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Extended-Spectrum Antimicrobial Activity of the Low Cost Produced Tilapia Piscidin 4 (TP4) Marine Antimicrobial Peptide

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ABSTRACT

Regarding the spread of antibiotic-resistant infections in recent years, many studies have been conducted on antimicrobial peptides as an alternative drug. Unfortunately, there are fewer studies on marine antimicrobial peptides than other living organisms.

In this study, Tilapia Piscidin 4 (TP4), one of the most efficient marine antimicrobial peptides that have been introduced so far, produced using the Pichia pastoris GS115 expression system. The TP4 sequence was first cloned to the pPIC9 vector and then transformed to the Pichia pastoris to integrate into the genome. The best expression conditions were obtained in a minimal protein-lacking medium of BMM, 1.5% methanol, pH of 6, and the time of 48 hours. Subsequently, the antimicrobial activity of the recombinant TP4 was evaluated on 12 gram-negative and 5 gram-positive common bacterial pathogens including sensitive and antibiotic-resistant strains and showed the wide-spectrum activity.

The TP4 antimicrobial peptide can be used as a competitive candidate for antibiotics in the post-antibiotic era due to the broad-spectrum antibacterial effects. The production of TP4 by the explained method in this study was a low-cost process and could pave the way for future studies and therapeutic applications.

Key words: Antimicrobial peptides, Marine, Heterologous expression, *Pichia pastoris*, Resistance

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INTRODUCTION

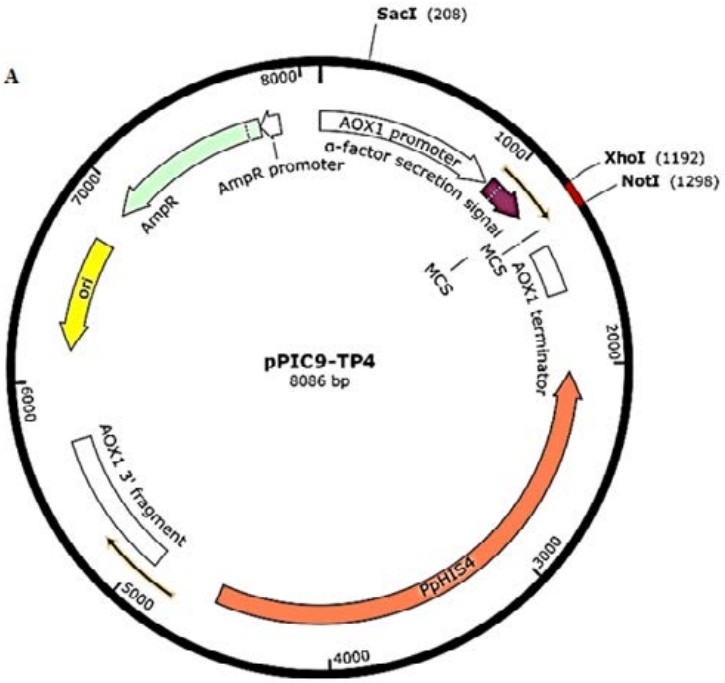
Antimicrobial peptides (AMPs) are a group of short peptides which are primarily known by their antimicrobial properties. AMPs often have a living origin and act as a part of the innate immunity of living organisms. Studies have shown that these peptides, in addition to their antimicrobial activity, have other effects such as anti-cancer, antioxidant, and wound healing. In recent years, with the spread of antibiotic-resistant microbial strains and the need to find an appropriate alternative to the post-antibiotic era, much attention has been paid to the study of AMPs. Although more than 2900 AMPs have been registered in antimicrobial peptide database, due to the novelty of this field, only about 17 peptides such as pexiganan, omiganan, and LL-37 have entered clinical trials until now [1-3].

One of the fields which are considered much lower is the marine AMPs. Studies have shown that the marine organisms are highly rich sources of AMPs. One of the most active marine AMPs which have been introduced so far is Tilapia Piscidin 4 (TP4) [4].

