Mapana Journal of Sciences
2020, Vol. 19, No. 4, 49-61
ISSN 0975-3303 | https://doi.org/10.12723/mjs.55.5

Expression, Purification and Functional Antibacterial Characterisation of Novel Antimicrobial Peptide Gene *Pediocin* against *Salmonella typhii*

Nandhini C*, Vinitha M† and Sudhakar Malla‡

Abstract

Salmonella is a major cause for serious food infections prevailing round the world. Growing resistance to most of the antibiotics makes the strain more problematic and threatening. Pediocin is known as antimicrobial peptides (AMPs) which were isolated from several Pediococcus strains. Peptides were designed to clone and express it in the commonly growing E.coli cells to produce rDNA proteins. The proteins were purified and used against the Salmonella strains. The disc diffusion assay proved the capable role of the protein in killing the *Salmonella* strains, especially typhii. This protein form pores within the membrane of the target, and so its ability with leakage assay was screened.

Keywords: *Salmonella*, Pediocins, AMPs, Antibacterial activity

* Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore, India; nandhusara142@gmail.com
† Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore, India; vinithaammu3@gmail.com
‡ Department of Biotechnology, Indian Academy Degree College-Autonomous, Bangalore, India; sudhasanmoon2000@gmail.com

1. Introduction

Bacteriocins, commonly called antimicrobial peptides (AMPs), are the proteins which are produced by several bacterial strains (Abbas, Ghadban, & Alghanim, 2017). These are also called toxin peptides which are generated from bacteria at the stationary phase or decline phase mostly to inhibit the growth of other bacterial species which are or not closely related (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008). Some strains of yeasts and *Paramecium* are also thought to be involved in this production. Some of the yeasts, like *Saccharomyces boulardii* are capable of producing bacteriocins called protein killer (Bhunia, Johnson, & Ray, 1988; Bhunia, Johnson, Ray, & Kalchayanand, 1991;).

Pediocin are low molecular weight (2.7-17KDa) cationic molecules which consist of a hydrophilic N-terminal part called pediocin box (YGNGV) motif and a hydrophobic C-terminal which is variable in nature (Biswas, Ray, Johnson, & Ray, 1991) and are produced by *Pediococcus* bacteria. It exhibits inhibition properties against most of the sensitive bacterias and usually acts on the cytoplasmic membrane via a pore forming mechanism. It also interferes with the amino acid absorption by the cytoplasmic membrane of the target cells (Castellano, Ibarreche, Massani, Fontana, & Vignolo, 2017). It is unique in nature by being more thermostable and with high resistance to many of the proteases. Pediocin is found to show inhibition activity against many food spoilage bacteria and even pathogenic bacteria like *Listeria*, *Clostridium*, *Bacillus*, *Aeromonas, Staphylocoocus, Lactobacillus* and *Enterococcus* (Chikindas et al., 1993; Kaur et al., 2014).

Pediocin is a class IIa group and is formed from the *Pediococcus* spp, which are Gram-positive, homofermentative belonging to the Lactobacillaceae family (Haakensen *et al*., 2009). The bacteriocin produced from these species is called pediocin. The cytoplasmic membrane of lactic acid bacterial strains are closely related and are able to target pediocins. Pediocin inhibits the growth of targets by making pores within their membranes, collapsing the pH of the intracellular environment and by inhibiting the proton motive force for producing energy. Such a protein is linked with the cell target

through active sites which are possibly the negative charges of the phospholipid groups (Parret, & De Mot, 2000).

Salmonella is the most common and serious cause of foodborne infections around the world and also seems to generate a negative impact on the economic status of the food industry (Fyfe, Harris, & Govan, 1984). Salmonellosis is a type of zoonotic infection primarily caused by *Salmonella* leading to gastroenteritis, typhoid fever and bacteremia. Transmission is usually by the oral and faecal route through the infected animal. However, human beings are also carriers of the *Salmonella* strains, but they are less likely to transmit it than animals (Combet, Blanchet, Geoujon, & Deleage, 2000).

There are many reasons which lead to several outbreaks within the food industry. Meat, dairy products, and fruits and vegetables are thought to be the primary mode of transmission (Tokuda, & Konisky, 1978). Undercooked meat allows *Salmonella* strains to survive and grow that leads to cross-contamination of other foods (Lazdunski, et al., 1998; Cascales, et al., 2007). Cross-contamination usually occurs through direct contact or indirectly by contaminated kitchen vessels. *Salmonella* transmission within the food processing plants is now a major threat to the food industry (Wong, Hald, Wolf, & Swnenburg, 2002).

