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Molecular cloning, expression and *in vitro* analysis of soluble cationic synthetic antimicrobial peptide from salt-inducible *Escherichia coli* GJ1158

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ABSTRACT

Antimicrobial peptides are the upcoming therapeutic molecules as alternative drugs to the existing antibiotics owing to their potent action against pathogenic microorganisms. In this study, to obtain an antimicrobial peptide with a broad range of activity, the synthetic cationic antimicrobial peptide was designed by using *in silico* tools viz., antimicrobial peptide database, protparam, hierarchical neural network. Later, the peptide was translated back into a core nucleotide sequence and the gene for the peptide was constructed by overlapping PCR. The amplified gene was cloned into pRSET–A vector and transformed into salt inducible expression host *E. coli* GJ1158. The expression results show high yields of soluble recombinant fusion peptide (0.52 g/L) from salt-inducible *E. coli.* The recombinant peptide was purified by the IMAC purification system and cleaved by enterokinase. The digested product was further purified and 0.12 g/L of biologically active recombinant cationic antimicrobial peptide was obtained. *In vitro* analysis of the purified peptide demonstrated high antimicrobial activity against both Gram positive and Gram negative bacteria devoid of hemolytic activity. Therefore, this synthetic cationic antimicrobial peptide could serves as an promising agent over chemical antibiotics. In this study, a synthetic cationic antimicrobial peptide was designed, cloned and expressed from salt-inducible *E. coli* GJ1158 using cost effective media in the large scale production of antimicrobial peptide and its biological activity was analysed against different Gram positive and negative organisms.

Key words: synthetic antimicrobial peptide, *in silco* techniques, pRSET-A, IMAC purification, entirokinase, antimicrobial activity

Introduction

The discovery, development and clinical exploitation of antibiotics are one of the most significant medical advances in twentieth century (Hancock, 1997). However, the usage of antibiotics towards potential pathogens have been over used or used improperly, owing to resistance or multidrug resistance, which is emerged rapidly in many microbial species (Robert & Moellering, 2003). The pharmaceutical industry has continuously met this need by modifying the existing antibiotics. Despite the success to date in antimicrobial development, the inexorable, ongoing

emergence of resistance worldwide continues to spur the search for novel anti-infectives to replace or supplement conventional antibiotics. So, there is an immediate demand to combat antibiotic-resistant microbes and scientists have begun to search for antimicrobial drugs in vertebrates, invertebrates and even bacteria and fungi living in the earth's most extreme environments (Bals, 2000).

Antimicrobial peptides are the upcoming therapeutic molecules as alternative drugs to the antibiotics. These peptides have a good scope in current antibiotic research (Raventos et al., 2005). Antimicrobial peptides work against a wide range of pathogenic organisms in different ways and mechanisms where there will be almost negligible of getting resistance by the organism against these peptides. Usually these antimicrobial peptides bear the size of 10-50 amino acids and show its antimicrobial activity by interacting, disturbing their cell wall, cell membrane and at various intracellular targets (Epand & Vogel, 1999). These antimicrobial peptides are identified in various prokaryotic and eukaryotic sources, but all these molecules are facing the problems viz., fast proteolysis, a poor absorption due to their hydrophilicity, and high cost of development, systemic and local toxicity (Andersons et al., 1991). To avoid these hurdles, a progressive research is going on to produce the peptide antibiotics at large scale using recombinant DNA technology. The most efficient method should be from *Escherichia coli* because of its well understood genetic nature and most inexpensive mode of production (Cipakova et al., 2004; Hwang et al., 2001; Lee et al., 2000; Miller et al., 1998; Pyo et al., 2004; Rao et al., 2004; Zhang et al., 1998).

The present work has been carried out to design, synthesize an array of antimicrobial peptides based on following parameters i.e., conformation (X), charge (Q), amphipathicity (A), hydrophobicity (H), polar angle (θ) (Yeaman & Yount, 2003). These peptides were analyzed by using various *in silco* techniques. The ideal peptide was amplified by PCR methodology and expressed in a non–pathogenic industrially feasible *E. coli* expression system and was analyzed by *in vitro* plate methods for its antimicrobial activity against different microorganisms.