In this study, we aimed for the low-cost production of the recombinant TP4 AMP using *Pichia pastoris* GS115. TP4 is a cationic AMP that is naturally produced by the mast cells of Nile Tilapia (*Oreochromis niloticus*). This peptide has an alpha-helix structure with 25 amino acids (FIHHIIGGLFSAGKAIHRLIRRRRR). Evaluation of the TP4 antimicrobial activity on some gram-positive and gram-

negative bacteria indicates that it has broad-spectrum antimicrobial effects that can compete with antibiotics. The reported minimal inhibitory concentrations (MICs) indicates the values of 0.03 to 8.39 µg/ml for some bacterial pathogens including *E. coli* (YT39), *V. vulnificus* 204, *E. faecalis* BCRC 10066, *A. baumannii* (Sk44) [5,6]. The study of the TP4 effect on resistant and sensitive strains of *Helicobacter pylori* shows that this AMP can cause micelles in the bacterial membrane. Micelle formation results in membrane depolarization and extravasation of cellular constituents and thus prevents bacterial growth [7]. Study of the TP4 effect on peritonitis and Methicillin-resistant *Staphylococcus aureus* (MRSA) wound infection shows that this AMP can effectively combat the infection without any toxic effect on the liver and kidney. TP4 can efficiently increase the survival rate of MRSA-infected mice with both antimicrobial and wound healing properties, mediated by transforming growth factor (TGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) [8]. Although strong antibacterial and wound healing effects of TP4 have been discovered yet [5,6,8], the study of scientific articles shows that the performed studies on the advantages and disadvantages of this AMP are few and there is a long pathway ahead to reach a candidate drug. One of the main factors limiting the study on TP4 and most of the AMPs is the lack of easy and cheap access to the peptides. As the AMP extraction from the original source (here, *Nile Tilapia*) is a difficult, time-consuming, and expensive labor, it usually forces researchers to ignore this method [9]. Another way is to use chemical methods to synthesize the desired peptide or to produce it recombinantly. The recombinant method is preferred to the chemical method in many aspects and is considered more in recent years for the peptide production. The recombinant systems are generally more cost-effective and provide an endless source of peptide production, both for research and large-scale production in pharmaceutical use [9-11].

In recent years, many recombinant expression systems have been introduced and each used based on the need of research. The two most common systems in this field are *E. coli* and *P. pastoris* [11]. Despite being common, *E. coli* expression system has limitations which require additional time and expense to be spent. These limitations include the bacterial LPS contamination in the final product, as well as the toxicity of final product for the recombinant *E. coli* [9,12,13]. In this study, *P. pastoris* GS115 expression system was used which is methylotrophic yeast. The toxicity of TP4 for this eukaryotic system was as low as we were able to produce appropriate concentrations of TP4. Also, the pPIC9 vector was used to transfer the nucleotide sequence of TP4 to *P. pastoris*. This vector contains three crucial components of the alcohol oxidase 1 promoter (PAOX1), the alpha factor secretion signal, and the *his4* gene. These three factors enabled us to use a minimal culture



B

*Xho*I restriction site	Digested sequence of alpha factor	Optimized TP4 sequence	Stop codons	*Not*I restriction site
CTCGAG	AAAAGAGAGGCTGAAGCT	TTTATTCATCATATTATTGGAGGTTTGTTTTCCGCTGGTAAGGCTATT CATAGATTGATTAGAAGAAGAAGAAGA	TGATAA	GCGGCCGC

Final seq: 5-CTCGAGAAAAGAGAGGCTGAAGCTTTTATTCATCATATTATTGGAGGTTTGTTTTCCGCTGGTAAGGCTATTCATAGATTGATTAGAAGAAGAAGAAGATGATAAGCGGCCGC-3

Figure 1: A) Map of *pPIC9* vector with the ligated TP4 sequence. The map is drawn by the SnapGene® software (from GSL Biotech; available at snapgene.com), B) TP4 sequence after optimization and added sequences to facilitate the cloning and expression

medium for the expression and to extract the TP4 from yeast cells in secreted form.

The aim of this study was the cost-effective production of crude TP4 along with the evaluation of the antibacterial effect of this recombinant peptide. It is hoped that this product can be used in basic studies and clinical applications.