The availability of the pediocin sequences enabled to predict the epitopes and transmembrane regions which could aid in the production of novel AMPS (Bhunia, Johnson, & Ray, 1988). In this study, we designed to overexpress the pediocin protein within the bacterial strains and further purify to screen its efficacy against *Salmonella* pathogens. This could further lead to the development of novel recombinant proteins which could be used in the food industry.

2. Materials and methods

2.1 Materials and chemicals

All the chemicals used were of molecular grade and purchased from different vendors. Vector was donated by Stellexer, Bangalore, restriction enzymes were purchased from HiMedia,

Tris, SDS, Glycerol, Bromophneol blue, Dithiothreitol (DTT) were purchased from HiMedia, Bangalore.

2.2 Sequence retrieval

Nucleotide and amino acid sequence of the pediocin protein (>AAT72009.2MNKTKSEHIKQQALDLFTRLQFLLQKHDTIEPYQ YVLDILETGISKTKHNQQTPERQARVVYNKIASQALVDKLHFTA EENKVLAAINELAHSQKGWGEFN) was isolated from the genebank in FASTA format. The Hierarchical Neural Network (HNN) was used to screen the secondary structures, and the SOSUI method was used to screen the trans-membrane protein prediction of the desired protein. The epitopes for the protein was screened by using bcepred tool. The BcePred server allows to predict B cell epitopes based on their physio-chemical properties (hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns) of amino acids. (Saha, 2004)

2.3 Bacterial strains

Salmonella strains were procured from the MTCC repository for the study of antibacterial activity *Salmonella typii* (MTCC 8767). All these cultures were maintained on nutrient agar plates at 4^0C.

2.4 Expression and protein purification

The pediocin gene (AY083244.3; pedA) 532bp, encoding 101aa) was synthesised (Sigma) and introduced into pTZ57R/T cloning vector (Fermentas, USA) by double digestion with EcoRI/HindIII followed by ligation and transformation into *E. coli* JM109. Overexpression of protein, PedA, was induced by adding 1mM IPTG and incubating for 3-4 hours until the culture reaches (OD600 = 0.6–1.0). Following expression, a single colony was isolated, and the recombinant plasmid was harvested and analysed for the total protein by SDS–PAGE. The recombinant protein was further purified by resuspending the cells in 1ml PBS with 10mM imidazole (pH 7.4) and further analysed by using ultrasonicator (Stellixir). Following centrifugation, the cell-free lysate was collected and purified on His Trap™ HP columns (HiMedia) through (Fast Protein Liquid Chromatography) FPLC. About 5ml of the fraction was allowed for desalting. Finally, the protein

fraction was eluted into sample buffer (Tris (1 M, pH 6.8) of 80mM, SDS 20%, Glycerol 10%, Bromophneol blue 0.1%, Dithiothreitol (DTT) 1M). The purity and concentration were measured by SDS–PAGE and Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951) respectively.

3. Antibacterial activity

3.1 Disc diffusion method

The strain was poured plated onto nutrient agar plates, and 20µl of the fraction was used for the study. It was added into a respective well. Ciprofloxacin (50mg/ml) was used as a positive control. The antibacterial activity was initially screened by disc diffusion and later by tube dilution method (Irayyif, Araghi, & Malla, 2015).

3.2 Tube dilution method

Tube dilution assay was done according to the protocol described. In brief, about 5ml of broth was added to the boiling tubes, and 20µl of inoculum was added to all the tubes except the negative control. 20µl of the fraction was used as a test sample. Ciprofloxacin (50mg/ml) was used as a positive control. The experiment was done in triplicates, and following incubation, the samples were observed for absorbance at 600nm in a spectrophotometer (Shimadzu 1800). Percentage Inhibition (%) = ((dc - dt)/dc) x 100, where dc and dt represent absorbance values of control and treated sample strains respectively.

3.3 Biofilm Cultivation

In brief, about 180µl of broth was added in their respective wells. 10µl of inoculum (diluted to 1: 200 with broth) was added to all the wells except the negative control. 10µl of the fraction was used as a test sample. Ciprofloxacin (50mg/ml) was used as a positive control. The plate was incubated at 37^0C for 24 hrs. Following incubation, the wells were washed with 200µl of PBS and stained with 2% crystal violet for 15 minutes. Destaining was done with 200µl of ethanol: acetone 80:20 and the absorbance was recorded at 590nm in a plate reader (Genetix, Germany). The experiment was done in triplicates (Irayyif, Araghi, & Malla, 2015).

