Exploring the role of FtsA fragment as a potent antimicrobial peptide

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In

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Submitted by

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"Exploring the role of FtsA fragment as a potent antimicrobial peptide"**, submitted by *Alok Chaudhary* (DTU/14/MTECH/099) in partial fulfillment of the requirement for the major project during M. Tech, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work which was carried out by him under our guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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DECLARATION

I declare that my major project dissertation entitled "**Exploring the role of FtsA fragment as a potent antimicrobial peptide**" submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at "IIT GUWAHATI" as Major project. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

Date: Alok Chaudhary

Place: Delhi

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I **Alok Chaudhary**, student of M. TECH BIOMEDICAL ENGINEERING, 2K14/BME/01 is presenting a project report on "**Exploring the role of FtsA fragment as a potent antimicrobial peptide**" under the supervision of **Dr. Nitin Chaudhary** (Assistant Professor), Department of Bioscience and Bioengineering, Indian Institute of Technology, Guwahati. He encouraged me to undertake this very interesting topic and even gave me valuable suggestion and information which were mandatory for the completion of the project.

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ABBREVIATION

Ac - Acetylated

AMP- Antimicrobial peptide

AFM- Atomic force microscopy

CPP - Cell penetrating peptide

DNA- Deoxyribonucleic acid

DMSO-Dimethyl sulfoxide

DIPEA- N, N-Diisopropylethylamine

HSV- Herpes simplex virus

HOBt- N-Hydroxybenzotriazol

HBTU-2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

NAG- N-acetyl glucosamine

NAM- N-acetyl muramic acid

O.D - Optical density

RNA- Ribonucleic acid

SPPS- Solid phase peptide synthesis

µM - micro molar

"Exploring the role of FtsA fragment as a potent antimicrobial peptide''

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ABSTRACT

The quick increase in drug-resistant infections has dispense a significant challenge to antimicrobial therapies. The collapse of the most potent antibiotics to eliminate "superbugs" emphasizes the critical need to develop other control agents. All organism like bacteria, insect, plant and human being produce antimicrobial peptides (AMPs) as vital part of their nonspecific and immediately effective immunity against infection. Antimicrobial peptides are a key component of the innate defense of all species of life. Antimicrobial peptide has different characteristic like antiendotoxic, antibacterial, antibiotic-potentiating or antifungal properties, so they can be utilizing to develop a novel class of antimicrobial agents. Moreover, their capability to destroy microbes, these peptides appear to have effective role in innate immunity and can modulate the expression of multiple genes in eukaryotic cells. Due to their broad spectrum of targeted microbes, AMPs have attracted increasing attention. With the increasing progress of antibiotic resistance between bacterial pathogens, there is a serious requirement to recognize or discover novel classes of antimicrobial peptide.

Keyword: Antimicrobial peptide, HPLC, MALDI, AFM.

2) INTRODUCTION

We are daily exposed to millions of pathogens through various mode like inhalation, contacted with infected surface and ingestion. The immune system (adaptive humoral and Cellular) have less impact on these pathogens. Bacteria are able to give fully grown infection within one day. On other hand, primary immune response involving B and T cells is very slow and it takes about seven days before it is even noticeable [1]. Only those animal and plant species prevent and overcome infection who developed and evolved host defence mechanism including antimicrobial peptide. In in-vitro experiments against microorganism, Antimicrobial peptide (AMPs) show impressive activity, comparison to conventional antibiotics. The antimicrobial peptide has advantages over existing antibiotics like activity against some of more serious antibiotic resistant pathogen, broad range activity, etc. Thus these peptides offer exciting possibilities in the face of the declining efficacy of conventional antibiotics owing to the rise of antibiotic resistant organisms. The best antimicrobial peptide kills vulnerable bacteria within the 4 microgram/ml concentration [2]. Antimicrobial peptide found in every organism whether they are bacteria, fungi, parasite, plant or even animal. Early work with insect, amphibian and human proved that they had straight activity against microbes. Recent study shows that they have variety of function in harmonize immunity which have impact in inflammation and infection [3-5].

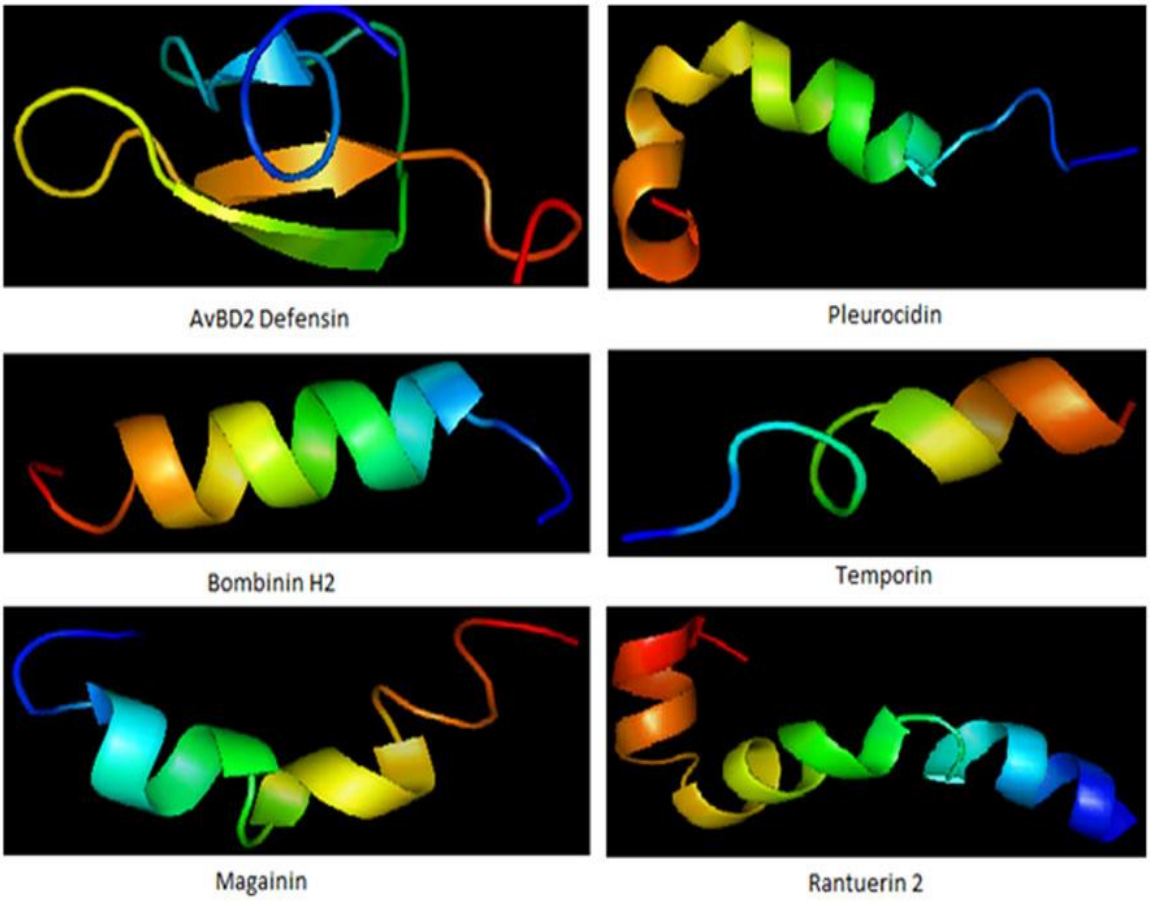


Figure-1: Different antimicrobial peptide,2014, Santi M Mandal *et al*

3) REVIEW OF LITERATURE

3.1) ANTIMICROBIAL PEPTIDE

Antimicrobial peptides (AMPs) are known as cationic host defence peptide [6], anionic, cationic amphipathic peptides [7], antimicrobial peptide [8] and alpha helical antimicrobial peptide [9]. The Antimicrobial peptide are generally oligopeptides and their size varying from five to over a hundred amino acids. Their target organism range varies from viruses to parasite. Natural AMPs are reported in both organisms i.e. prokaryotic(bacteria) and eukaryotic (fungi, plant and animals). Till now up to 5000 AMPs has been discovered and synthesize [10-13]. Multicellular organism produced AMPs as a defence mechanism against pathogen [14-15]. AMPs are mostly found in those tissue and organ which are exposed to air borne pathogen in the animals and it is assume that they are the first line of defence against bacteria, virus and other microbes [16-17]. Thus the key role of AMPs is to prevent infection. For example, Frog skin consist of AMPs more than 300 in number [18-19]. Most of the time AMPs are produced by specific cell but some time their production is inducible. For example, P9A and P9B production can be induced by the vaccination of *Enterobacter cloacae* hemolymph [20]. Several type of eukaryotic cells such as phagocytes [21], lymphocyte of immune system [22] and lymph's, epithelial cells in gastrointestinal and genitourinary systems respectively [23,24]. The first AMPs was discovered by Dubos in 1939, he extracts an antimicrobial strain from soil bacillus strain [25]. This extract was indicating to protect mice from pneumococci infection [26.]. 1n 1940, Hotchkiss and Dubos separated this extract and identified an AMP which was named gramicidin [27]. Gramicidin was found toxic in intraperitoneal application but was very useful in treatment of ulcer and wounds [28]. Tyrocidine was discovered in 1941 and was effective against Gram positive and Gram negative bacteria but exhibit toxicity against human blood cell [29,30]. In the same year Purothionin was isolated from plant *Triticumaestivum* and is effective against fungi [31,32]. Defensin was the first animal originated AMP and is isolated from rabbit leukocyte in 1956 [33]. In the following years, bombinin from epithelia [34] and lactoferrin from cow milk [35] were both reported. During the same time, it was also demonstrating that human leukocytes contain AMPs in their lysosomes [36]. But the actual research related with AMPs originated in 1980 when Hans boman reported that humoral immune system of silk moths (*Hyalophora cecropia*) contained peptide with broad spectrum activity [37].

3.2) STRUCTURE OF AMPs

Most of AMPs found till date can be categories into four basic types viz α-helix, β-sheet, loop and extended. This categorization based on the secondary structure of peptide. Among all type α-helix and β-sheet are most common studied [38]. In α-helix, the distance between two amino acids is about 0.15 nm while in β-sheet, it is about .35nm. In the top view, the angle between two amino acids is 100 degrees in α-helix. Both α-helix and β-sheet can form transmembrane channel in plasma membrane. The core of plasma membrane is roughly about 3.5 nm, so it takes about 20-21 amino acids to span the membrane either by α-helix or β-sheet. Protegrin, Magainin, cyclic indolicin, and coiled indolicin are some common example of α-helix. β-sheet consist of at least two β-strand joined together by disulphide bond [39]. B-sheet can be arranging in parallel or anti-parallel fashion. Almost all AMPs belong to all the above classes but some contain two different structural components for example indolicin [40,41].