MATERIALS AND METHODS

Design of recombinant vector

The sequence of TP4 with 25 amino acids was extracted from the study by Peng et al. (FIHHIIGGLFSAGKAIHRLIRRRRR) [5]. Then, due to the codon usage bias between *Nile tilapia* and *Pichia pastoris*, the TP4 nucleotide sequence was optimized for the expression in *P. pastoris* using Optigene™ software (the Generay Codon Optimization Analysis Platform). This action stabilizes DNA fragments and improves gene expression efficiency. In this process, the codon adaptation index (CAI) was used to show the optimization rate of the sequence for the expression in *P. pastoris*. Two restriction enzymes of *NotI* and *XhoI* were used to enter the optimized sequence to the *pPIC9*. To facilitate the cloning and expression steps, several other sequences were added to the optimized sequence, including two stop codons, restriction sites for NotI and XhoI, and part of the alpha factor sequence which is removed from *pPIC9* after *XhoI* digestion (Figure 1A).

Construction and multiplication of pPIC9-TP4

The final sequence designed in the previous step (Figure 1B) was constructed in the pGH cloning vector by Generay Biotechnology Company (Shanghai, China). Then, both pGH (containing TP4 sequence) and *pPIC9* vectors were separately digested with *XhoI* and *NotI* restriction enzymes. The product of pGH plasmid digestion was electrophoresed, and finally, the related band for the TP4 sequence was extracted from agarose gel. Then for the ligation of gene cassette to the *pPIC9* plasmid, a mixture of 30 ng of the gel-extracted fragment, 90 ng of digested *pPIC9* plasmid, 1 µl of 10X ligation buffer, and 1 U of T4 DNA ligase was prepared and incubated at 14°C for 16 hours. Finally, the ligation accuracy was confirmed by PCR reaction using AOX primer pairs and eventually by sequencing. The preparation of all used reagents and also the steps of this part were performed with the help of Generay Biotechnology Company (Shanghai, China).

Next, the recombinant pPIC9-TP4 vector was transformed into *Escherichia coli* TOP10 using heat shock method according to the study by Froger et al. [14]. To select the transformed colonies, Luria-Bertani (LB) agar medium containing 100 µg/ml ampicillin was used with the overnight incubation at 37°C. Then, a pure colony was selected and inoculated to 5 ml of LB broth containing 100 µg/ml ampicillin and incubated at 37°C for 5-6 hours along with 200 rpm shaking. The suspension was then centrifuged for 5 minutes at 6000 rpm. The pellet was used for plasmid extraction according to the instructions by GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia).

pPIC9-TP4 vector preparation for efficient integration into the Pichia genome

Previous studies have shown that the vector linearization by some of the restriction enzymes such as SacI would make it easier to integrate the recombinant vector to the *P. pastoris* genome [15], so we used the SacI enzyme (Cat No.1078A, Takara, Japan) by the ratio of 1 µl enzyme/1 µg DNA for one hour at 37°C. Then, linearized vector purification was performed by the Silica Bead DNA gel extraction kit (Fermentas, São Paulo, SP, Brazil). The purity and concentration of the linear DNA obtained in this step were measured with the Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Electroporation

