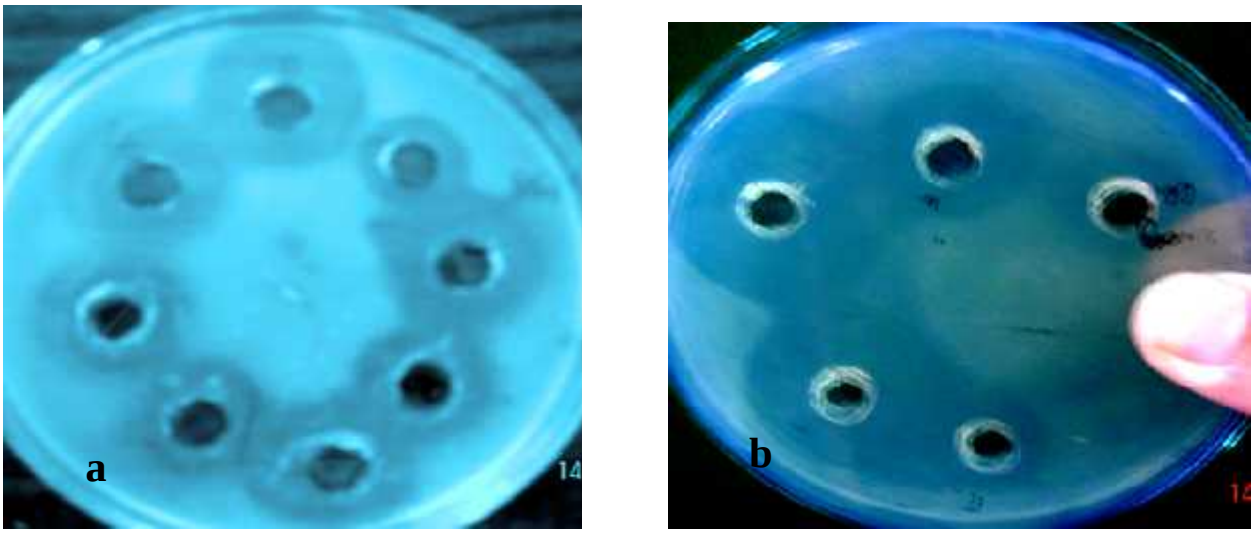

Figure 2: (a) Inhibitory zone of antimicrobial peptides after optimization (b) Inhibitory zone of antimicrobial peptides after partial purification

Characterization and antibacterial spectrum: Crude ammonium sulfate precipitates were characterized for its approximate molecular weight through dialyzing membrane, < 20 kda molecular weight cut off. During dialysis, the crude precipitates lost the antimicrobial activity. So, it indicates that the molecular size of peptides was roughly estimated to be less than 20 kda. The ammonium sulfate precipitates treated with proteinase K enzyme lost their antimicrobial activity confirming the protein nature of the antimicrobial peptides. The crude antimicrobial peptide showed strong antimicrobial activity against sensitive strains.

The antibacterial spectrum of partially purified crude peptides obtained from NK 127, showed activity against *Shigella*, *Salmonella*, *Staphylococcus aureus, E. coli* and *Streptococcus* by agar well diffusion assay (Table 3).

Table 2. Antibacterial spectrum of partially purified crude peptide antibiotic.

Bacterial Strain	Spectrum of partially purified peptide antibiotics
Shigella	+++
Salmonella	+++
Staphylococcus aureus	+++
Escherichia. coli	+++
Streptococcus	----

Conclusion: The study confirmed that soil ecological habitats contain various microbial species having potential of producing antimicrobial peptide. These species can be used as clinical probiotics. Such antimicrobial agents are considered to have low side effects on environment and consumers and high activity against clinically isolated pathogenic microorganisms. The study revealed that optimum conditions such as pH= 7, 16 h incubation at 35 ˚C in nitrogen rich medium) can increase the productivity of antimicrobial peptides that can inhibit the sensitive strain as well as other aforementioned pathogens

REFERENCES

Ahmed, S., and S.A. Rasool (2003). Isolation and biochemical characterization of mutacin VSM43 isolated from human oral streptococcus mutans VSM43. Pakistan J. Pharm. Sci. 16. 2:43-50.

Akbar, A. and K. A. Anal (2014). Occurrence of *Staphylococcus aureus* and evaluation of anti staphylococcal activity of *Lactococcus lactis* subsp. *lactis* in ready-to-eat poultry meat. Ann Microbiol 64 (1):131-138.

Akbar, A. and K.A. Anal (2013). Prevalence and antibiogram study of *Salmonella* and *Staphylococcus aureus* in poultry meat. Asian Pac. J. Trop. Biomed 3(2):163-168.