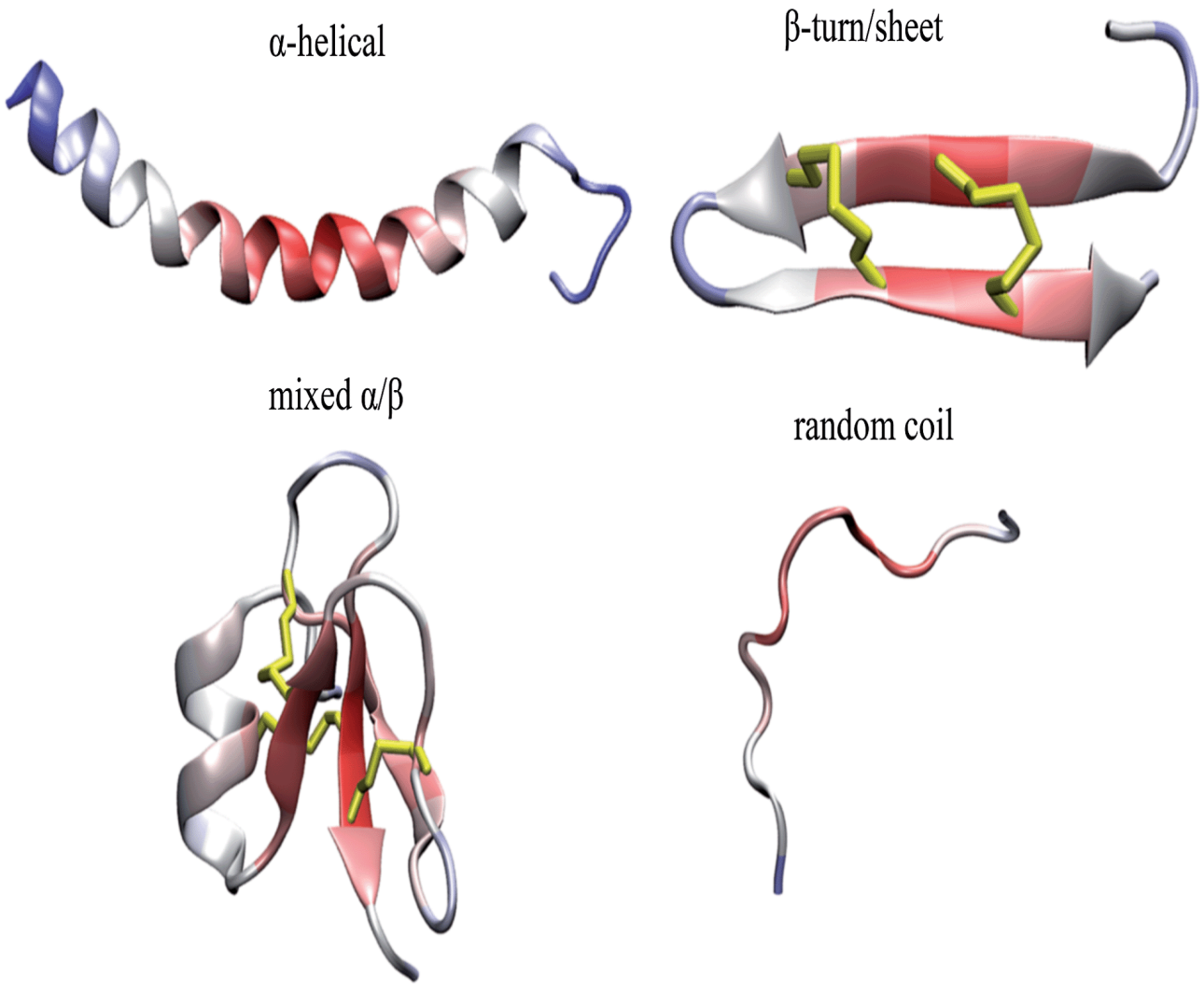


Figure-2: Different type of structure of Antimicrobial peptide, 2014, Mingzhen Zhang,10,7425-7451

3.3) MAJOR CATAGORIES OF AMPs

In general, AMPs do not kill bacteria though enzymatic mechanism. On the basis of basis of target eukaryotic AMPs can be divide into four categories.

3.3.1) ANTIVIRAL PEPTIDE

Antiviral peptide binds to the viral envelope or host membrane and then neutralizes viruses effect. They are effective against viruses. Previous studies shown that they target both i.e. RNA and DNA of the viruses [42,43]. AMPs bind to the viral envelops and cause membrane instability which further inhibit viruses to bind with host cells [44-46]. For example, the attachment of herpes simplex viruses (HSV) to the host cell surface is inhibited by Defensin [47]. Apart from viral envelope disruption and blocking viral receptor, some AMPs occupying specific mammalian receptor and thus prevent viral particle entry into host cell [48, 49]. For example, Lactoferrin, an α-helical cationic peptide binds to the negatively charge glycosaminoglycan and thus prevent binding of HSV infection [50, 51].

3.3.2) ANTIBACTERIAL PEPTIDE

Antibacterial peptide are the most common and studied AMPs. They are mostly cationic in nature i.e. positively charge. They integrate to the bacterial membrane and cause disintegration [52, 53]. They are amphipathic in nature i.e. they have both hydrophilic and hydrophobic domains. Due to such amphipathic nature, these AMPs are able to bind with lipid component and phospholipid group of cell membrane.

3.3.3) ANTIFUNGAL PEPTIDE

Antifungal peptide is found to be effective against fungi and can kill fungi by targeting cell wall or intracellular component [54-56]. Some AMPs are able to binds with chitin, which is main component of fungal cell wall. Such binding capability help AMPs to target fungal cells [57, 58]. Most of the antifungal AMPs consist of polar and neutral amino acid [59]. But still there in no relation appears between structure of AMPs and its cell type target.

3.3.4) ANTIPARASITIC PEPTIDE

Antiparasitic peptide consists less not number of peptide as compared to above three class. They are found to be effective against parasite like *Paramecium caudetum, Leishmania parasite* etc. Magainin was the first antiviral peptide reported against *Paramecium caudetum* [60].

3.4) MODE OF ACTION OF AMPs

AMPs can kill microbes by different mode of action. It includes disrupting membrane integrity inhibit DNA, RNA and peptide synthesis or by combine with intracellular targets.

3.4.1) MEMBRANE ACTIVE AMPs

The membrane active AMPs interact with the cell membrane for their activity and interaction decide the spectrum of target cell [61]. Membrane active AMPs are mostly amphipathic and cationic. Amphipathic means they have both hydrophobic and hydrophilic group. This characteristic is beneficial for electrostatic interaction between negatively charge cell membrane and positively charge peptide. After initial electrostatic interaction, the AMP inserted into cell membrane though its hydrophobic portion [62]. Cationic state and hydrophobicity of the peptide are two major factor for such interaction.

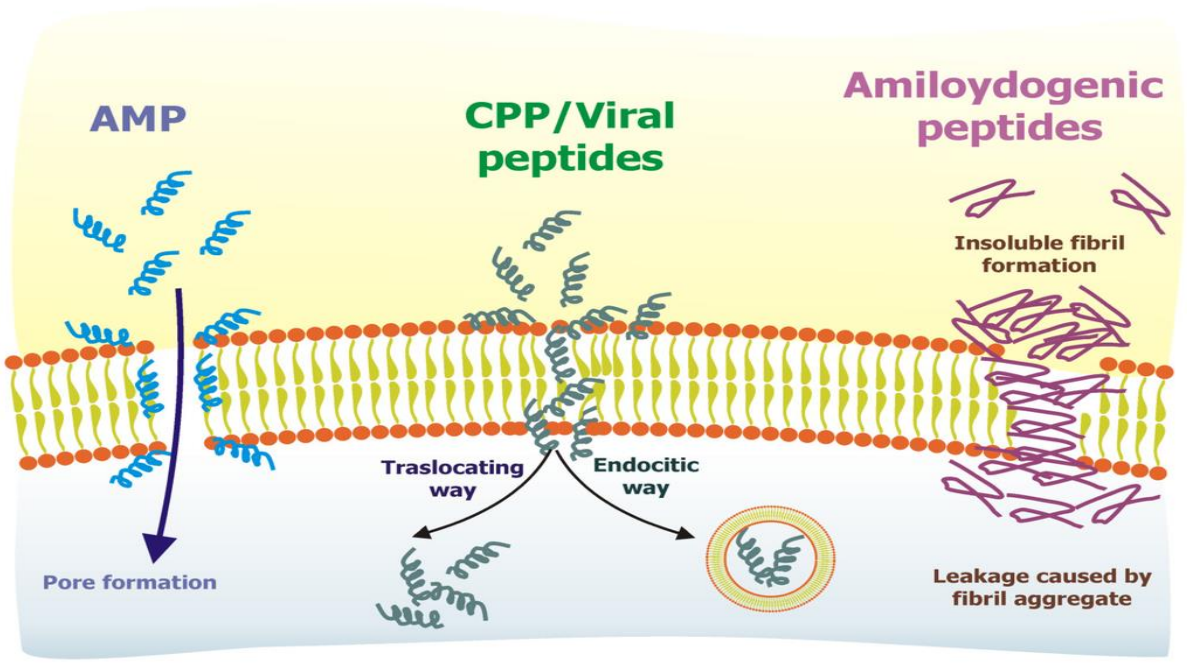


Figure-3: Mechanism of action of membrane-active peptides **2013,** Stefania Galdiero *et. al* 14,18758-18789

3.4.2) INTRACELLULAR ACTIVE AMPs

Rather showing the activity on cell membrane, some AMPs show intracellular activity by various ways like inhibit DNA, RNA and Peptide synthesis, binding to specific DNA sequence etc. [64,64]. For example, indolicin bind to specific DNA sequence and kill bacteria. PR-39 from pig intestine, kill bacteria by inhibits DNA and protein synthesis [65].

3.5) CATIONIC AMPs

Cationic peptide is most studied peptide till date and found in every complex organism [66]. Their length varies from 12 to 50 amino acid with 50% hydrophobic amino acid. The positive charge lies between 2-9 with relative abundance of lysine and arginine residue. These peptide form variety secondary structure like α-helix and β-sheet in which charge, polar and hydrophobic residue from patches on the surface of molecule. Earlier studies show that these peptides have role in modulating immunity in variety of organism like insect, mammal and amphibian. After injury or infection, the expression of cationic peptide rises. These peptides when interact with membrane, the hydrophobic domain integrate in core part of membrane while hydrophobic part will reside on surface. Cationic peptide has wide range of activity which include Gram positive, Gram negative, fungi and other organism.

3.6) BACTERIAL MEMBRANE

Bacterial cell consists of cell membrane, surrounded by complex cell wall which protect the bacteria from external harsh environment. Bacterial cell lack membrane bound organelle, hence look morphologically similar. Cell wall is buildup of peptidoglycan (also known as murein). In Gram positive bacteria, the thickness of peptidoglycan varies from 20-80 nm while in Gram negative it is 2-7 nm thick. NAG (N-acetylglucosamin) and NAM (N-acetyle muramic acid) are joined together by β-1,4 linkage and act as monomeric unit for peptidoglycan. Many Gram positive bacteria contain acidic substance known as teichoic acid. Teichoic acid are polyol phosphate polymers which is responsible for giving strong negative charge to cell wall.