To provide competent yeast cells, a *P. pastoris* GS115 vial was provided from the -70°C stock and inoculated to 20 ml of yeast extract peptone dextrose (YPD) medium (with 72 hours incubation at 30°C along with 200 rpm shaking). Then, 100 µl of the suspension was added to 100 ml of YPD broth medium (with 18 hours incubation at 30°C along with 200 rpm shaking). From this step, all the work was carried out on ice or 4°C. The yeast cells were detached from the culture medium, and the yeast pellet was rewashed twice with sterile cold distilled water and once by cold 1M sorbitol (with 4000 rpm centrifugation, 5 minutes). In the last step of competent cell preparation, yeast pellet was suspended in 300 µl of 1M sorbitol. 80 µl of the competent cells were added to 40 µl of the pure linearized pPIC9-TP4 vector (containing 10 µg DNA), mixed, and transferred to electroporation cuvette of 0.2 cm with 1 hour incubation at 4°C. Electroporation was performed using a Gene Pulser apparatus (Bio-Rad, USA) with the parameters of 2 kv, 25 µF, 200 Ω, 5 ms. Then immediately 1 ml of cold 1M sorbitol was added to the cuvette and incubated for one hour at 30°C. The cells which were transformed by pPIC9-TP4 vector contained the *his4* gene so that they could grow well on a histidine-free medium. Hence, 100 µl of cell suspension was diffused on minimal dextrose (MD) plates (1.34% YNB, 4×10−5% biotin, 2% dextrose, and 1.5% agar) and incubated at 30°C for 3-5 days [16]. To confirm the transformation, each colony was collected with a sterile needle and suspended in sterile distilled water. To remove the extracellular DNA that may have remained from the previous step, the colonies were washed three times with sterile distilled water (by 8000 rpm centrifugation for 5 minutes) and finally, a suspension with the OD of 2-3 was prepared. 1 µl of this suspension was applied as a template for the colony-PCR using AOX primer pairs.

Selection of best transformants

In order to find the best colony regarding the TP4 production, the number of 30 larger colonies grown on MD plates was selected and each was separately inoculated into 5 ml of buffered minimal glycerol (BMG) medium. It contained 4×10−5% (w/v) of biotin,

100 mM phosphate buffer (pH 6), 1.34% (w/v) yeast nitrogen base and 1% (v/v) glycerol. The BMG medium was used for biomass production (with 24 hours of 200 rpm shaking at 30°C). Then, all the culture media were centrifuged, and after the supernatant removal, the yeast pellet was washed twice with 100 mM phosphate buffer (with 6000 rpm centrifugation for 5 minutes). The final pellet was suspended in 10 ml of buffered minimal methanol (BMM) medium (200 rpm shaking at 30°C). This medium is similar to BMG medium except for the presence of 0.5% methanol instead of glycerol. After 24 hours, another 0.5% methanol was added to each medium, and after 48 hours, 100 µl of the supernatant was tested on *Staphylococcus aureus* ATCC 25923 by well diffusion method to determine the antimicrobial effect. The well diffusion test was carried out by providing wells on Mueller-Hinton agar medium using the bottom of 1 ml pipette tip (blue). After inoculation of 0.5 McFarland suspensions by swab, 100 µl of BMM medium supernatant was sampled and loaded into the wells, followed by overnight incubation at 37°C. From the 30 selected colonies, the yeast colony with the largest zone of growth inhibition after the expression was selected as the best transformant for the following steps of the study.

Determination of the best expression conditions

In this step, the effect of methanol concentration, pH, and time on the TP4 expression was investigated using the selected colony of the previous step on *S. aureus* ATCC 25923. At first, the zone of growth inhibition was compared after 48 hours of expression in BMM medium containing 0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% methanol. Then, the zone of growth inhibition was compared in buffer pH of 4, 5, 6, 7 (100 mM phosphate buffer) and also a medium without buffer. Finally, the antimicrobial effect was evaluated at the times of 0, 24, 48, 72, 96 hours under the optimal conditions of pH and methanol, and the best expression time was determined.

Partial purification

In this part, the expression was first performed in one liter of BMM medium according to the optimal conditions of methanol, buffer, and time (after biomass production in BMG). To separate the supernatant containing TP4 from yeast cells, 10000 rpm centrifugation for 15 minutes was first performed, and the supernatant was separated. Then, to remove the maximum yeast residue, the supernatant was filtered using vacuum filtration with a 0.45 µm pore size membrane filter (Whatman® membrane filters nylon).

Until this part, a clear solution was obtained which contained a small amount of methanol, phosphate buffer, and YNB (containing trace elements, vitamins, and salts). Since *P. pastoris* GS115 naturally lacks any significant secreted proteins [17-21], so the total protein of the solution can be partially attributed to the amount of secreted TP4 in the environment. Nevertheless, to ensure the removal of few extra proteins including the proteins released from disrupted yeasts during the expression, the proteins larger than 5 kDa were separated from the solution using Vivaspin 20 (5,000 MWCO PES) (Sartorius). As the molecular weight of TP4 is lower than 5 kDa (about 2.98 kDa), it remained in the solution. Then, the TP4 concentration was determined using Bradford assay [22], and it was lyophilized for easier storage.