Materials and Methods

Strains, plasmids and enzymes

E. coli DH5α (MTCC 1652), *Pseudomonas putida* (MTCC 102), *Micrococcus luteus* (MTCC 106), *Bacillus subtilis* (MTCC 511), *Staphylococcus aureus* (MTCC 87) and *E. coli* K12 (MTCC 1302) are procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *E. coli* GJ1158 is kindly donated by Prof. J. Srinivasa Rao, Department of Chemical Engineering, Bapatla College of Engineering, Bapatla. *E. coli* DH5α is used as the primary host for construction and propagation of plasmids. *E. coli* GJ1158 is used for peptide expression. Industrial feasible vector pRSET-A is procured from Invitrogen. All molecular biology grade enzymes, oligonucleotides and purification kits are procured from Sigma-Aldrich, Bangalore, India.

Media preparation and culture conditions

LBON (LB without NaCl) was used as seed culture. The expression studies were carried out in Glucose yeast extract medium (GYEON): Na_2HPO_4 – 6 g/L, KH_2PO_4 – 3 g/L, NH_4Cl – 1 g/L, Yeast extract – 5 g/L, Glucose – 5 g/L, 1M $MgSO_4$ – 2 mL, TMM – 1 mL ($Al_2(S0_4)_3.7H_2O$ – 10 mg/L, $CuSO_4.H_2O$ – 2 mg/L, H_3BO_4 – 1 mg/L, $MnCl_3.4H_2O$ – 20 mg/L, $NiCl_2.6H_2O$ – 1 mg/L, $Na_2MoO_4.2H_2O$ – 50 mg/L, $ZnSO_4.7H_2O$ – 50 mg/L, $FeSO_4$ – 50 mg/L). The initial pH of the medium was not adjusted to any value before autoclaving at 121°C for 15 to 20 min resulting in an initial pH value in the range of pH 6.9 to 7.2. The autoclaved medium was inoculated aseptically with 4% of overnight fresh culture. The flask was kept on rotary shaker at 37°C. NaCl was excluded while working with *E. coli* GJ1158.

Computational tools

Online computational tools viz., PROTPARAM, HNN and ANTIMICROBIAL PEPTIDE DATABASE were used to check various physicochemical properties, secondary structure and antimicrobial property of designed peptides. Initially, we considered factors like conformation, charge, hydrophobicity and polar angle are chosen randomly to analyze five peptide sequences. Often the peptide sequences were submitted to PROTPARAM and it computes various physicochemical properties. Later, the secondary structure of the peptide was predicted using HNN, which plays a crucial role in antimicrobial action of the peptide. At last, antimicrobial peptide database was used to predict the antimicrobial property of the chosen peptide.

Construction of synthetic gene by PCR

The chosen peptide sequence was translated back to get the nucleotide sequence, which is the prerequisite for constructing the synthetic gene to the peptide by PCR methodology. The nucleic acid sequence corresponding to the

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synthetic peptide cassette was synthesized according to the codon bias of *E. coli.* As per the design a 78 bp sized core nucleotide with two forward and reverse overlapping primers carrying the enzyme sites *Bam* HI and *Hind* III at 5' regions were used to amplify the synthetic gene. PCR program was carried out at 95°C for 5 min, 32 cycles at 95°C for 1 min, 64°C for 1 min, 72°C for 1 min and a final extension cycle at 72°C for 10 min. Later, the PCR product was purified by using Qiagen PCR purification kit.

Cloning of synthetic gene into expression plasmid pRSET-A

The plasmid vector pRSET-A, a 2.9 kb cytosolic bacterial expression vector with T7 promoter was used for cloning the gene of interest. The vector DNA (pRSET-A) and the insert DNA (Synthetic gene) were digested with *Bam*HI and *Hind*III restriction enzymes and ligated using T4 DNA ligase. The ligation mixture was transformed into the *E. coli* DH5α competent cells for propagation of the recombinant plasmid. A positive control plasmid (pGEX) was used in the experiments to verify the transformation efficiency. Cells with no DNA added to serve as a negative control. The recombinant plasmid (pRSET-A – cationic antimicrobial peptide) was confirmed by using PCR with gene specific primers and restriction digestion.