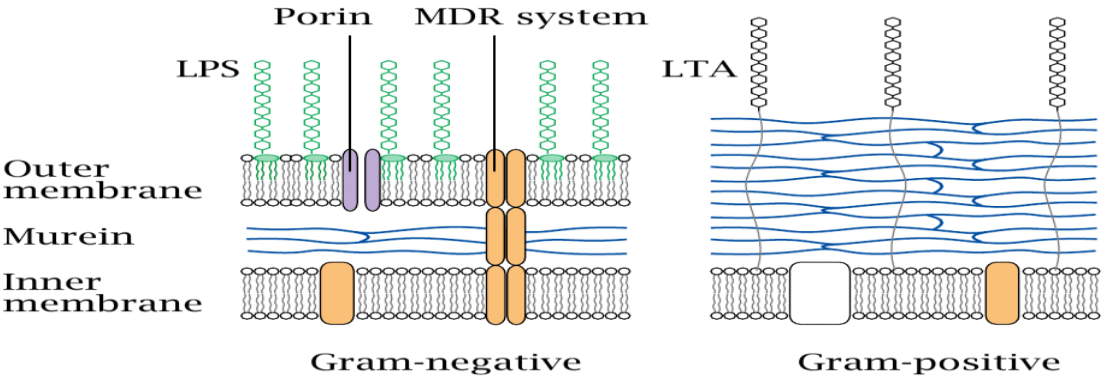


Figure-4: Bacterial membrane

(http://watcut.uwaterloo.ca/webnotes/Pharmacology/microbesBacterialCellWall.html)

3.7) FtsA PROTEIN

Most Prokaryotic divide by binary fission in which they give rise to two identical similar daughter nuclei. In bacteria for example *Escherichia coli* almost tens of protein involved in cell division [67]. Earlier studies shown that for septation, protein like FtsZ FtsA FtsI FtsK FtsL FtsN FtsQ FtsW and ZipA are localize to division plane [68]. Out of these nine, FtsA, FtsA and FtsQ are well studied [69]. The ftsZ gene encode a 40 kDa protein which play key role during cell division. FtsZ protein which is homologous to tubulin, form a dynamic ring like structure at the edge of constriction during cell division. The product of ftsA gene assume to be function after ftsZ in cell division. The product of ftsA gene is 45 kDa protein which is associated with cytoplasmic membrane. FtsA assist FtsZ protein during septum formation in cell division. Some studies reveal that the ratio of all the protein should be appropriate especially FtsA and FtsZ. Increase in level of FtsZ to that of FtsA could inhibit cell division and vice-versa.

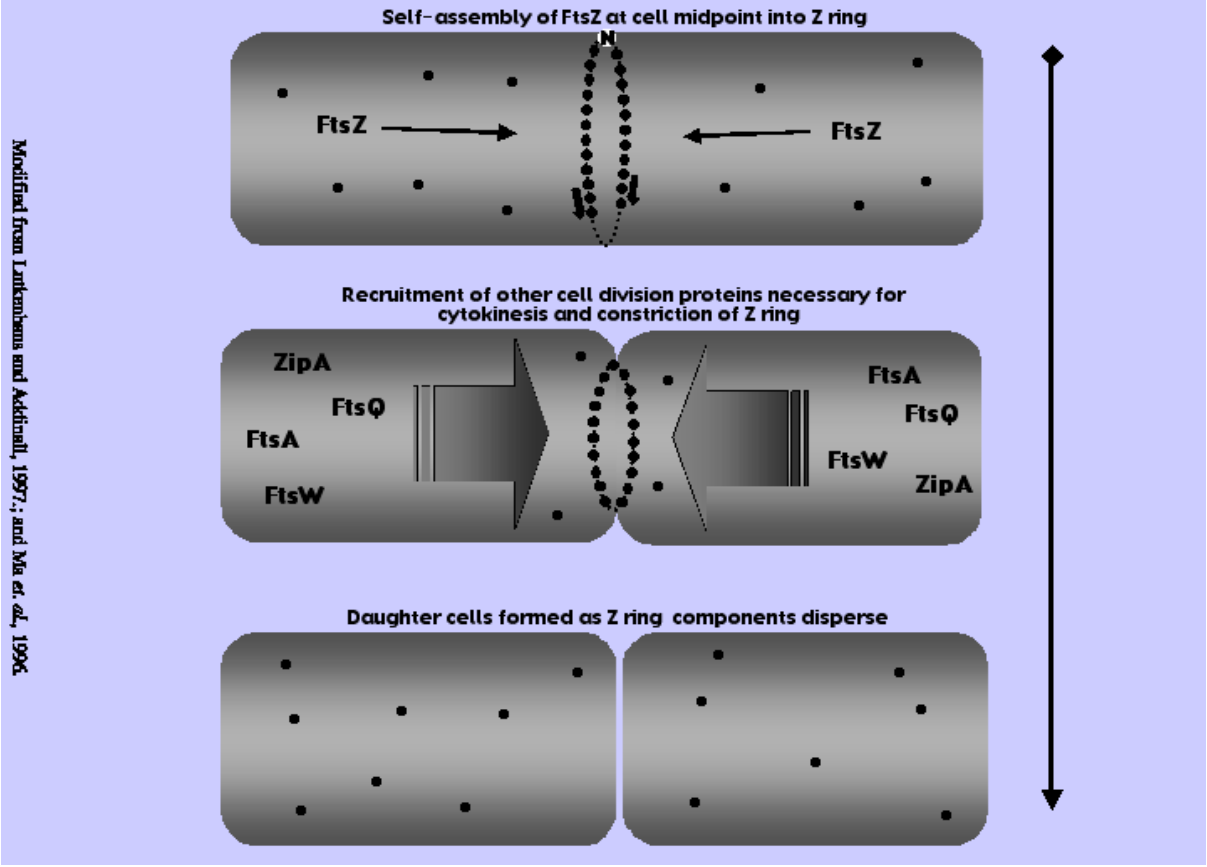


Figure-5: Self-assembly of FtsZ at cell midpoint into Z ring (Lamkenbens and Acktinall, 1997); Ma *et. Al*, 1996

4.) MATERIALS AND METHODOLOGY

4.1) CHEMICALS AND REAGENT

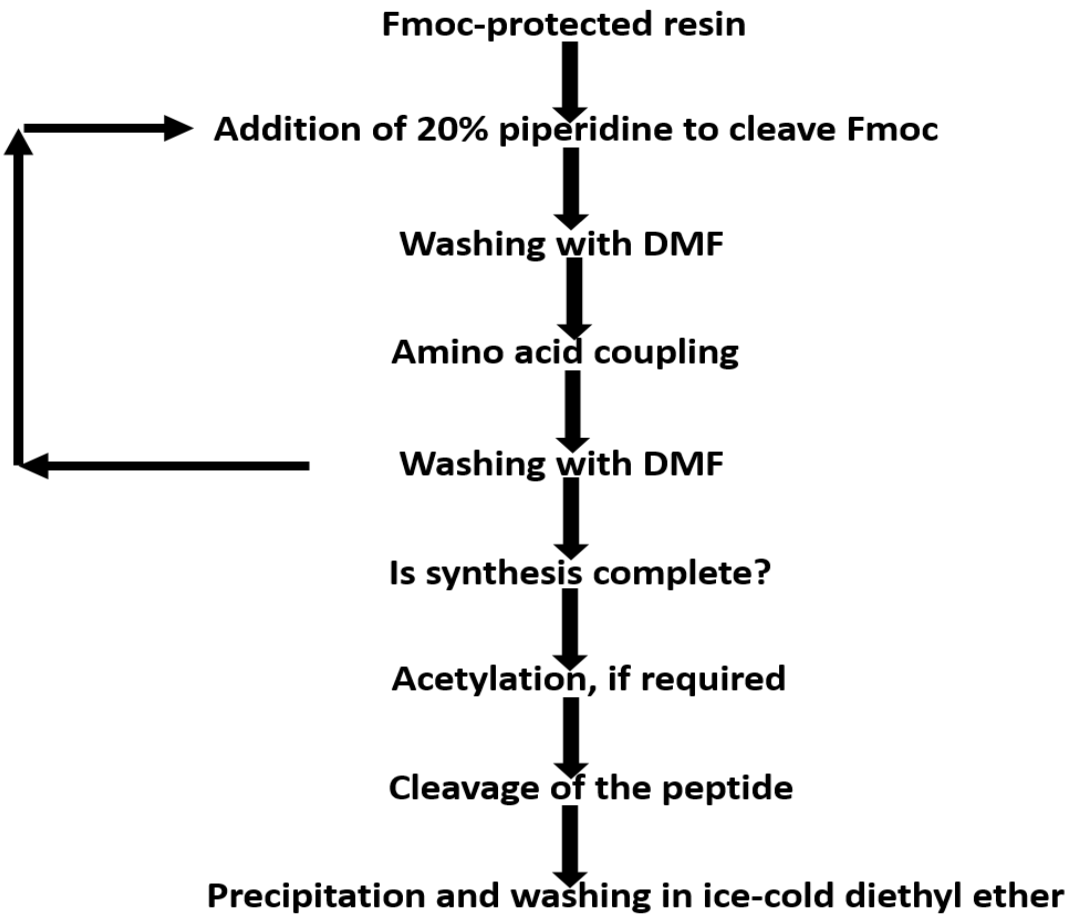
HBTU, HOBt, DMF, Rink amide resin, L-amino acids, DIPEA, nitrogen gas for purging, 20% piperidine, Ether, acetic anhydride, TFA, EDT, m-cresol, thioanisol, m-cresol, Ice, DMSO, acetone, miliq water were used during peptide synthesis. Phosphate buffer, nutrient broth, agar, autoclave water, 70% ethanol, methanol was used during antimicrobial assay.

4.2) MATERIALS

Eppendorf, Reaction vessel, tissue paper for cleaning, pipettes, tips, parafilm for covering, Silver foil, glass wool, pH paper, scissor, gloves, rubber band, vortex machine, centrifuge tube, aluminum foil, needle, test tube holder, centrifuge, ice bucket, plastic rod spatula.

4.3) METHODOLOGY

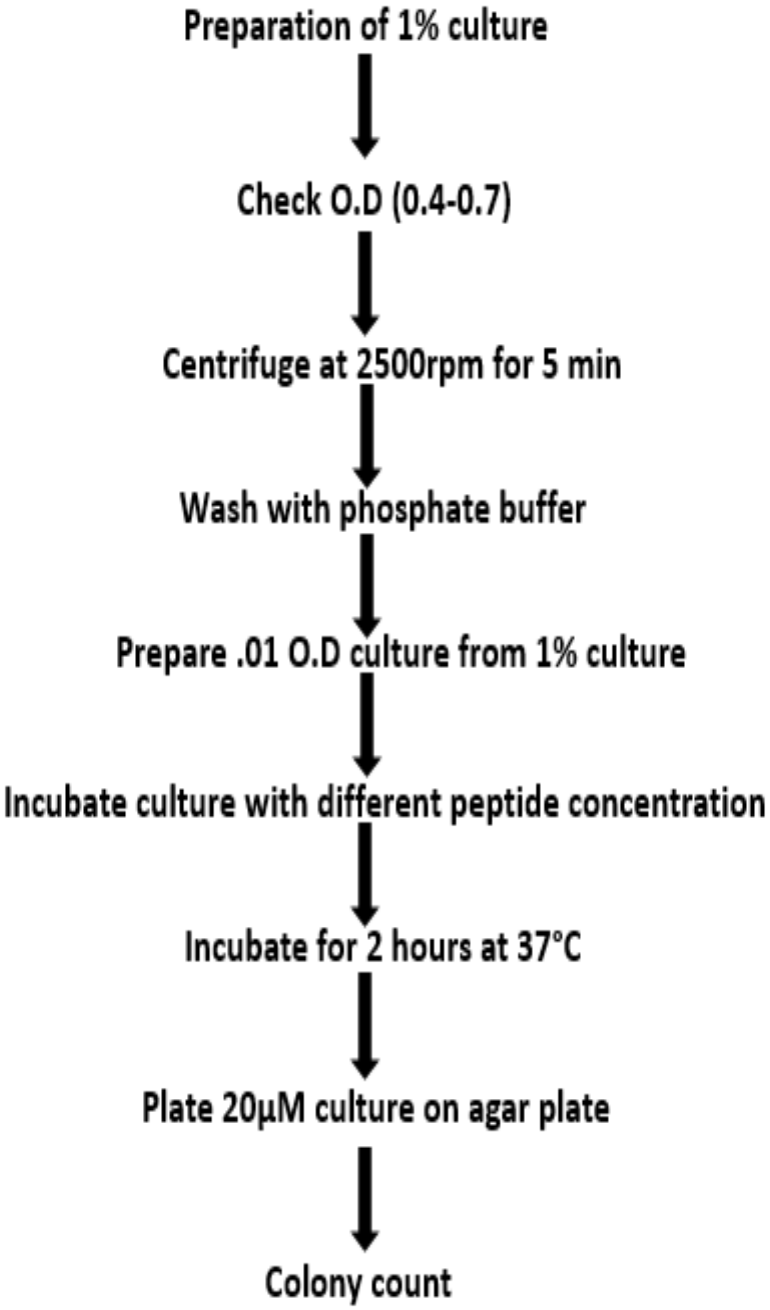
4.3.1) PEPTIDE SYNTHESIS



Flow chart of Peptide synthesis

For the synthesis of peptide solid phase peptide support method was used. In SPPS, the amino acid that will be at one end of the peptide is attached to a water-insoluble polymer and remains protected throughout the formation of the peptide, meaning both that fewer protection / deprotection steps are necessary and that the reagents can easily be rinsed away without losing any of the peptide**.**