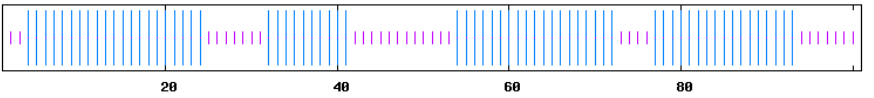
3.4 Antimicrobial assay by ATP release

To screen for leakage of the nucleic acids (Ling, Saeidi, Rasouliha, & Chang, 2010) from the bacterial cells after the treatment, the absorbance was recorded at 260nm (A260) in the cell-free lysate in a UV spectrophotometer (Shimadzu, 1800). The cells without treatment served as a negative control.

4. Results and discussion

4.1 Expression and purification of proteins

From the HNN server, around 67 alpha-helixes were seen in the secondary structure. The transmembrane region prediction from the SOSUI program indicates that the protein is more soluble in nature. As such, it is more selectable in terms of purification during downstream processing. The killing domain obtained was sharing 78% homology with that of penicillin acylase family protein. This similarity index proposes the desired protein as bacteriocin.



Sequence	1AATPEDICINPARTIALPEDICCCSACIDILACTICIMNK TKSEHIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILET GISKTKHNQQTPERQARVVYNKIASQALVDKLHFTAE ENKVLAAINELAHSQKGWGEFN138
Flexibility	1AATPEDICINPARTIALPEDICCCSACIDILACTICIMNK TKSEHIKQQALDLFTRLQFLLQKHDTIEPYQYVLDILET GISKTKHNQQTPERQARVVYNKIASQALVDKLHFTAE ENKVLAAINELAHSQKGWGEFN138

Figure 1: Images of the Transmembrane model as shown from HNN server and possible epitopes (shown in blue) as predicted by bcepred.

Following transformation and expression purification step yielded a protein fraction which was quantified using Lowry's method. His

tag-S tag (about 4.8kDa) was fused onto the N-terminal region to facilitate the purification of protein. Following purification, about 3.06 mg/ml of pediocin protein was obtained and showed a single visible band at about 11.8kDa (Fig. 2).

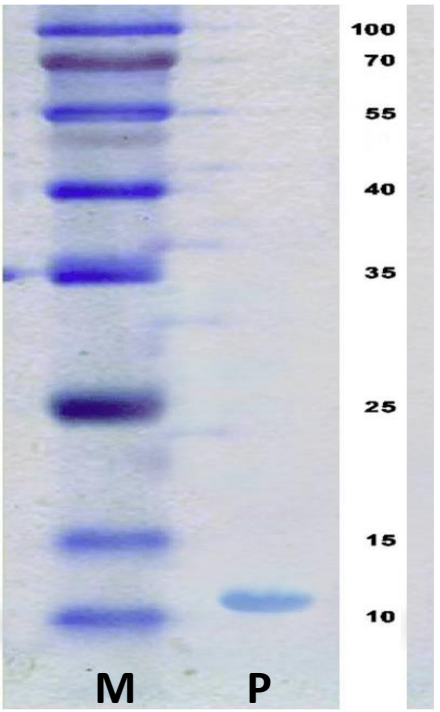


Figure 2: SDS PAGE gel showing the protein band of interest at about 11.2KDa. M: marker; P: Protein of Interest.

4.2 Bactericidal activity against *Salmonella typhii*

In order to estimate the antimicrobial spectrum of the purified fraction, we examined the susceptibility against the salmonella strain. A zone of inhibition was found towards the strain and was equally effective when compared to the positive control. The diameter of the inhibition zone was found to be dose dependant. The percent inhibition results also exhibited the same significant results in accordance with the disc diffusion assay. The percent inhibition for *Salmonella typhii* was found to be 45.32 ± 0.23, 56.43±

0.11, 64.32± 0.05 and 87.67± 0.10 for 3.5, 7,28 and 112µg/ml respectively. The inhibition of positive control was found to be 100% at 112µg/ml. Negative control was found to be 0.02.