Heterologous expression using E. coli GJ1158

The recombinant DNA was isolated and transformed into *E. coli* GJ1158 for further expression studies. The recombinant bacterial expression host was grown in LBON media supplemented with ampicillin (100 μg/ml) at 37°C. When the OD reaches to 0.6 to 0.8, 5% of grown culture was aseptically transferred to GYEON medium with antibiotic (100 μg/ml) and incubate on shaker to an OD_{600} of 1.0. The culture broth was induced with 200 mM NaCl and incubated at 37°C for the production of recombinant peptide. After 4 h of induction, the induced culture was harvested by centrifugation at 13,800 rpm for 10 min. The pellet thus obtained was dissolved in PBS and equal concentration of SSB. Later analyze the peptide expression using 18% tricine–SDS–PAGE and peptide concentration was determined by Lowry method using BSA as a standard.

Soluble form of the peptide

In order to know the soluble form of the peptide, after induction, 10 ml of the induced cell culture was pelleted down and STE buffer (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA) was added to the pellet and mixed well. The samples were centrifuged at 4°C at 13,800 rpm for 20 min and supernatant labelled as S-1. In continuation, NTE buffer was added to the pellet and subjected to sonication (10 cycles on time, 10 cycles off time and 5 min total time). The sonicated samples were centrifuged at 13,800 rpm for 20 min at 4°C and the supernatant labelled as S-2. NTET buffer (100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, 1% Triton X-100 and 2 M Urea) was added to the pellet and mixed well and centrifuged at 13,800 rpm for 20 min at 4°C. The supernatant was labelled as S-3 and solubulizing buffer (50 mM NaH_2PO_4, 20 mM Tris, 100 mM NaCl and 8 M Urea) was added to the pellet and labelled as S-4. Peptide analysis was done using 18% separating gel to all the tubes labelled S1, S2, S3 and S4.

Purification of recombinant cationic antimicrobial peptide

The recombinant peptide carrying an N-terminal 6x His tag was expressed in *E. coli* GJ1158 and purified using affinity chromatography with Ni^{2+}- resin under hybrid conditions. In order to purify recombinant peptide, 400 mg wet cells were obtained from a freshly grown 50 ml culture in LBON and GYEON, containing the appropriate antibiotic. The cells were harvested and centrifuged at 13,800 rpm for 20 min at 4°C and the pellet attained was suspended in 6 ml of guanidinium lysis buffer containing 7 M guanidine HCl, 22 mM sodium phosphate, 510 mM NaCl with pH 7.8 at 4°C for 1 h. The cell lysate was again centrifuged at 13,800 rpm for 20 to 15 min at 4°C. A volume of 5 ml cell lysate was purified on a column containing 2 ml resin equilibrated with denaturing binding buffer, incubated for 45 min at room temperature with gentle hand shaking for several times. The column was washed with 2 ml of denaturing wash buffer and twice with 2 ml of native wash buffer. The bounded peptide was eluted with 2 ml of native elution buffer and analysed on 18% separating gel against to protein marker.

Antimicrobial assay and determination of minimal inhibitory concentration (MIC)

Antimicrobial activity was tested by top agar assay and also by the radial diffusion method on both Gram-positive (*Staphylococcus aureus)* and Gram-negative *(Pseudomonas putida* and *E. coli* K12) bacteria (Asoodeh et al., 2004). Bacteria were first grown in LB (Luria-Bertani) broth to an OD_{600} of 0.8. A 10 μL of the bacterial culture was added to 8 mL of LB broth with 0.7% agar and poured in petri dish containing 25 mL of 1.5% LB agar. After the top agar harden, a 10 μL of the test sample was added on the surface of the top agar and completely dried before incubating overnight at 37°C.

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If sample containing antimicrobial activity, a zone of clearance was formed on the surface of the top agar representing the inhibition of bacterial growth and ampicillin was used as a control.

The peptide was further tested for its minimal inhibitory concentration (MIC) on various bacterial strains (Xu et al., 2006). Various concentrations ranging from 5 µg to 25 µg/ml of peptide (5 µg, 10 µg, 15 µg, 20 µg and 25 µg/ml) was added to the optimal diluted bacterial cultures of both Gram positive and Gram negative bacteria and incubated at 37°C for 3 h. Further, the three bacterial cultures of above mentioned concentrations were transferred, spread on agar plates and incubated at 37°C for overnight. The MIC of peptide concentration was recorded.