Antibacterial activity

In this part, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined on 12 gram-negative and 5 gram-positive bacteria, to assess the spectrum of antimicrobial activity for the recombinant TP4. To achieve this goal, the lyophilized powder of TP4 was used in Broth Microdilution Method [23]. Standard strains were obtained from the microbial bank of antimicrobial resistance research center of Buali Institute of Mashhad, and the clinical isolates were obtained from the microbiology laboratory of Ghaem University Hospital in Mashhad [24].

RESULTS

Selection of best transformants

Since *P. pastoris* GS115 transformed with pPIC9 (negative control) didn't show any antibacterial effect, the diameter of the zone of growth inhibition in the same conditions, indirectly indicated the TP4 expression rate in pPIC9-TP4 transformed colonies. From the 30 colonies evaluated for TP4 expression, the highest antimicrobial activity was related to three transformants which produced a 24 mm inhibition zone after 48 hours (Table 1). One of these three colonies was selected for the next steps.

Determination of best expression conditions

Indirect investigation of TP4 expression using well diffusion method showed that the largest zone of growth inhibition occurred in the 1.5% methanol. However, the expression rate at the concentrations of 2% and 2.5% was also high and close to the expression rate in 1.5% methanol (Figure 2A). Also, the effect of medium's pH on the expression was studied and the best pH was attributed to the pH of 6 (100 mM phosphate buffer) according to the zone of inhibition (Figure 2B). To investigate the most favorable time, we observed that

Table 1: Investigation of TP4 expression in 30 transformed colonies with pPIC9-TP4 using well diffusion test on *Staphylococcus aureus* ATCC 25923

Zone of growth inhibition (Diameter)	Frequency between transformants
No zone	13
About 12 mm	9
About 19 mm	5
About 24 mm	3

under the optimal conditions (pH=6 and 1.5% methanol), the highest expression rate occurred in the first 48 hours and decreased significantly after that. Accordingly, there was no increase in the zone of growth inhibition after this time (Figure 2C).

Partial purification

Measuring the amount of TP4 expression under optimal conditions after partial purification showed that about 71 mg of TP4 was produced per 1 liter of culture medium.

Antibacterial activity

The lowest concentration of TP4 which inhibited the observable growth of a microorganism after overnight incubation at 37°C was reported as the MIC and the lowest concentration of TP4 which was able to kill the bacteria and no growth was observed after subculture on antibiotic-free Mueller Hinton agar was reported as the MBC (Table 2).

DISCUSSION

One of the topics that have always been interesting is the discovery of new drugs for the treatment of disease. The work on AMPs is also a fascinating field that has been reconsidered by the spread of microbial resistance in recent years [2,3,25-28]. These peptides are usually a part of innate immunity in various living organisms and have evolved over a million years. One of the fields which are less studied than the others is the AMPs with the origin of marine organisms. While, among the reported AMPs, some of them have strong antimicrobial effects and some also have other effects such as anti-cancer, immune regulation, and wound healing. The TP4 AMP isolated from *Nile tilapia* (*Oreochromis niloticus*) was considered by our team for its broad-spectrum antimicrobial activity as well as the excellent MIC and MBC (0.03-8.39 µg/ml) reported in earlier studies [5,6]. This peptide has an alpha-helical structure and exerts its antimicrobial effect

Figure 2: Investigation of the effect of methanol, pH, and time on TP4 expression using well diffusion test on *S. aureus*, (A) The methanol content of the expression supernatant loaded in wells 1 to 6 was 0.5%, 1%, 1.5%, 2%, 2.5%, and 3% respectively. In the 7th well, the expression supernatant of pPIC9-transformed *P. pastoris* without TP4 (in methanol 1.5%) was used as the negative control, (B) The pH of phosphate buffer used in the loaded expression supernatant in wells 1 to 4 was 4, 5, 6, and 7, respectively. The expression supernatant of the non-buffered medium was used for the 5th well, (C) The expression supernatant in optimal expression conditions (pH=6, 1.5% methanol) was collected after 24, 48, 72, 96 hours and loaded in the wells 1 to 4. The 5th well was regarded as the zero time (negative control)