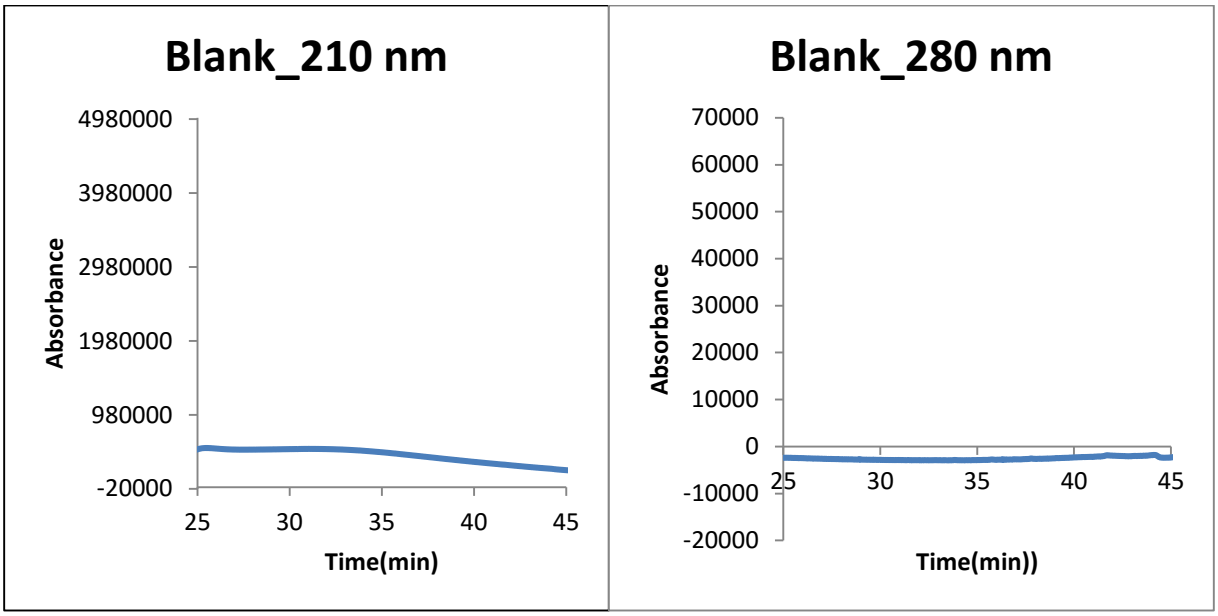
4.3.2) ANTIMCIROBIAL ASSAY



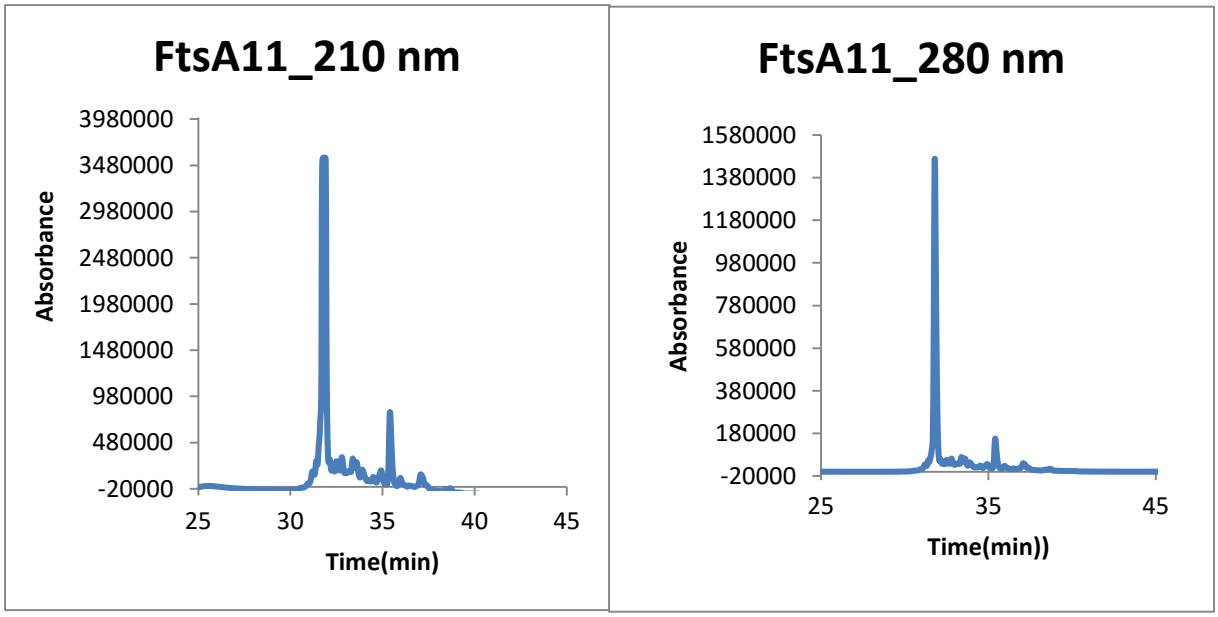
Flow chart of antimicrobial assay method

5) RESULT

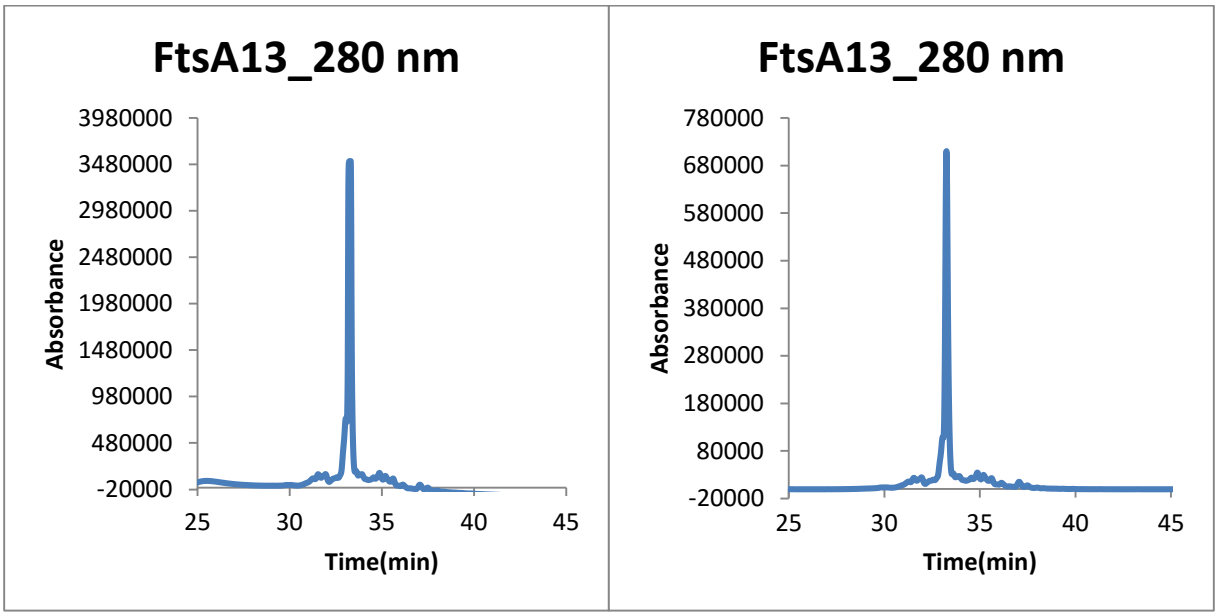
a) HPLC Chromatogram



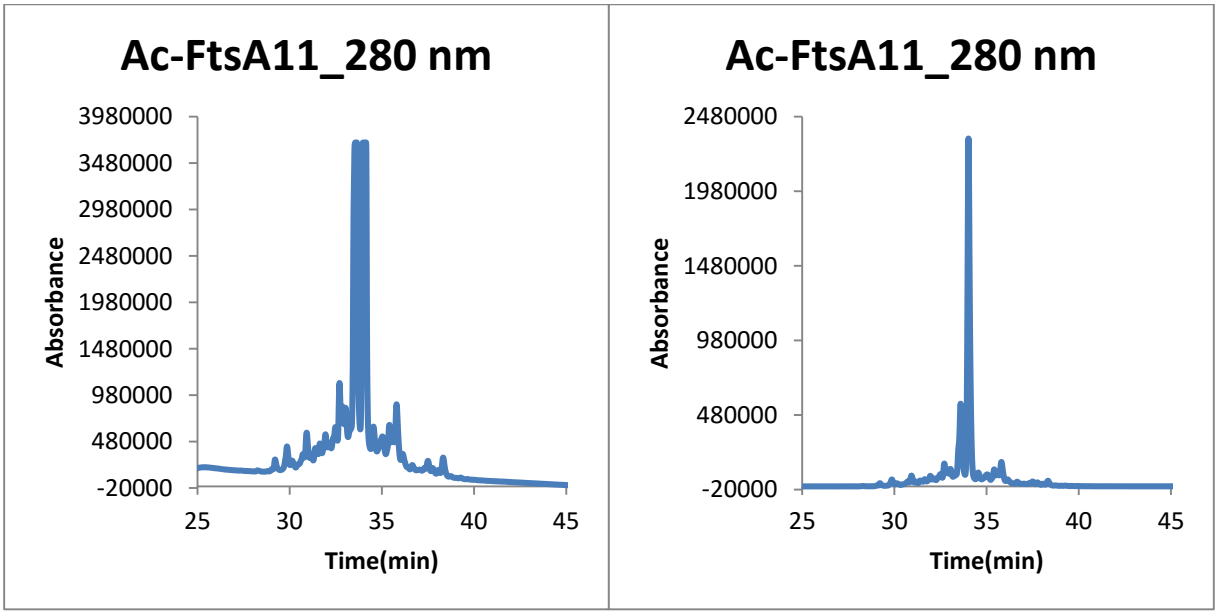
Graph-1: Chromatogram for blank (deionized water)