Hemolytic assay

The hemolytic activity of the cationic antimicrobial peptide was determined with sheep erythrocytes based on radial diffusion assay and the release of hemoglobin from the cells or the lysis of erythrocytes describes the hemolytic activity of the peptide (Conceição et al., 2006). The well punched on the solidified blood agar medium was filled with 20 µl cationic antimicrobial peptide and 0.2% triton X-100, which was 100% hemolytically active. The plates were incubated at 37°C for overnight to determine the hemolytic activity of the peptide on the erythrocytes.

Results

Designing and analysis of the cationic antimicrobial peptide

The factors chosen for designing cationic antimicrobial peptide as conformation, charge, hydrophobicity and polar angle are selected randomly for analyzing five peptide sequences. Often one stable peptide sequence was obtained from the PROTPARAM for the input sequence of NH2-CLKVRIWFK-COOH (molecular weight 1192.5) based on various computed parameters. Theoretical pI 10.06, N-terminal of the sequence considered is C (Cys), estimated half-life of 1.2 hours (mammalian reticulocytes, *in vitro*), >20 hours (yeast, *in vivo*); >10 hours (*Escherichia coli*, *in vivo*) and instability index (II) is computed to be 5.28. The predicted secondary structure of a peptide from HNN shows that sequence length was 9, α–helix (Hh) 0.00%, 310 helix (Gg) 0.00%, Pi helix (Ii) 0.00%, β–bridge (Bb 0.00%, extended strand (Ee) 77.78%, β–turn (Tt) 0.00%, bend region (Ss) 0.00%, random coil (Cc) 22.22%, ambigous state 0.00% and other states 0.00%. This shows that the peptide has extended strand structure in solution, which is homologous with tigerinin.

After binding to lipid membrane, the structural conformation differs from solution structure. Based on the antimicrobial properties like total hydrophobic ratio 6 %, total net charge +3, peptide binding potential (Boman index) 0.61 kcal/mol of peptide through the antimicrobial peptide database. This peptide may interact with membranes and has a chance to be an antimicrobial peptide.

Construction of the synthetic gene

The sequence of the peptide chosen for investigation is NH_2-CLKVRIWFK-COOH. For effective expression and to avoid the detrimental effects of the synthesized cationic peptide in host system, a 26 amino acid length expression cassette containing anionic spacer was designed as "**M**" (as a cleavage site for cyanogen bromide) - "**CLKVRIWFK**" (as cationic peptide) - "**M**" - "**EAED**" (as an anionic spacer peptide) - "**M**" - " **CLKVRIWFK**" - "**M**". As per the design the nucleotide sequence was translated back from the peptide sequence and synthetic gene construction (5'-ATGTGCCTTAAAGTCCGTATTTGGTTTAAAATGGAG GCGGAGGACATGTGCCTGAAGGTGCGCATCTGGTTC AAGATG-3') was carried out using overlapping PCR. Primers were designed as per the strategy, which includes forward primer: 5'-CGC**GGATCC**ATGTGCCTTAAAGTC-3' (24 mer, *Bam*HI cleavage site is in bold) and reverse primer: 5'-GGG**AAGCTT**TTACATCTTGAACCAGAT-3' (27 mer, *Hind*III cleavage site is in bold) for large scale amplification. A number of amplification reactions were performed to optimize the annealing temperature and at the temperature of 64°C, an efficient amplification the synthetic gene was observed. The results of the PCR methodologies were analyzed and confirmed the 99 bp amplified synthetic gene product on 2% agarose gel by running against a 100 bp DNA ladder (Figure 1).

Generation of recombinant DNA

The resulting PCR product was digested with *Bam*HI and *Hind*III and ligated into the pRSET-A plasmid that was digested with the same restriction enzymes to generate the complex cationic antimicrobial peptide/pRSET-A (Figure 2). The ligation mixture was transformed into *E. coli* DH5α competent cells for propagation of the recombinant plasmids (Figure 3). The recombinant plasmid was confirmed by PCR with gene specific primers and the desired product size of 99 bp DNA was identified against a 100 bp DNA ladder (Figure 4).

