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Cloning and High Level Production of Engineered Synthetic Cationic Antimicrobial Peptide using Methanol Inducible *Pichia pastoris* GS115

Seetha Ram Kotra[1], K. Sobha[2], V. Viharika[3], A. Pavan Kumar[2], P. Vengala Rao[4], M. Mary Vijaya Kumari[5], Kona Prasad[2], G. Ravi Teja[2], K. V. Rajesh[6] and JB Peravali[6]

[1]*Department of Biotechnology, Acharya Nagarjuna University, Guntur – 522510, India*
[2]*Department of Biotechnology, R.V.R.& J.C. College of Engineering, Guntur – 522019, India*
[3]*Department of Biology, Bharathi Junior College, Chirala – 523155, India*
[4]*Department of Biotechnology, Alagappa University, Karaikudi – 630003, India*
[5]*Department of Microbiology, M.V.R. P.G. College, Gajuwaka – 530026, India*
[6]*Department of Biotechnology, Bapatla Engineering College, Bapatla – 522101, India*

Corresponding author: JB Peravali. E-mail: jbperavali@gmail.com; +919491475860

Abstract

Engineered synthetic cationic antimicrobial peptides are the potential alternative drugs to existing antibiotics. In the present study, a novel attempt for the intracellular production of engineered synthetic cationic antimicrobial peptide (escAMP) using Pichia pastoris was studied. The engineered synthetic cationic antimicrobial peptide gene was synthesized using overlapping PCR. An entirokinase and hydroxylamine hydrochloride cleavage sites are incorporated at N- and C- terminal end of escAMP respectively for easy purification. Later the gene was inserted into the MCS region of pPICZ-B vector. The synthetic peptide under the AOX1 promoter was integrated into the Pichia pastoris GS115 genome and the recombinant clones were screened by using antibiotic resistance. Expression profiles of recombinant peptide were done using glycerol and methanol based synthetic medium and analysed on 18 % Tricine-SDS-PAGE. Purification of the expressed peptide was done after cell disruption (10 cycles on time, 10 cycles off time and 10 min of total time) using 6X histidine tag followed by enzymatic cleavage. In this study, 67 gm of dry cell weight/L and 580 mg/L of purified escAMP was produced. The purified peptide is analysed for its anti microbial activity against different Gram positive and Gram negative microbes. For the first time smallest engineered synthetic cationic peptide was designed, cloned and expressed from methanol inducible Pichia pastoris GS115 and production ranges are encouraging.

Keywords: *pPICZ-B, Pichia pastoris, AOX1 promoter, 18% Tricine-SDS-PAGE, 6X histidine tag, enzymatic cleavage*

1. Introduction

Multi-cellular organisms consist of several mechanisms to protect from the infectious microbes. On the other hand antibiotics are in the market to control the various infectious

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diseases. The increased availability due to bulk production of various antibiotics has led to widespread and improper use, leads to the several side effects viz., fever, seizures, amnesia, nervous disorders, *etc.* As an alternative, novel potential source of antimicrobial drugs are being sought. These peptide antibiotics are produced by various species of bacteria, insects, plants and vertebrates [1]. In the present day scenario, antimicrobial drugs are the upcoming therapeutic molecules to the existing antibiotics. The production of these peptides has a good scope in current antibiotic research [2].

In general, the antimicrobial peptides consists of 10–50 amino acids and work against a wide range of pathogenic organisms in different ways by interacting and disturbing the bacterial cell wall, cell membrane and at various intracellular parts directly or indirectly [3]. Native production was not up to the industrial needs and also cost of purification is also high. Hence, production using recombinant hosts is encouraging. The mostly used host is *Escherichia coli* because of its well understood genetic map and cost effective production [4, 5, 6]. But problems like fast proteolysis, poor absorption, cost of the drug and toxic nature are the major hurdles [7, 8, 9, 10]. To avoid such hindrance an engineered peptide was designed and cloned in eukaryotic host results the production of peptide devoid of side effects [11]. Eukaryotic host like *Pichia pastoris* is the prominent host for the production of recombinant therapeutics [12, 13].