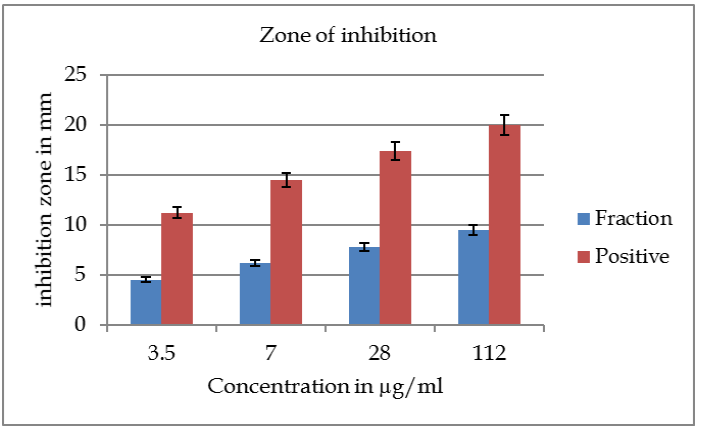


Figure 3: Graph showing the zone of inhibition values in mm. All the values are the averages of triplicates. The values are expressed as value ± s.d.

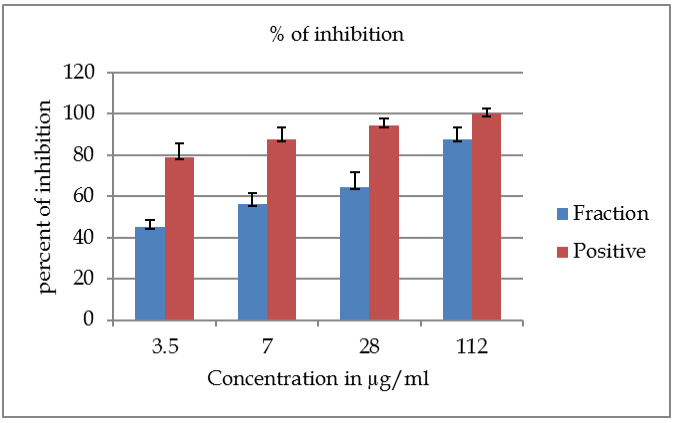


Figure 4: Graph showing the percent of inhibition by broth dilution assay. All the values are the averages of triplicates. The values are expressed as value ± s.d.

4.3 Biofilm Cultivation

The biofilm assay also showed significant results on treating with the fraction. Biofilm inhibition was expressed as a percentage (%) in relation to the positive control. The biofilm inhibition in *Salmonella typhii* was found to be 0.42± 0.38, 0.61± 0.23, 0.74± 0.13 and 0.87± 0.3 for 3.5µg/ml, 7µg/ml, 28µg/ml, 112µg/ml respectively. On the other hand, for the positive control, it was found 100% at 28µg/ml. Negative control was found to be 0.08± 0.21.

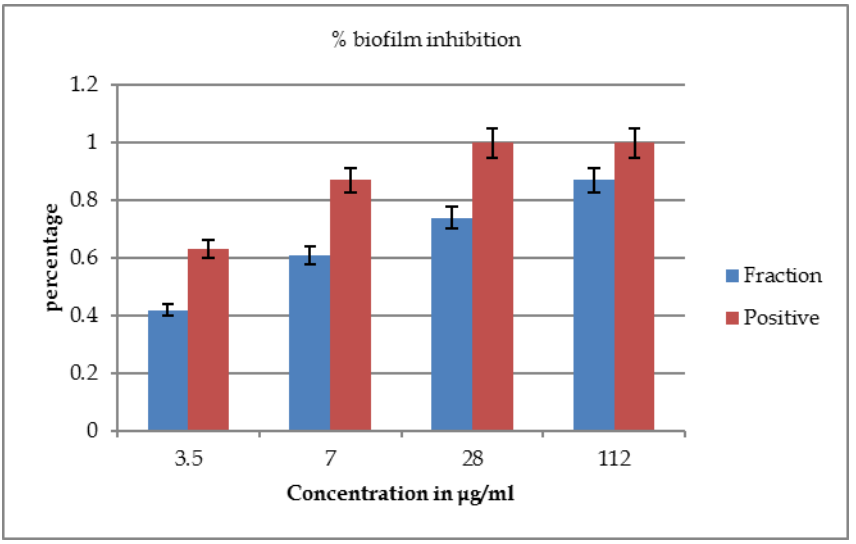


Figure 5: Graph showing the percentage inhibition values of biofilm cultivation assay of Salmonella strains. All the values are the averages of triplicates. The absorbance values were recorded at 590nm, and the values were expressed as value ± s.d.