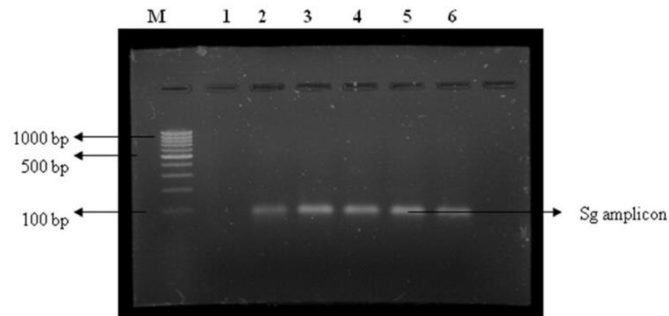

Figure 1. *PCR optimization for synthetic antimicrobial peptide.* ***M****: 100 base pair DNA ladder;* ***1****: Ve;* ***2****: Annealing temperature at 58°C;* ***3****: Annealing temperature at 61°C;* ***4****: Annealing temperature at 64°C;* ***5****: Annealing temperature at 66°C;* ***6****: Annealing temperature at 69°C.*

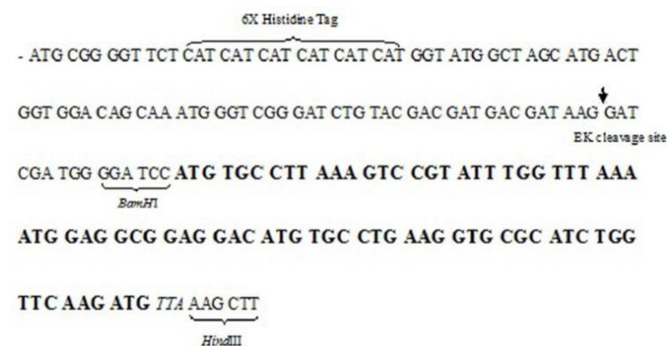

Figure 2. *Schematic representation of the construction of* cationic antimicrobial peptide*–pRSET-A gene. Bold letters represents synthetic cationic antimicrobial peptide. Italic letters indicates termination codon.*

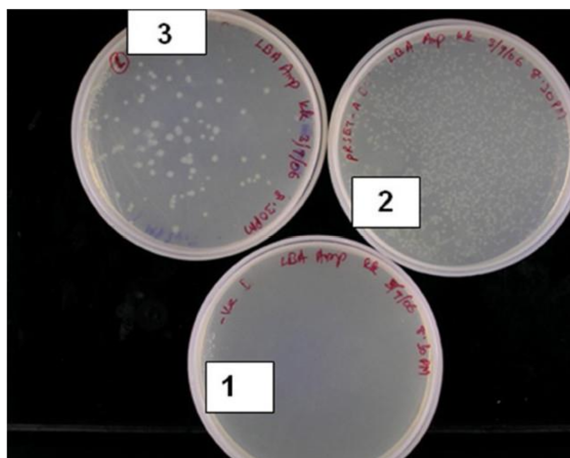

Figure 3. *Transformation of SG-pRSET-A into E.coli GJ1158 bacterial cells.* ***1****: –Ve plate;* ***2****: pRSET-A (+Ve plate);* ***3****: SG – pRSET-A (Ligation sample).*

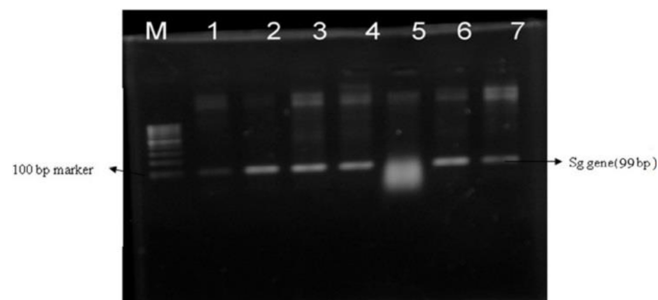

Figure 4. *PCR for the plasmids from transformed plate with gene specific primers.* ***M****: 100bp DNA ladder;* ***1*** *to* ***7****: PCR for the plasmid DNA from colonies*

Expression and purification of synthetic cationic antimicrobial peptide

For the expression studies, the *E. coli* expression host GJ1158 was used for NaCl induction. The confirmed recombinant plasmid DNA was transformed into competent *E. coli* expression host GJ1158 using heat shock method. To enhance the expression levels of heterologous peptide, LBON medium was used as seed medium and GYEON medium was used for the expression of recombinant peptide. Cells containing the recombinant DNA were grown up to 0.8–1 OD_{600} and induced with appropriate inducer NaCl (200 mM), for 4 h at 37°C. The induced peptide expression profiles were resolved on 18% tricine–SDS–PAGE. The synthetic peptide was visualized on the gel at the position corresponding to the reference or standard protein (Figure 5).