The present work has been carried out to design the engineered cationic antimicrobial peptide using *in-silico* tools followed by cloning and expression using eukaryotic system *Pichia pastoris*. Purification was carried out using 6X histidine tag and activity analysis of the peptide was studied using different Gram positive and Gram negative pathogenic microorganisms.

2. Materials & methods

2.1. Strains and media

E. coli DH5α was used as maintenance host and was procured from MTCC, Chandigarh. Recombinant transformants were selected on solidified Luria–Bertani plates supplemented with 25 µg of antibiotic (zeocin) per mL.

P. pastoris GS115 used as expression host was purchased from Invitrogen and was propagated on yeast extract peptone dextrose (YEPD) agar plates containing yeast extract – 10 g/L, peptone – 20 g/L, dextrose – 20 g/L and agar – 20 g/L.

Recombinant *P. pastoris* strains were selected on regeneration dextrose medium (RDB) agar plates containing yeast nitrogen base – 13.4 g/L, dextrose – 20 g/L, 1 M sorbitol, amino acid mix (L-glutamic acid, L-methionine, L-lysine, L-leucine and L-isolucine) – 50 mg/L, biotin – 400 µg/L and agar – 20 g/L.

For growth and induction, buffered methanol-complex medium (BMMY) or buffered glycerol-complex medium (BMGY) media (yeast extract – 10 g/L, peptone – 20 g/L, yeast nitrogen base – 13.4 g/L, 100 mM potassium phosphate (pH 6.0), biotin – 400 µg/L, glycerol – 10 mL or methanol – 5 mL) were used.

2.2. Computational tools used

The desired synthetic cationic antimicrobial peptide was designed by using *in-silico* tools, *i.e.*, hierarchical neural network (HNN), antimicrobial peptide database (AMPDB) and protparam (PP).

2.3. PCR amplification

The synthetic cationic antimicrobial peptide gene was engineered using over lapping PCR with the following primers (Sigma-Aldrich, Bangalore, India). Forward primer (72 mer) sequence was 5' CCG**GAATTC**ATGGACGATGACGATAAGATGTGCCTTAAAGTCCGTATTTGGTTTA AAATGGAGGCGGAGGAC 3' and reverse primer (77 mer) sequence was 5' TGC**TCTAGA**GCCGTTCATCTTGAAGCAGATGCGCACCTTCAGGCACATGTCCTCC GCCTCCATTTTAAACCAAATAC 3'. Enterokinase (*EK*) cleavage site is incorporated at 5' end of forward primer and hydroxylamine hydrochloride (*Asn-Gly*) cleavage site was incorporated at 5' end of reverse primer. The PCR reaction set up with an initial denaturation at 95 ^{0}C for 5 min, followed by 25 cycles of 95 ^{0}C for 30 s, 60 ^{0}C for 45 sec and 72 ^{0}C for 45 sec and a final extension at 72 ^{0}C for 10 min. Later the amplified PCR product was purified using Gen Elute PCR clean-up kit (Qiagen) as per the manufacturer's instruction.

2.4. Construction of recombinant vector

All molecular cloning steps were performed as described in Sambrook *et al.*, 1989 [14]. The purified PCR product was double digested with *Eco*RI and *Xba*I. The purified double digested PCR product was ligated to double digested pPICZ-B vector at 1:3 ratio. Later the ligation mixture was introduced into chemically competent *E. coli* DH5α cells and the highest concentrations of zeocin resistant transformants were screened for the presence of synthetic cationic antimicrobial peptide gene. On the other hand, colony lysate PCR was done using gene specific primers and double digestion of recombinant vector is carried out to confirm the ligation, followed by DNA sequencing.

2.5. Transformation to *P. pastoris*

The recombinant plasmid escAMP/pPICZ-B was linearized using *Sac*I and transformed to *P. pastoris* by electroporation using an electroporator at 2000 V with a 0.2 cm cuvette. After electroporation, immediately 1 mL of ice-cold 1 M sorbitol was added to the cuvette and allowed for incubation for 1 hr at 30 ^{0}C. Later the cells were plated on RDB agar plates with different concentrations (ascending concentration) of zeocin and incubated at 30 ^{0}C for 2–3 days until the colonies appeared. The genomic DNA was isolated from the recombinant transformants using Qiagen genomic DNA isolation kit and the integration of the gene was confirmed with gene specific primers.