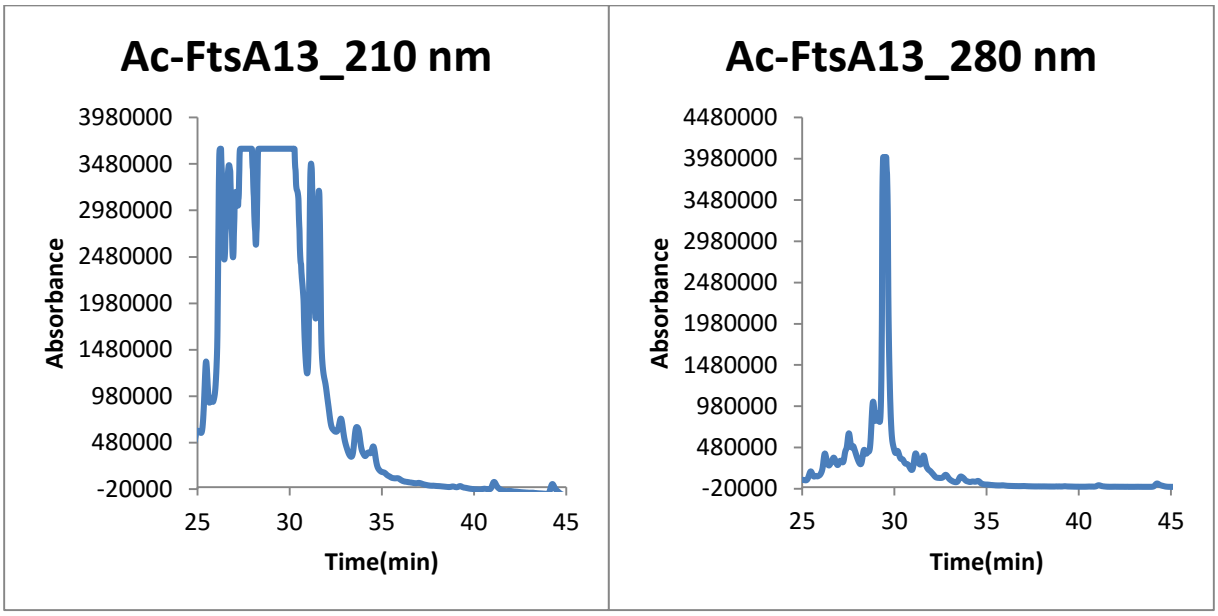
Graph-2: Chromatogram of FtsA11



Graph-3: Chromatogram of FtsA13



Graph-4: Chromatogram of Ac-FtsA11



Graph-5: Chromatogram of Ac-FtsA13

b) MALDI spectrum

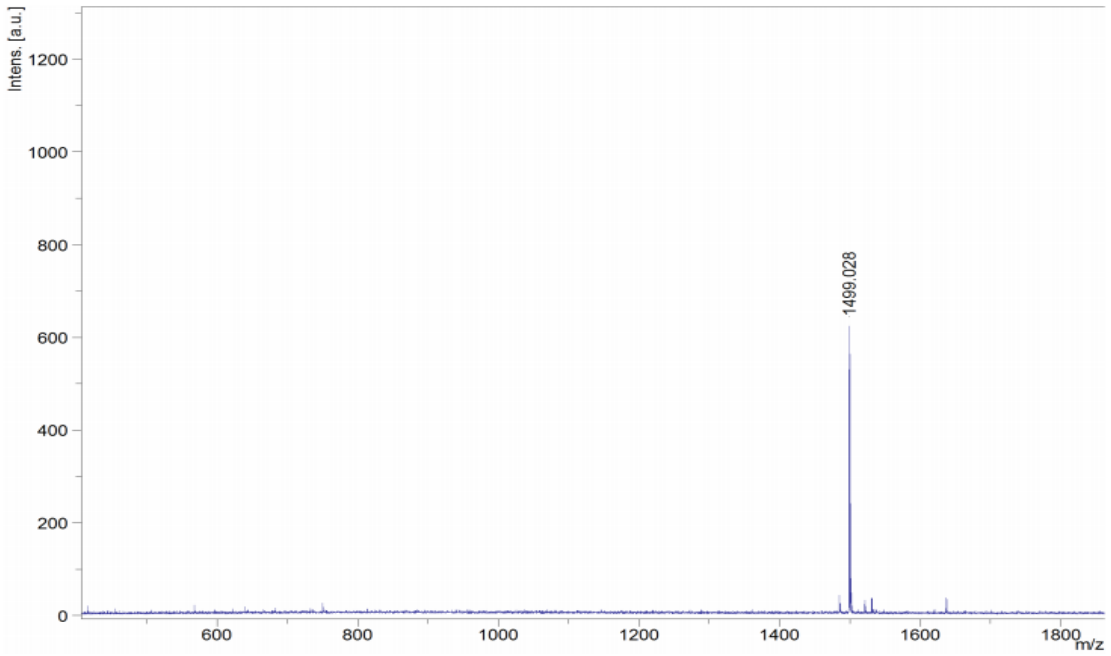


Figure-6: MALDI spectrum of FtsA11

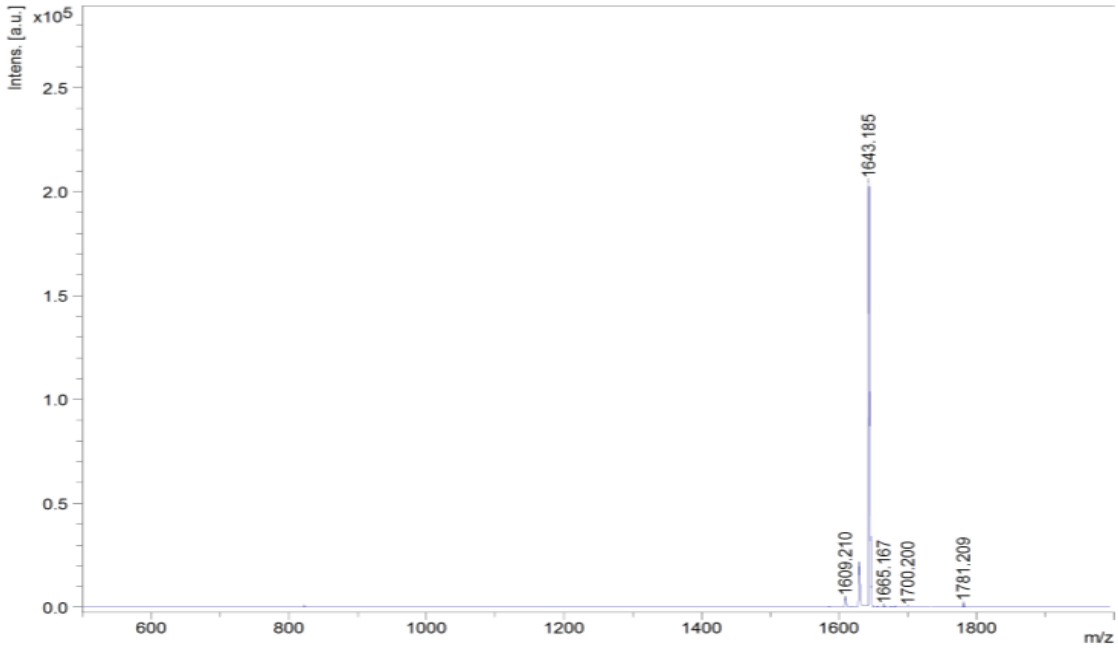


Figure-7: MALDI spectrum of FtsA13

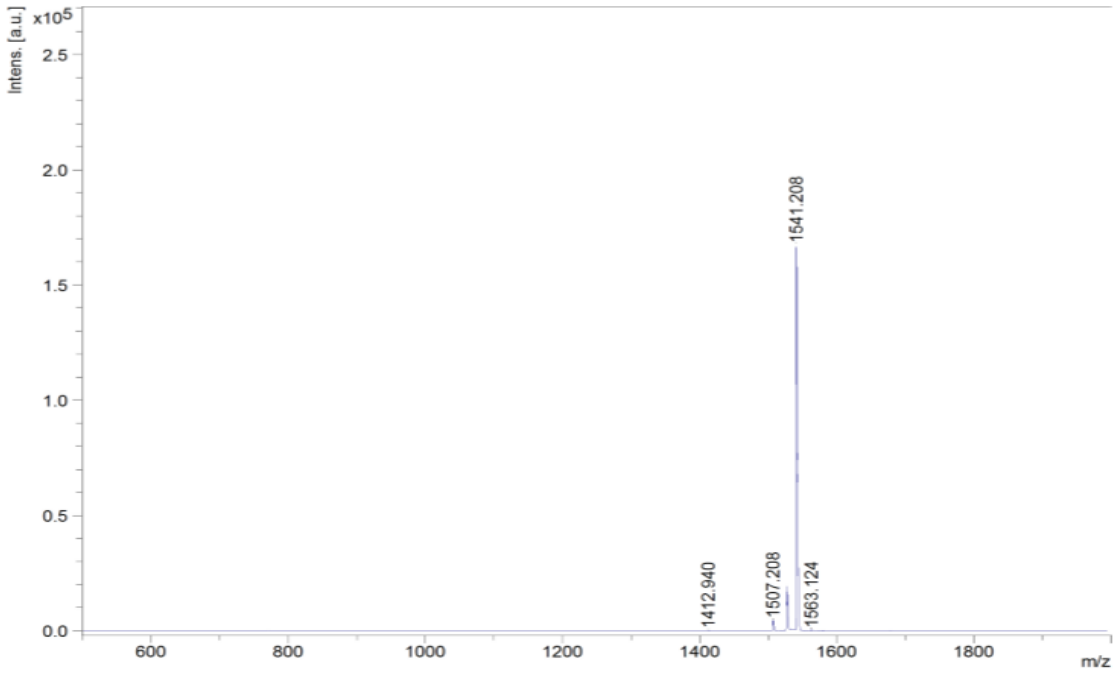


Figure-8: MALDI spectrum of Ac-FtsA11

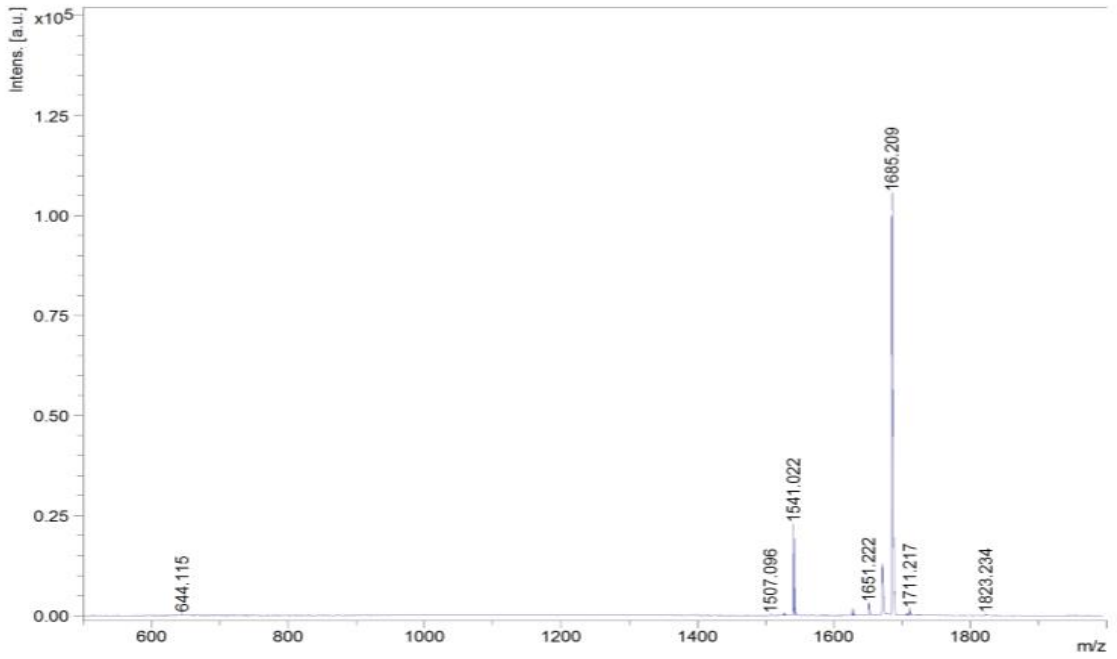


Figure-9: MALDI spectrum of Ac-FtsA13

Peptide	Expected mass (g/mole)	Observed mass(g/mole)
FtsA11	**1498**	**1499.028**
FtsA13	**1642**	**1643.185**
Ac-FtsA11	**1540**	**1541.208**
Ac-FtsA13	**1684**	**1685.209**