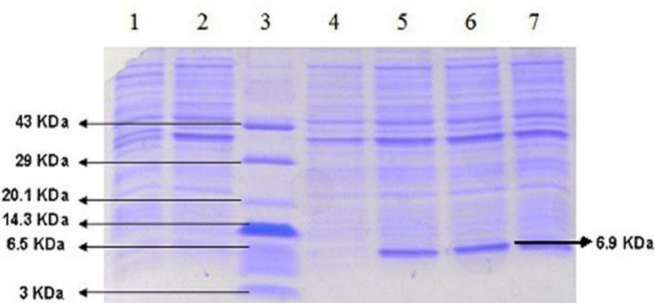

Figure 5. *Tricine SDS-PAGE analysis of recombinant protein expression (antimicrobial peptide) at different time intervals.* ***1****: GJ 1158 non-induced;* ***2****: GJ 1158 induced;* ***3****: low molecular weight protein marker;* ***4****: Sg pRSET-A GJ 1158 non-induced;* ***5****: Sg pRSET-A GJ 1158 induced–induced 1;* ***6****: Sg pRSETA GJ 1158 induced–induced 2;* ***7****: Sg pRSET-A GJ 1158 induced–induced 3*

Out of four samples, S-2 fraction shows that the synthetic peptide was highly soluble (i.e. 96% of the total fusion

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peptide) and accounted for 0.52 g/L of the total soluble protein. Later, the recombinant peptide was purified by the IMAC purification system and cleaved by enterokinase. The digested product was further purified and achieved about 0.12 g/L of biologically active recombinant cationic antimicrobial peptide. To check the recombinant purified peptide 18% tricine–SDS–PAGE was used. Results showed that 2.9 kDa band corresponding to low molecular weight protein marker (Figure 6).

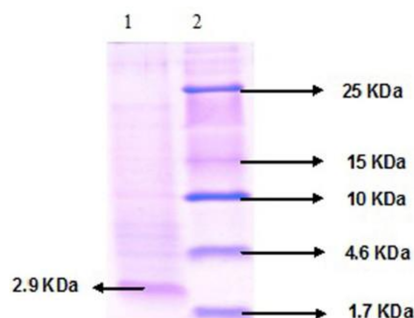

Figure 6. *Tricine SDS-PAGE analysis of IMAC purified antimicrobial peptide. Lane 1: purified peptide; Lane 2: low molecular weight protein marker.*

Antimicrobial and hemolytic activity

The antimicrobial activity assay was performed to determine the activity of the purified recombinant synthetic cationic antimicrobial peptide. Figure 7 shows that zone of clearance around the well containing recombinant antimicrobial peptide. The control well shows no zone of inhibition.

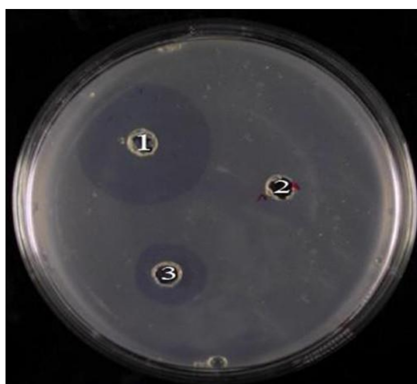

Figure 7. *Inhibitory zone evaluations of recombinant synthetic antimicrobial peptide on growth of Staphylococcus aureus.* ***1****: Ampicillin (10 µg/ml);* ***2****: 1 mM PPB (pH 7) as negative control;* ***3****: Recombinant synthetic peptide (10 µg/ml).*

The minimal inhibitory concentration (MIC) of the purified recombinant antimicrobial peptide against Gram-positive and Gram-negative bacteria are shown in Table 1. The MIC values were in the range of 15–25 µg/ml. The antimicrobial effect of different concentration of the cationic antimicrobial peptide on *E. coli* K12 growth was illustrated in Figure 8. Some antimicrobial peptides exhibit hemolytic activities but no hemolytic activity was observed after overnight incubation using the concentration of 5 – 50 µg/ml, indicating that the cationic antimicrobial peptide was not toxic to red blood cells.