2.6. Expression of escAMP

Clones of highest zeocin resistance was chosen and inoculated into baffled shake flask containing 25 mL BMGY and were grown at 30 ^{0}C for 18-24 hrs until the culture OD_{600} reaches between 2–6. Later, cells were harvested by centrifugation at 2,000 X g for 5 minutes and resuspend in BMMY with methanol as an inducer. 100 % methanol was added to a final concentration of 0.5% every 12 hours to maintain induction. To prevent glycosylation of escAMP, expression in another flask was carried out with 20 µg tunicamycin/mL in the induction medium as described by Sebban-Kreuzer *et al.*, 2006 [15]. Expression of escAMP was analyzed on a 18% Tricine–SDS–PAGE. After sonication, escAMP concentration was estimated by Lowry method [16].

2.7. Purification of escAMP

The engineered peptide containing an C–terminal 6X His tag was expressed and purified using affinity chromatography with Ni^{2+} resin In order to purify the peptide, 800 mg of wet cells were obtained from a freshly grown culture. The cells were harvested and centrifuged at 13,800 rpm for 10 min at room temperature. Later the pellet was suspended in 12 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 hr. The cell lysate was again centrifuged at 13,800 rpm for 10 to 15 min at room temperature. A volume of 10 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 6 mL of denaturing wash buffer and twice with 6 mL of native wash buffer. The bounded peptide was eluted with 5 mL of native elution buffer and analysed on 18% Tricine–SDS–PAGE against standard protein marker.

After purification, escAMP was incubated in hydroxylamine cleavage buffer (2 M hydroxylamine hydrochloride, 0.2 M Tris HCl, pH 9.0) for 4 hrs at 45 ^{0}C followed by cleavage with entirokinase. The reaction was terminated by cooling the samples and adjusting the pH to acidic. After enzymatic cleavage the peptide samples was dialyzed twice with 10 mM Tri Hcl, pH 8.0 for 16 hrs at 4 ^{0}C using 2.5 kDa (initially) and 3.5 kDa (finally) cutoff membranes and later dialyzed sample was loaded on Q Sepharose column matrix equilibrated with the same buffer. The bound peptides were eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The elute containing escAMP were dialyzed against 100 volumes of 20 mM sodium acetate buffer, pH 4.5 and passed through a SP-Sepharose column matrix. The bound escAMP was eluted using a linear gradient of NaCl (0–1 M) in the same buffer. The purity of the peptide was analyzed using 18 % Tricine – SDS – PAGE.

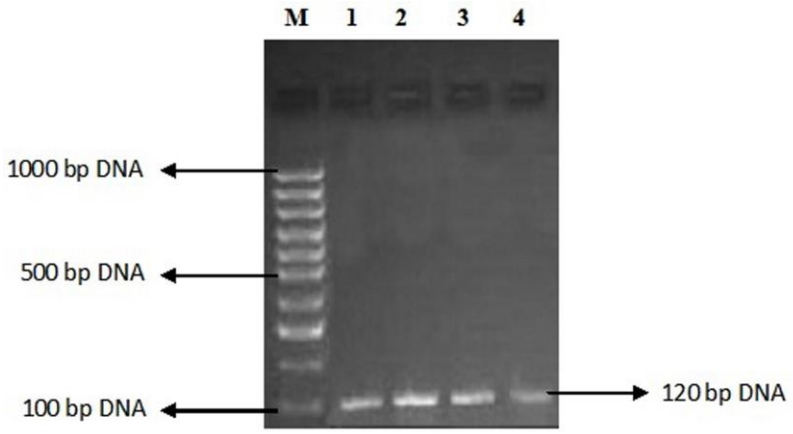
2.8. Antimicrobial activity assay

The antibacterial activity of engineered recombinant synthetic cationic antimicrobial peptide was tested by well diffusion method using *E. coli*. On the other hand, minimum inhibitory concentration (MIC) of the purified and unpurified escAMP was determined against different Gram negative and Gram positive microbes viz., *Klebsiella pneumoniae*, *Haemophilus influenza, Staphylococcus aureus, Micrococcus luteus* and *Bacillus subtilis* by a liquid growth-inhibition assay [17].