4.4 Membrane leakage and permeability

To screen the leakage of intracellular materials like DNA and RNA from the bacterial cells upon exposure to treatment with fraction and positive absorbance values at 260nm of the cell-free lysate was measured. From the experiment, it was found that positive control showed complete leakage at 112µg/ml. On the other hand, the fraction showed a significant effect when compared to the positive control. The absorbance at 260nm for the sample was found to be six times more than the negative control (0.02).

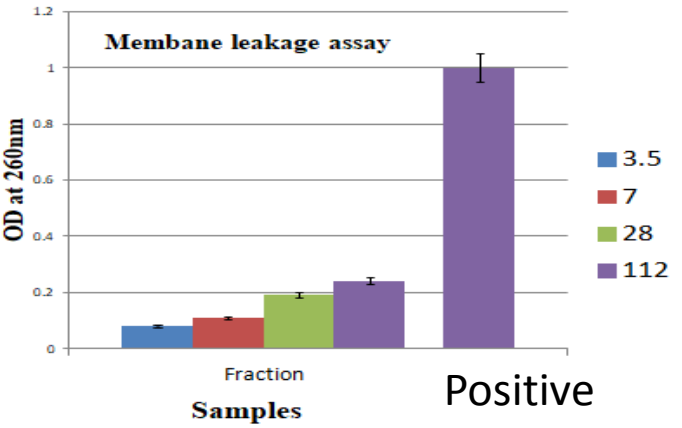


Figure 6: Graph showing the leakage of nucleic acids from the cell into the lysate. A positive control was shown as 1 at 112µg/ml. All the values were average of triplicates.

To summarise, this study provides fundamental evidence that the gene sequences of the protein pediocin encode a bacteriocin protein which could be used as AMPs. We designed to study its effect on the *Salmonella* strains, which are a major threat to the food industry. The cloning and expression were carried out wherein we purified the protein of interest by SDS PAGE and used the purified fraction in the study. The zone of inhibition obtained was almost very promising when compared to a positive control (ciprofloxacin) (Girish, R, 2013). Even the bacterial inhibition studies using the broth dilution method also showed the same results. The biofilm inhibition studies resulted in about 0.87± 0.3 inhibition for the fraction, which is very significant in terms of positive control (Brunner, H¸1988).

As stated earlier in the context, pediocin protein works by making pores within the target cell membrane (Gaussier, 2003). This was confirmed by the leakage assay, which showed very high significant results even. The positive control showed complete leakage at 112µg/ml, which was almost similar to our fraction, which showed leakage of 6 times more when compared to the negative control (0.02).

5. Conclusion

Given such significant results and possible activity of AMPs against *Salmonella*, this study might prove capable for the potential use of pediocon in the food industry. This novel AMPs protein-peptide could be used in the rDNA technology to further improve the residues and capability. This could also be used to study several other food pathogens and could play a vital role in reducing the fatal food industry infections.

Conflicts of interest: The authors declare no conflict of interest.

Acknowledgement: We wholeheartedly extend our thanks to the STAR college program scheme for funding the present study.

References

[1] Abbas, B.A.; Ghadban, M.K. & Alghanim, A. M. (2017). Microbial evaluation of milk and milk products during a past two decades, in Basrah southern Iraq, a review. Annual Research & Review in Biology, 14(2): 1-8.

[2] Anastasiadou, S.; Papagianni, M.; Filiousis, G.; Ambrosiadis, I. & Koidis, P. (2008). Pediocin SA-1, an antimicrobial peptide from *Pediococcus acidilactici* NRRL B5627: Production conditions, purification and characterization. Bioresource Technol., 99(13): 5384-5390.

[3] Bhunia, A.K.; Johnson, M.C. & Ray, B. (1988). Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. Appl. Microbiol., 65(4): 261-268.

[4] Bhunia, A.K.; Johnson, M.C.; Ray, B. & Kalchayanand, N. (1991). Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. Appl. Microbiol., 70(1): 25-33.

[5] Biswas, S.R.; Ray, P.; Johnson, M.C. & Ray, B. (1991). Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. Appl. Environ. Microbiol., 57(4): 1265-1267.