Table 1. *Minimal inhibitory concentrations (MICs) of synthetic cationic peptide on Gram positive and Gram negative microorganisms.*

Microorganisms	MIC[a] (µg/ml)
Gram positive	
Micrococcus luteus	15
Bacillus subtilis	20
Staphylococcus aureus	15
Gram negative	
Pseudomonas putida	15
E. coli DH5α	25

[a] Experiments were carried out in duplicates.

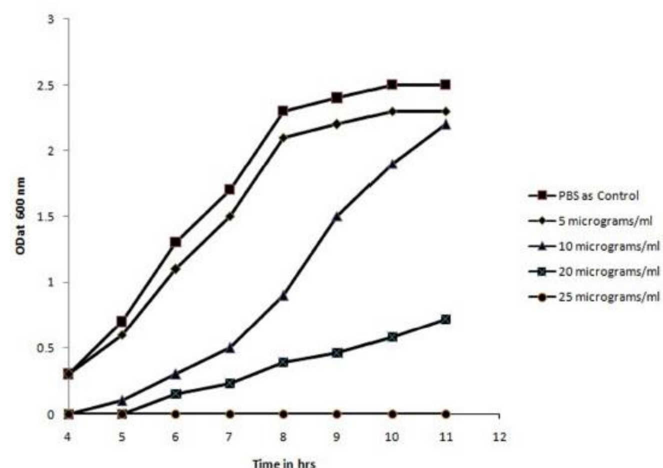

Figure 8. *The effect of cationic antimicrobial peptide on the growth of E. coli K12 monitored by measuring the OD_{600} nm. Experiments were performed in duplicates.*

Discussion

Nowa days, antimicrobial peptides are extensively studied owing to their possible medical applications. Researchers are always in hunt for effective production of biologically active

antimicrobial peptides in large quantities with affordable prices (Robert & Moellering, 2003; Hancock, 2001). Out of different antimicrobial peptides available in the market, cationic synthetic antimicrobial peptides are promising agents (Projan, 2003). Owing to the requirement for large quantity of antimicrobial peptides, we required a production method using economically feasible *E. coli* GJ1158 with low cost of medium instead of using LBON. To express the synthetic antimicrobial peptides in prokaryotic host *E. coli*, anionic spacer peptide and cleavage site for cyanogen bromide are used to neutralize the innate toxic activity to host bacterial cells and to increase their expression levels (Xu et al., 2006; Cao et al., 2005; Lai et al., 2005; Sanz et al., 2005; Shlyapnikov et al., 2008).

In the present study a 9 amino acid synthetic antimicrobial peptide was designed by using the bioinformatics tools viz., ProtParam, Hierarchical Neural Network, Antimicrobial peptide database. The designed peptide was translated back into a core nucleotide sequence and the gene for the peptide was constructed by overlapping PCR methodology. Synthesized gene was cloned into pRSET-A vector and transformed into *E.coli* host system GJ1158. The transformation of *E.coli* GJ1158 by pRSET-A–cationic antimicrobial peptide was successful, as demonstrated by PCR analysis. Three colonies which generated well-amplified products were used for a small-scale expression trial, and the amount of recombinant peptide was determined by tricine–SDS polyacrylamide gel electrophoresis (tricine–SDS–PAGE). The expression levels of antimicrobial peptide in this study was 20 – 30% where in other studies it is ranged from 11 to 44% of total protein (Cao et al., 2005; Huang et al., 2008; Li et al., 2007). The bactericidal assay of cationic antimicrobial peptide peptide showed high activity to Gram-positive and Gram-negative bacteria. The peptide was devoid of toxic nature to red blood cells and had high activity against bacteria indicates that it has potent antibacterial agent. Further clinical trials could prove this synthetic cationic antimicrobial peptide to be an effective antimicrobial agent and it can be effectively used on a commercial scale for control of several severe human pathogenic microorganisms.

Acknowledgement

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