3. Results & Discussion

3.1. Synthesis of escAMP

The desired 102 bp length DNA was synthesized by using overlapping PCR. The result of the PCR methodologies was analyzed and confirmed the amplified product on 2 % agarose gel against a 100 bp DNA ladder. Further this gene is used as template to amplify the escAMP with a different set of primers containing restriction sites at 5’ end and the desired product size of 120 bp DNA is confirmed on 2 % agarose (Figure 1).



M = Low range DNA ladder
1 = First elute of 120 bp DNA
2 = Second elute of 120 bp DNA
3 = Third elute of 120 bp DNA
4 = Fourth elute of 120 bp DNA

Figure 1. Analysis of PCR amplified engineered synthetic cationic antimicrobial peptide (escAMP) gene against 100 bp DNA ladder

3.2. Construction of pPICZ-B-escAMP expression vector

The double digested purified PCR product was cloned in pPICZ-B vector between *Eco*RI and *Xba*I sites using T_4 DNA ligase. After PCR confirmation of the recombinant plasmid (pPICZ-B-escAMP) (Figure 2), electroporation was carried out into *P. pastoris* produced more than 30 colonies in highest zeocin concentration 100 µg/mL instead of normal concentration 25 µg/mL.

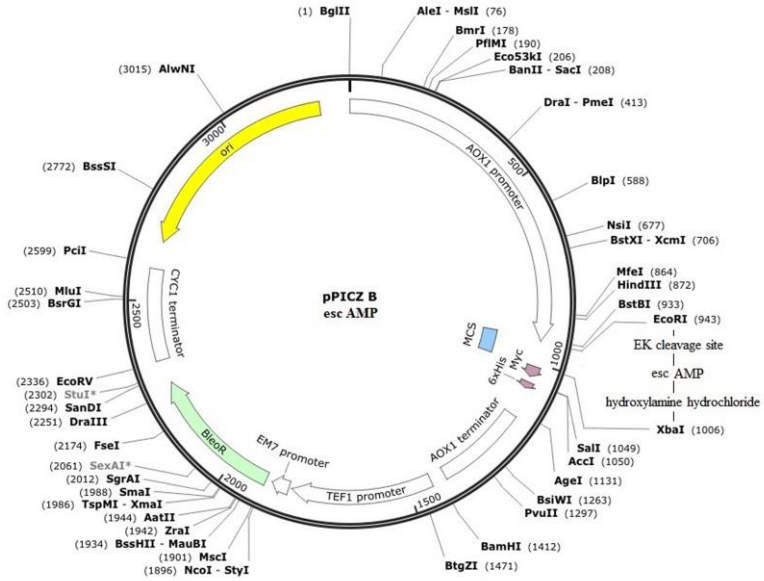


Figure 2. Schematic representation of the recombinant expression vector pPICZ-B -escAMP

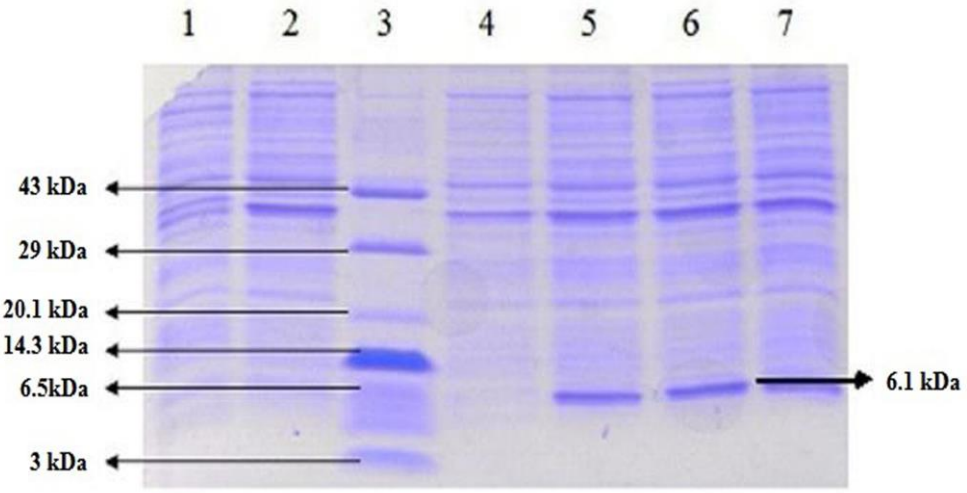
The recombinant clone was isolated and linearized with *Sac*I and introduced into *P. pastoris* using electroporation. The recombinant *Pichia pastoris* transformants gave expected size (202 bp) of PCR product using gene specific reverse primer and vector specific forward primer (Figure 3) and followed by sequencing.

5' GACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAA
GATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGACGATGACGATAAGATG
TGCCTTAAAGTCCGTATTTGGTTTAAAATGGAGGCGGAGGACATGTGCCTGAAGGTGC
GCATCTGCTTCAAGAACGGCTCTAGAGCA 3'

Figure 3. DNA sequencing of recombinant synthetic cationic antimicrobial peptide amplified using 5' *AOX1* sequencing primer and gene specific reverse primer

3.3. Expression of escAMP

For the expression studies, methanol inducible *Pichia pastoris* GS115 was used. This expression system was chosen owing to its simplicity. As mentioned earlier all peptide expressions were analyzed by running on 18 % Tricine-SDS-PAGE against standard protein marker and a very clear 6.1 kDa band was identified against a low molecular weight protein ladder (Figure 4). The observed results in the present investigation were coincided with the similar expression patterns in *E. coli*.



1 = Uninduced *Pichia pastoris* GS115
2 = Induced *Pichia pastoris* GS115
3 = Protein marker
4 = Uninduced recombinant *Pichia pastoris* GS115
5 = Induced recombinant *Pichia pastoris* GS115 (24 hrs)
6 = Induced recombinant *Pichia pastoris* GS115 (36 hrs)
7 = Induced recombinant *Pichia pastoris* GS115 (48 hrs)

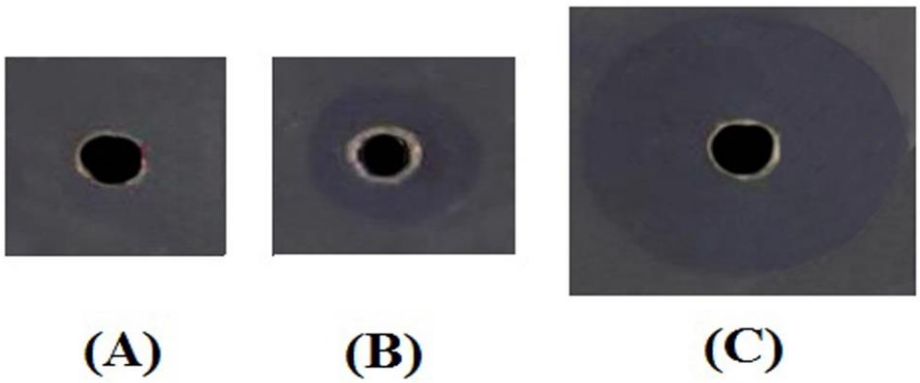
Figure 4. Tricine SDS PAGE analysis of engineered synthetic cationic antimicrobial peptide (escAMP)

3.4. Purification of the peptide

Purification of the intracellular peptide was carried out using 6X histidine column by loading the supernatant after sonication followed by enzymatic cleavage. Tricine–SDS–PAGE analysis of the purified sample showed single band slightly at 3 kDa, which is consistent with the molecular weight of escAMP and was also confirmed by western blot analysis. Upon quantification, 580 mg/L of purified peptide was achieved.

3.5. Antimicrobial activity assay

Antimicrobial assay was performed to determine the activity of the purified and unpurified escAMP. Figure 5 shows the zone of clearance around the treated well of different concentration of purified and unpurified samples, but no zone of clearance is observed around the control well.



(A). –Ve (PBS), (B) 25 µl of unpurified escAMP, (C) 4 µl of purified escAMP

Figure 5. Antimicrobial activity of escAMP on *E. coli*

The MICs of the purified and unpurified escAMP against several microorganisms were shown in Table 1. These results demonstrated the recombinant peptides were functional and active after enzymatic cleavage.