Table: -1 Showing observed and expected mass of peptides

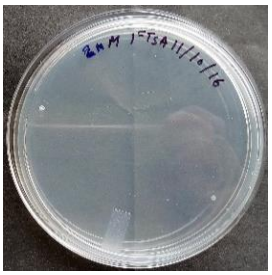
c) Antimicrobial assay on *E. coli*

***i) Escherichia coli treated* with FtsA11**

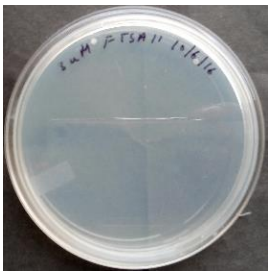
Negative control

0.5 µM

1 µM

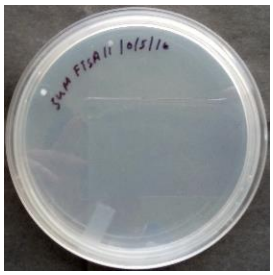


2 µM

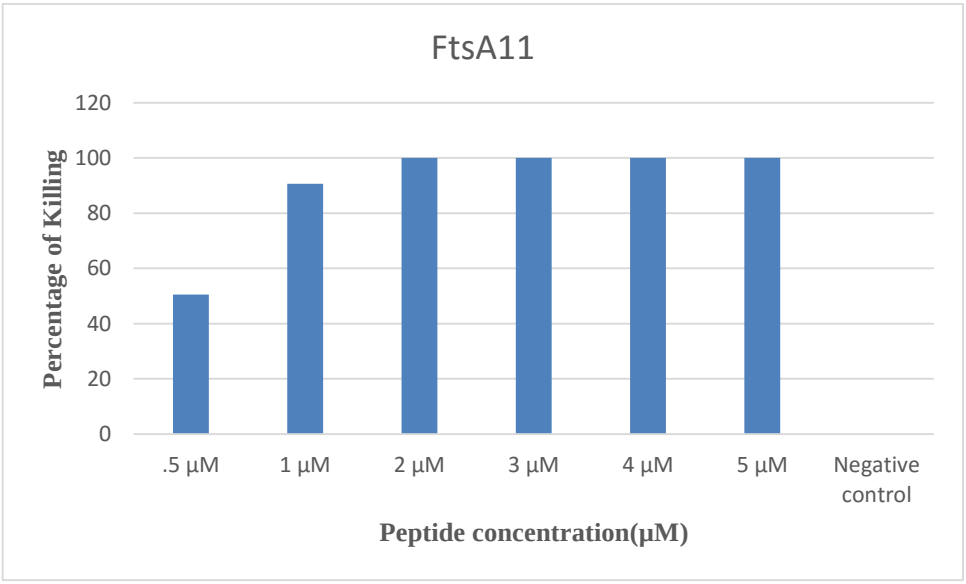


3 µM

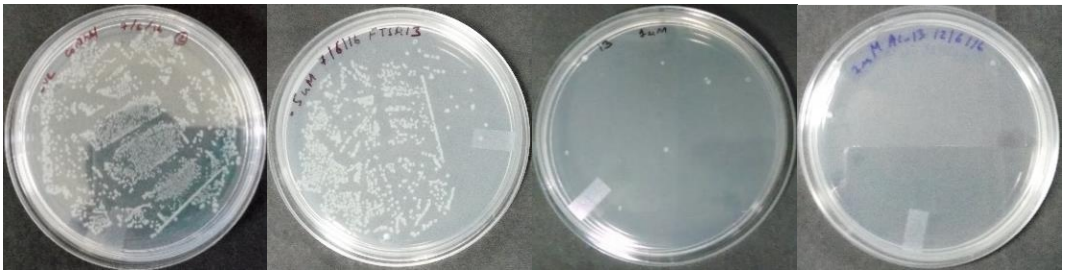
4 µM



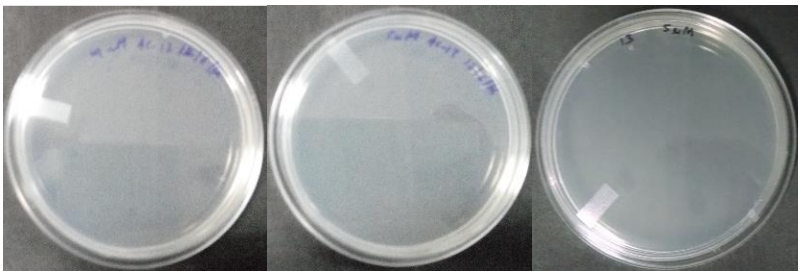
5 µM



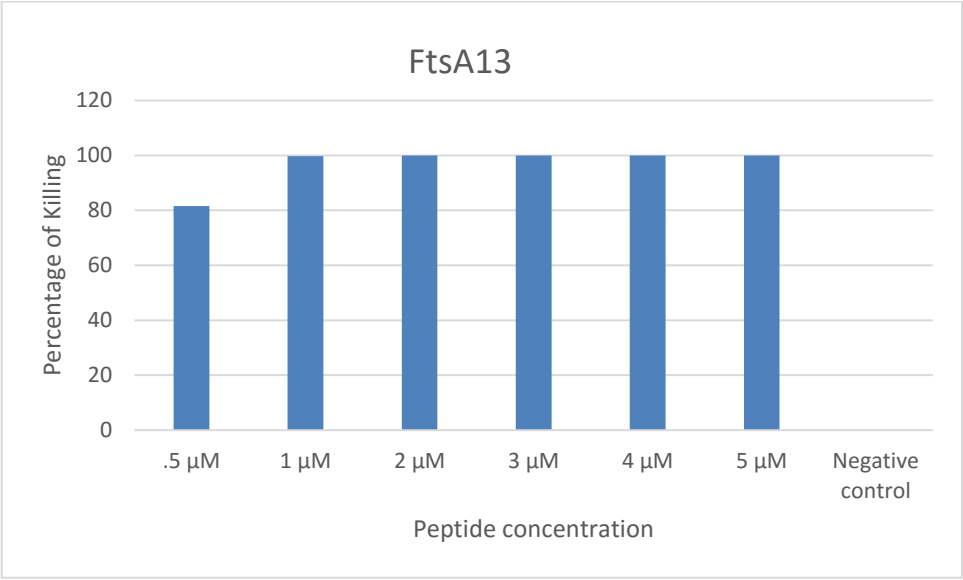
***ii) Escherichia coli* treated with FtsA13**