Ananou, S., M. Garriga, A.Jofré, T. Aymerich, A. Gálvez., M. Maqueda., M. Martínez-Bueno and E. Valdivia (2010). Combined effect of enterocin AS-48 and high hydrostatic pressure to control food-borne pathogens inoculated in low acid fermented sausages. Meat Science 84:594–600.

Anthony, T., T. Rajesh, N. Kayalvizhi and P. Gunasekaran (2009). Influence of medium components and fermentation conditions on the production of bacteriocin(s) by Bacillus licheniformis AnBa9. Bioresource Technol., 100: 872-877.

Bizaani, D., S.A. Motta, J.A.C. Morrisy, R. M. S. Terra, A.A. Souto and A. Brandelli (2005). Antibacterial activity of cerein 8A, A bacteriocin like peptide produced by *Bacillus cereus*. Int. Microbiol. 8: 125-131.

Bowdish, D.M., D.J. Davidson and R.E. Hancock (2005). A re-evaluation of the role of host defence peptides in mammalian immunity. Curr. Protein. Pept. Sci. 6:35–51.

Cotter P.D, C. Hill, R.P. Ross (2005). Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3:777-778.

Hardy, K.G. (1987). Plamid-A Practical Approach, IRL press, Oxford, England :42.

Heu, S., J. Oh, Y. Kang, S. Ryu, K. S. Cho, Y. Cho and M. Cho. (2001). glygene cloning and expression and purification of glycinecin A, A bacteriocin produced by *Xanthomonas campestris* PV. glycines 8RA. Appl. Environ. Microbiol. 67(9): 4105-4110.

Holt, J., N.R. Krieg, P.H.A. Sneath, J.T. Staleand and S.T. Williams (1994). Bergey's Manual of Determinative Bacteriology, 9th ed. William and Wilkins, London. : 528-538.

Iqbal, A., S. Ahmed, S.A. Ali and S. A.Rasool (1999). Isolation and partial cheracterization of Bac201: A plasmid associated bacteriocin like inhibitory substancefrom *S. aureus* AB201, J. Basic Microbiol. 39:325-329.

Kang, J. H. and M.S. Lee (2004). Characterization of a bacteriocin produced by *Enterococcus faecium* GM-1 isolated from an infant. J. Appl. Microbiol. 98:1169-1176.

Kim, M.H., Kong, Y.J. Baek and H., Hyun (2006). Optimization of cultural conditions and medium composition for the production of micrococcin GO5 by *Micrococcus* sp. GO5. J. of Biotech. 121: 54-61.

Klaenahmmer T.R. (1998). Bacteriocins of lactic acid bacteria. Biochemie, 70: 337-349.

Leroy, F. and L. De Vuyst (2002). Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. Int. J. Food Microbiol. 72: 155-164.

Messaoudi, S., M. Manai, G. Kergourlay, H Prévost, N.Connil, J.-M.Chobert (2013). *Lactobacillus salivarius*: Bacteriocin and probiotic activity. *Food Microbiol.* 36, 296-304.

Muriana, P.M. and T.R. Klaenhammer (1991). Purification and partial characteriation of lactacin F, a bacteriocin produced *by Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57(1):114-121.

Nel, H.A., R. Bauer, E. J. Vandammeand and L.M.T. Dicks (2001). Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1 J. Appl. Microbiol. .91:1131-1138.

Parente, E. and A. Ricciardi (1994). Influence of pH on the production of enterocin 1146 during batch fermentation, Lett. in Appl. Microbiol. 19: 12-15.

Riley, M.A. and J. E. Wertz (2002). Bacteriocin: evolution, ecology and application. Ann. Rev. Microbiol. 56:117-137.

Savadogo A, C.A.T. Ouattara, I.H.N. Basssole, S.A. Traoer (2006) Bacteriocins and lactic acid bacteria- a minireview. Afr J Biotechnol 5:678-683.

Settanni, L., A. Corsetti, (2008). Application of bacteriocins in vegetable food biopreservation. Int. J. Food Microbiol. 121, 123-138.

Talpur A. D., A. J. Memon, M. I Khan, M. Ikhwanuddin, M. M. Danish., Daniel and A.B. Abol-Munafi (2012). Inhibition of pathogens by lactic acid bacteria and application as water additive multi isolates in early stages larviculture of *P. pelagicus* (LINNAEUS, 1758). The J. Anim. Plant Sci., 22(1):54-64.

Yousef, A. E., J. B. Luchansky, A. K. Degnan and M. P. Doyle (1991). Behavior of *Listeria monocytogenes* in Wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin ACH during storage at 4 ºC or 25 ºC. Appl. Environ. Microbiol. 57: 1461-1467.

Zendo, T., F. S. Eungruttanagorn, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki and K. Sonomoto. (2005). Identification and production of a bacteriocin from *Enterococcus mundii* QU 2 isolated from soyabean. J. Appl. Microbiol. 99: 1181-1190.