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

Microorganism	Unpurified peptide (µg/mL)[a]	Purified peptide (µg/mL) [a]
Klebsiella pneumoniae	25	4
Haemophilus influenzae	20	3
Bacillus subtilis	40	5
Micrococcus luteus	30	4
Streptococcus aureus	35	3

[a] Results are ± of two independent experiments

The effect of antimicrobial activity of different concentrations of escAMP on growth of pathogenic bacteria viz., *Staphylococcus aureus* and *Pseudomonas aeruginosa* was illustrated in Figure 6 (A) and (B). 25 µg/mL of unpurified escAMP is enough to inhibit the growth of *Staphylococcus aureus* and 20 µg/mL of unpurified escAMP shows the inhibition of *Pseudomonas aeruginosa.* These purified engineered synthetic cationic AMP does not exhibit

any haemolytic activity even at the highest concentrations (50 µg/mL) over night, indicating the cationic antimicrobial peptide was devoid of toxic nature to RBC.

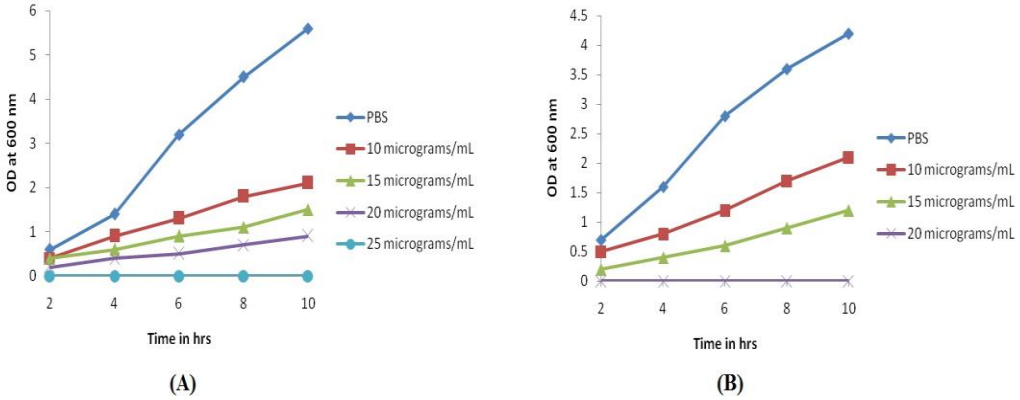


Figure 6. (A). Concentrations of escAMP on propagation of *Staphylococcus aureus;* (B). Concentrations of escAMP on propagation of *Pseudomonas aeruginosa*

4. Conclusion

The clinical applications of antimicrobial peptides in treatment of several infectious diseases have attracted the attention of researchers [18, 19]. Different antimicrobial peptides are produced using recombinant DNA technology [20, 21, 22]. Hypersensitivity and poor absorption are the drawbacks of major peptides [23, 24]. The present study focussed on engineered synthetic cationic antimicrobial peptide consists of 26 amino acids is a promising molecule synthesized using codon bias of *Escherichia coli* [25, 26]. The production level of recombinant synthetic cationic antimicrobial peptide was relatively high when compared with PG-1 (1.1 mg/L) [27], active recombinant CM4 (1.4 mg/L) [28], recombinant PR-39 (1.9 mg/L) [27], cationic recombinant thanatin analog (13.2 mg/L) [29], cationic antimicrobial peptide lactoferricin (60 mg/L) [30], plectasin (92 mg/L) [31], mouse beta defensin-1 mature peptide (140 mg/L) [32], synthetic cationic antimicrobial peptide (520 mg/L and 258 mg/L) [33, 34], and slightly lower when compared to mature hBD4 (689 mg/L) [35]. Production levels are very far away from HBD5 (1.49 g/L) and HBD6 (1.57 g/L) [36].

In the present work, we constructed a recombinant system for the enhanced production of escAMP using the pPICZ-B expression vector. Still now no expression methods were reported using eukaryotic system and engineered peptides. For the first time *Pichia pastoris* was used for the production of small length engineered antimicrobial peptides.

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