Negative control **0.5 µM** **1 µM** **2 µM**

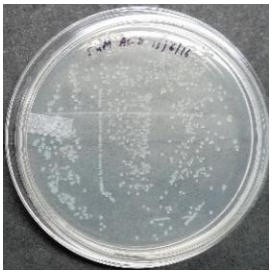


3 µM **4 µM** **5 µM**

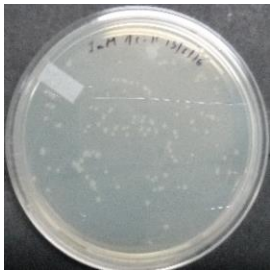


iii) Escherichia coli treated with Ac-FtsA11

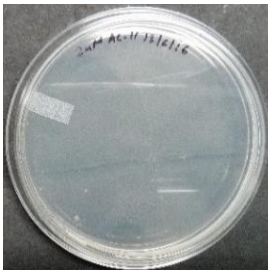
Negative control



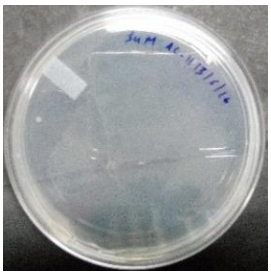
0.5 µM



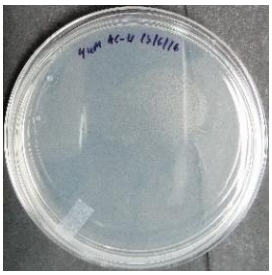
1 µM



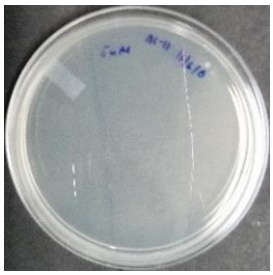
2 µM



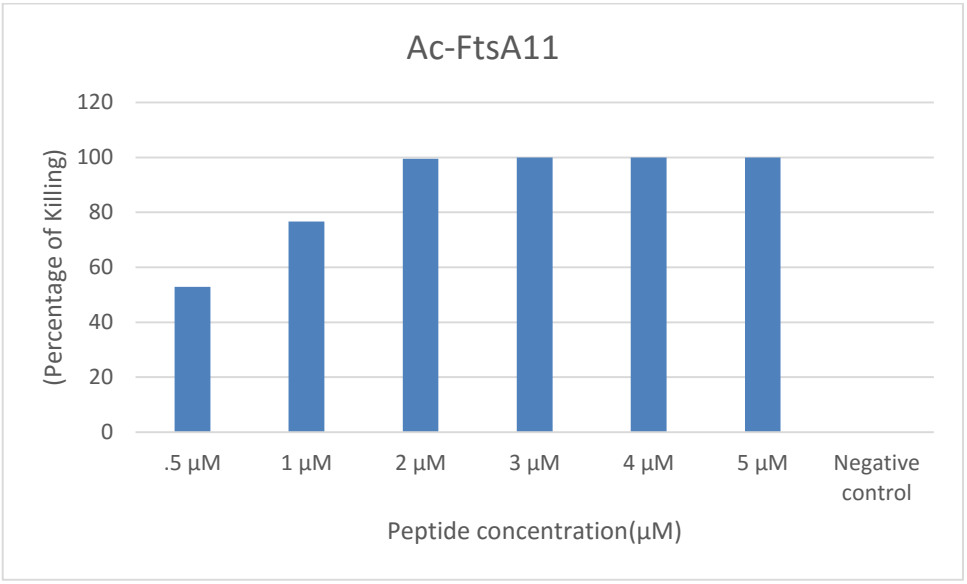
3 µM



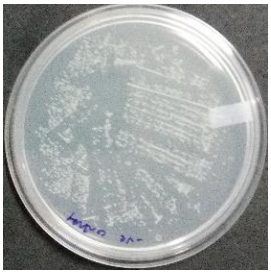
4 µM



5 µM

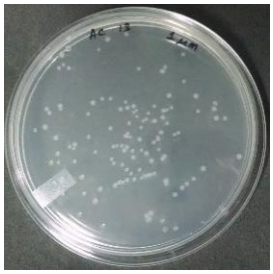


***iv) Escherichia coli* treated with Ac-FtsA13**

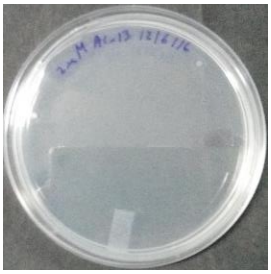


Negative control

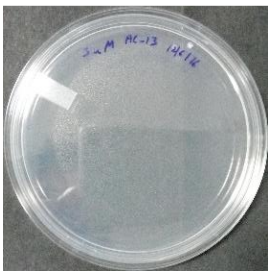
0.5 µM



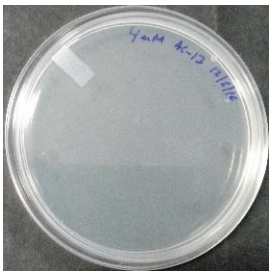
1 µM



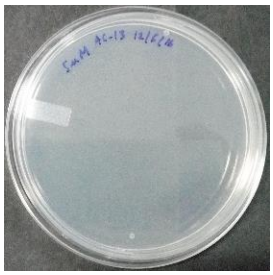
2 µM



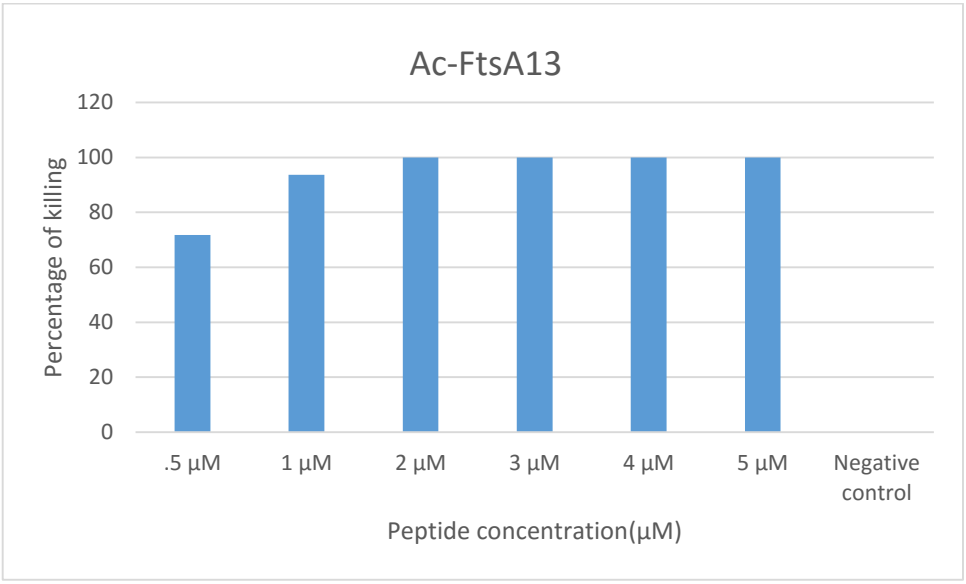
3 µM



4 µM



5 µM

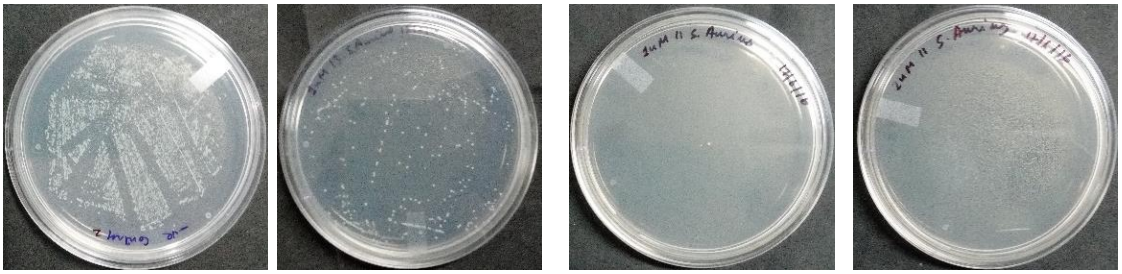


Colony Count for *E. coli*

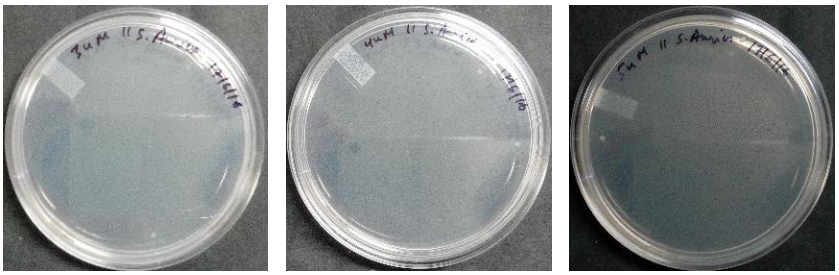
	No of colonies (E. coli)
Negative control 1	2064 approx.
Negative control 2	2039 approx.

No. of Viable colonies (count) for *E. coli*						
	0.5 µM Peptide concentration	1 µM Peptide concentration	2 µM Peptide concentration	3 µM Peptide concentration	4 µM Peptide concentration	5 µM Peptide concentration
FtsA11	510	160	0	0	0	0
Ac-Ftsa11	670	190	0	0	0	0
FtsA13	400	2	0	0	0	0
Ac-FtsA13	650	143	0	0	0	0

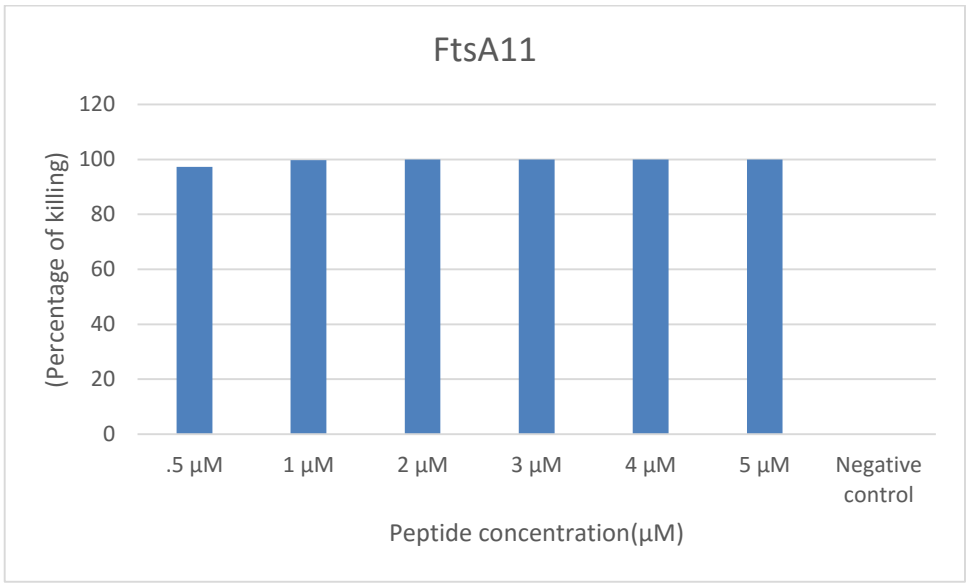
***v) Staphylococcus aureus* with FtsA11**