[6] Sajjad Mohsin Irayyif, Asal Mohseni Sajadi Araghi and SudhakarMalla. silver nanoparticles and their effect on the biofilm formation in food borne salmonella species. International Journal of Recent Scientific Research.Vol. 6, Issue, 5, pp.4343-4346, May, 2015

[7] Wong, D. M. A.L. F., Hald, T., Wolf, P.J.v.d. & Swanenburg, M. (2002). Epidemiology and control measures for Salmonella in pigs and pork,

Livestock Production Science, Vol.76, No.3, (September 2002), pp. 215-222, ISSN 0301-6226

[8] Castellano, P.; Pérez Ibarreche, M.; Blanco Massani, M.; Fontana, C. & Vignolo, G.M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: A focus on meat ecosystems and industrial environments. Microorganisms, 5(3): 38.

[9] Chikindas, M.L.; García-Garcerá, M.J.; Driessen, A.J.; Ledeboer, A.M.; Nissen- Meyer, J.; Nes, I.F.; Abee, T.; Konings, W.N. & Venema, G. (1993). Pediocin PA- 1, a bacteriocin from *Pediococcus acidilactici* PAC1. 0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol., 59(11): 3577-3584.

[10] Kaur, G.; Singh, T.P.; Malik, R.K.; Bhardwaj, A. & De, S. (2014). Antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 against *L. monocytogenes, E. faecium and E. faecalis* and bacteriocin cross resistance and antibiotic susceptibility of their bacteriocin resistant variants. J. Food Sci. & Tech., 51(2): 233-244.

[11] Parret, A. and De Mot, R. (2000) Novel bacteriocins with predicted tRNase and pore-forming activities in Pseudomonas aeruginosa PAO1. Mol. Microbiol. 35, 472–473.

[12] Fyfe, J.A., Harris, G. and Govan, J.R. (1984) Revised pyocin typing method for Pseudomonas aeruginosa. J. Clin. Microbiol. 20, 47–50.

[13] Combet, C., Blanchet, C., Geourjon, C. and Deleage, G. (2000) NPS@: network protein sequence analysis. Trends Biochem. Sci. 25, 147–150.

[14] Lazdunski, C.J., Bouveret, E., Rigal, A., Journet, L., Lloubes, R. and Benedetti, H. (1998) Colicin import into Escherichia coli cells. J. Bacteriol. 180, 4993–5002.

[15] Cascales, E. et al. (2007) Colicin biology. Microbiol. Mol. Biol. Rev. 71, 158–229.

[16] Tokuda, H. and Konisky, J. (1978) In vitro depolarization of Escherichia coli membrane vesicles by colicin Ia. J. Biol. Chem. 253, 7731–7737.

[17] Hua Ling, Nazanin Saeidi, Bahareh Haji Rasouliha, Matthew Wook Chang. A predicted S-type pyocin shows a bactericidal activity against clinical Pseudomonas aeruginosa isolates through membrane damage. FEBS Letters 584 (2010) 3354–3358.

[18] Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. (1951). "Protein measurement with the Folin phenol reagent" (PDF). *Journal of Biological Chemistry*. **193** (1): 265–75. PMID 14907713.

[19] Saha.S and Raghava G.P.S. BcePred:Prediction of Continuous B-Cell Epitopes in Antigenic Sequences Using Physico-chemical Properties. In G.Nicosia, V.Cutello, P.J. Bentley and J.Timis (Eds.) ICARIS 2004, LNCS 3239, 197-204, Springer,2004.

[20] Girish, R., Kumar, A., Khan, S., Dinesh, K. R., & Karim, S. (2013). Revised Ciprofloxacin Breakpoints for Salmonella: Is it Time to Write an Obituary?. *Journal of clinical and diagnostic research : JCDR*, *7*(11), 2467–2469. https://doi.org/10.7860/JCDR/2013/7312.3581

[21] Brunner, H., & Zeiler, H. J. (1988). Oral ciprofloxacin treatment for Salmonella typhimurium infection of normal and immunocompromised mice. *Antimicrobial agents and chemotherapy*, *32*(1), 57–62. https://doi.org/10.1128/aac.32.1.57

[22] Gaussier, H., Lefèvre, T., & Subirade, M. (2003). Binding of pediocin PA-1 with anionic lipid induces model membrane destabilization. *Applied and environmental microbiology*, *69*(11), 6777–6784. https://doi.org/ 10.1128/aem.69.11.6777-6784.2003