Negative control **.5 µM** **1 µM** **2 µM**

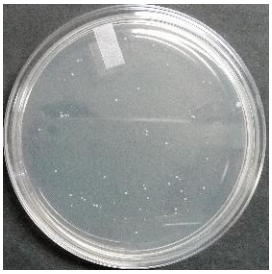


3 µM **4 µM** **5 µM**

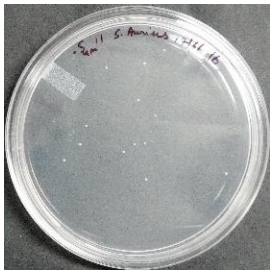


vi) Staphylococcus aureus treated with FtsA13

Negative control

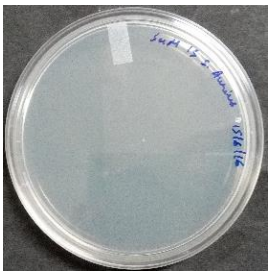


0.5 µM

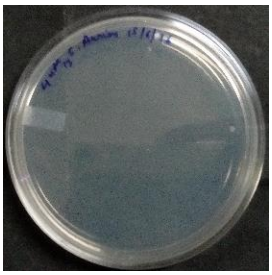


1 µM

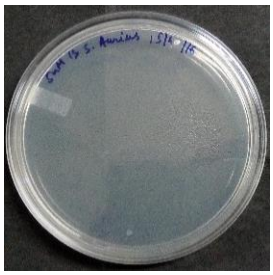
2 µM



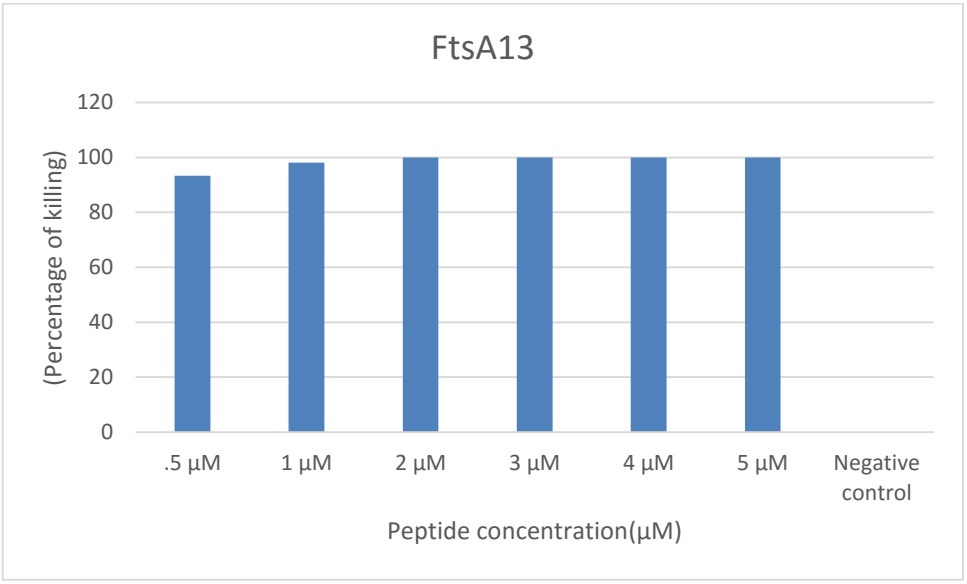
3 µM



4 µM



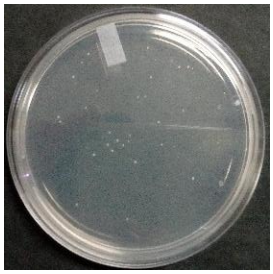
5 µM



***vii) Staphylococcus aureus* s treated with Ac-FtsA11**

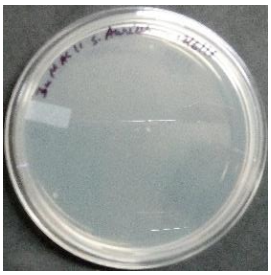
Negative control

0.5 µM

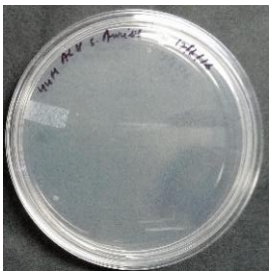


1 µM

2 µM

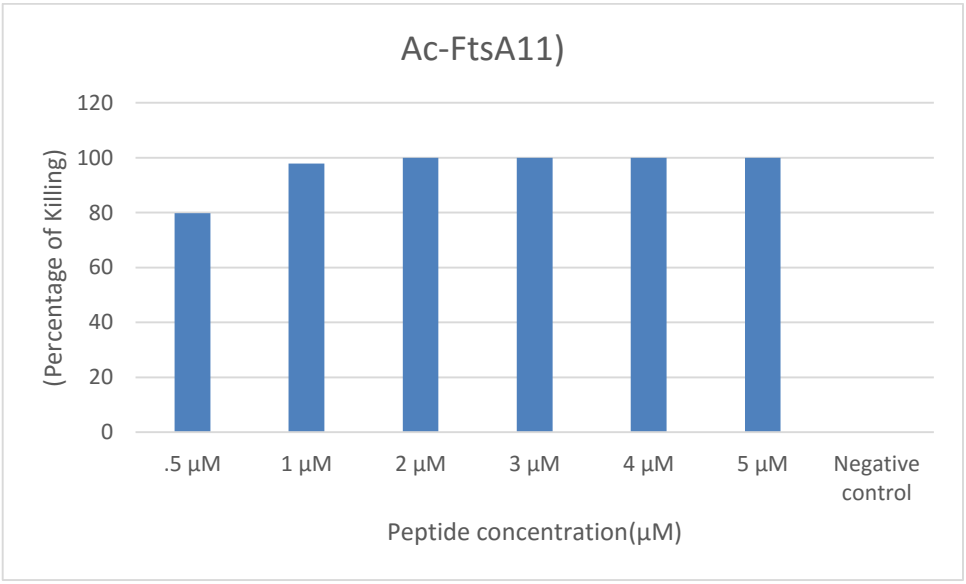


3 µM

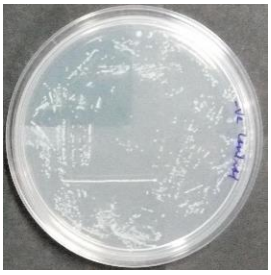


4 µM

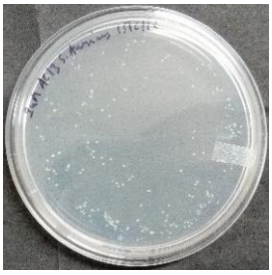
5 µM



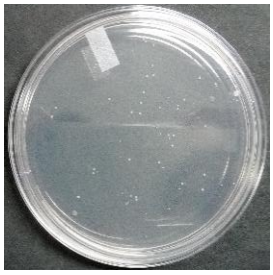
***viii) Staphylococcus aureus* treated with Ac-FtsA13**



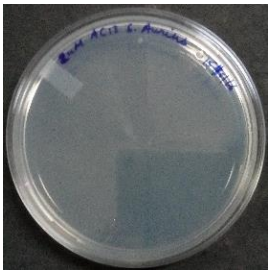
Negative control



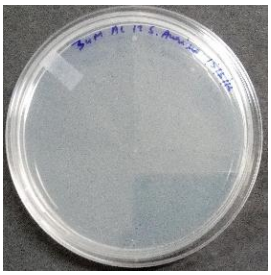
0.5 µM



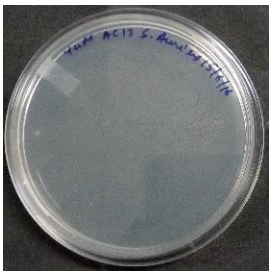
1 µM



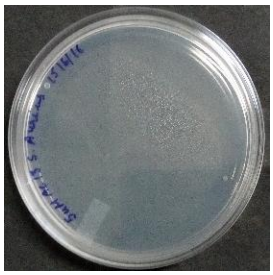
2 µM



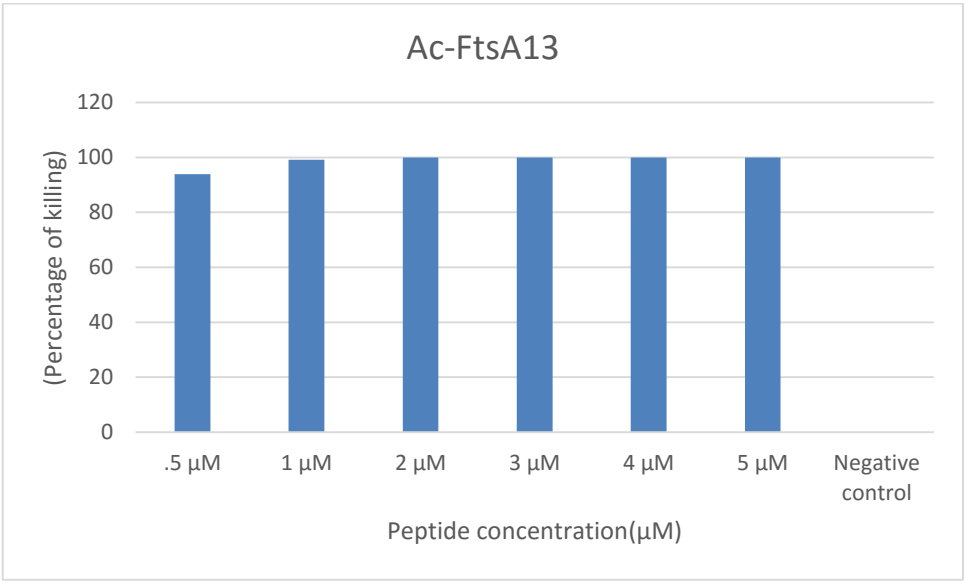
3 µM



4 µM



5 µM



Colony Count for *S. aureus*

	No of colonies (*S. aureus*)
Negative control 1	1990 approx.
Negative control 2	1995 approx.

No. of Viable colonies (count) for *S. aureus*						
	0.5 µM Peptide concentration	1 µM Peptide concentration	2 µM Peptide concentration	3 µM Peptide concentration	4 µM Peptide concentration	5 µM Peptide concentration
FtsA11	135	3	0	0	0	0
Ac-Ftsa11	260	35	0	0	0	0
FtsA13	30	14	0	0	0	0
Ac-FtsA13	60	32	0	0	0	0

d) AFM analysis

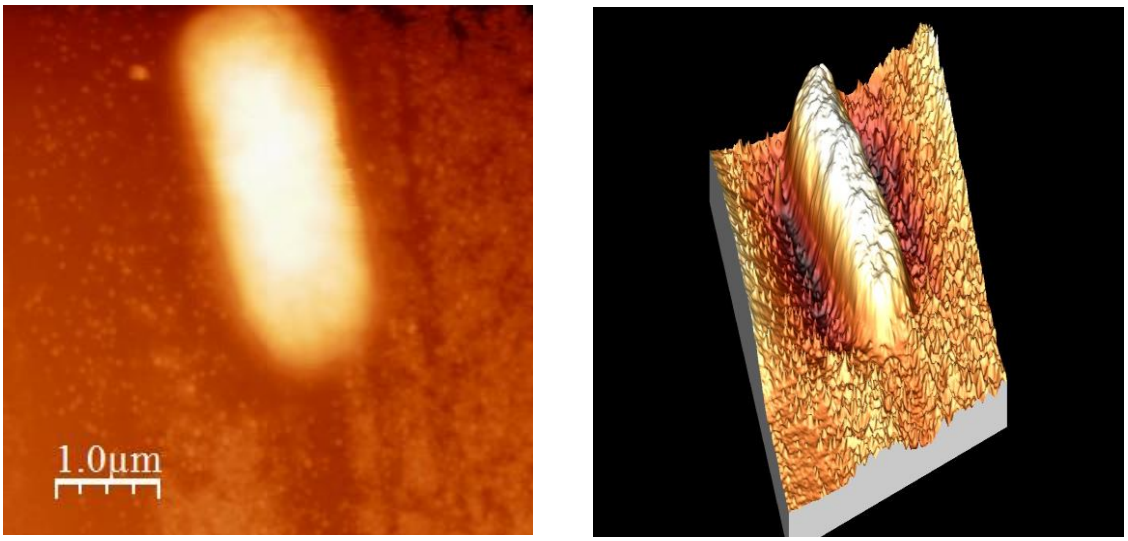


Figure-10: AFM images of untreated *E. coli* bacteria

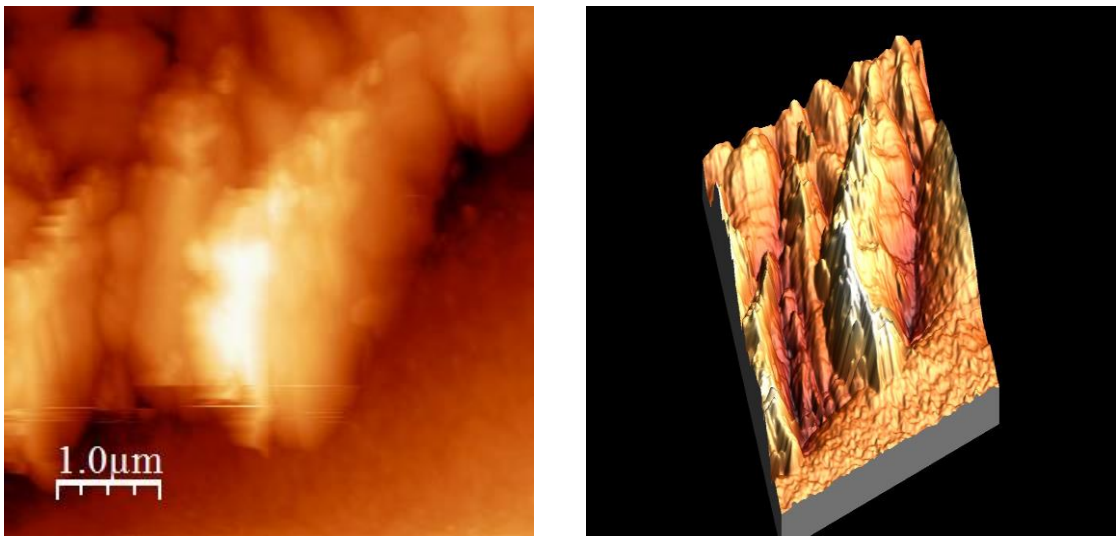


Figure-11: AFM imaged of Peptide(FtsA13) treated *E. coli* bacteria

6) DISCUSSION

FtsA peptide fragment that we have design have ability to form amphipathic helical structure. Antimicrobial activity was studied against Gram-negative (*E. coli)* and Gram-positive (Staphylococcus *aureus).* All four peptides (FtsA13, FtsA11, Ac-FtsA11, Ac-FtsA13) showed significant antimicrobial activity against both Gram-positive and Gram-negative bacteria. Among all the four peptides, higher charge containing FtsA13 showed higher antimicrobial activity compared to less charge containing Ac-FtsA11 and Ac-FtsA13 peptide. Earlier studies have shown that highly charged peptide bind more efficiently comparison to less charged containing peptide. Peptide fragments that we have synthesized has +5 charge for the peptides with free amino terminus while +4 for the acetylated ones. Therefore, the reason behind higher activity of FtsA13 is relatively higher binding capacity due to the greater electrostatic interaction. Arginine has ability to form bidentate hydrogen bond with phosphate group and other negatively charge moieties in membrane. Furthermore, presence of Tryptophan in peptide fragment enhance binding of peptide due to its hydrophobic nature.

7) CONCLUSION

Amphipathic cationic AMPs are the most widely distributed and found in almost all living organism like bacteria, fungi, amphibian and humans. Amphipathic nature allows the hydrophobic residue towards the lipid membrane and promotes its permeabilization. We identified an amphipathic stretch in the *E. coli* FtsA protein and designed four sequences for the antimicrobial assays. All the designed peptides showed significant antimicrobial activity. The minimal inhibitory concentration of all the peptides (FtsA11 FtsA13 Ac- FtsA11 Ac-FtsA13) were within 2 μM range. We conclude that the amphipathic FtsA peptide fragments that we have designed can act as potential antimicrobial agents. This suggests that the bacterial sequences could be developed as the potent weapons against them.

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