Table 2: MIC and MBC values of TP4 on standard strains and clinical isolates

Type	Standard strains/Clinical isolates	MIC (µg/ml)	MBC (µg/ml)
Gram (-)	*Escherichia coli* (ATCC 25922)	2	4
	Escherichia coli DH5-Alpha	2	4
	Pseudomonas aeruginosa (Schroeter) Migula (ATCC 27853)	4	4
	Pseudomonas aeruginosa IMP+, VIM+ (clinical isolate)	4	4
	Klebsiella pneumoniae subsp. *pneumoniae* (ATCC 700603)	4	8
	Klebsiella pneumoniae subsp. *pneumoniae* (ATCC 13883)	4	8
	Listeria monocytogenes (ATCC 7644)	<1	4
	Acinetobacter baumannii NCTC 13304	4	4
	Salmonella enterica subsp. *enterica serovar Typhimurium* (ATCC 14028)	4	8
	Salmonella enteritidis (RITCC 1624)	2	4
	Proteus vulgaris (clinical isolate)	<1	4
	Proteus mirabilis (clinical isolate)	<1	4
Gram (+)	*Staphylococcus aureus* subsp. *aureus Rosenbach* (ATCC 29213)	1	2
	Staphylococcus aureus subsp. *aureus Rosenbach* (ATCC 25923)	2	4
	Methicillin-resistant *Staphylococcus aureus* (MRSA clinical isolate)	4	4
	Enterococcus faecalis (ATCC 29212)	<1	4
	Vancomycin-resistant *enterococci* (VRE clinical isolate)	1	4

Table 3: MIC values of some marine AMPs (µg/ml)

	EC-HEPCIDIN3	SA-hepcidin1	MoroPC-NH2	Mytimacin-AF	SpHyastatin	Acipensin1
S. aureus	4.2-8.4	150	12	18.4	8.8-17.5	4.7
E. coli	ND	150	12	ND	>140	3.7

The results are converted from µM to µg/ml for better comparison; ND: not defined

through the disruption of membrane potential. An interesting point to be noted is that it showed the same lethal effects on both sensitive and antibiotic-resistant strains of *Helicobacter pylori* [7]. In our study, these results were true not only for gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae*), but also for gram-positive bacteria (*Enterococcus, Staphylococcus aureus*). Some other advantages previously mentioned for TP4 include synergistic effect with conventional antibiotics and inducing host adaptive immune responses [6,7,25].

One of the limiting factors in the process of research and the commercialization of AMPs are the relatively high costs of their production. In this study, we tried to achieve a low-cost method to produce the TP4 for research or even local therapeutic applications. To attain this goal, some strategies were considered including: 1) Production of TP4 in the form with a minimum requirement of purification, 2) Impurities of medium would not have toxic effects on the living organism, 3) No need to complex tools along with as low as possible production costs.

For this purpose, the *P. pastoris* expression system was used for TP4 production. This expression system, due to the lack of LPS and sensitivity to TP4, would make it possible to produce a non-toxic product. In addition, the pPIC9 vector was used to transfer the TP4 sequence to the *P. pastoris* GS115 genome. This vector despite the similarities with other common shuttle vectors for *P. pastoris*, has a trivial but unique underestimated feature of simultaneous presence of secreted alpha factor and the *his4* gene (the sequence is available at: www.snapgene.com/resources/plasmid_files/yeast_plasmids/pPIC9/)

1. **Alpha factor secretion signal:** This sequence causes the recombinant peptide to be expressed in a secreted form and released to the environment. The *P. pastoris* naturally lacks any significant secreted protein [17-21], so if a culture medium without peptide and protein is used, about all the total protein of supernatant after the expression can be attributed to our recombinant peptide.
2. ***his4* gene:** This sequence causes the production of an enzyme which plays a role in the histidine biosynthesis (as a major growth factor). The presence of *his4* allows the *P. pastoris* to grow in a medium lacking amino acid and peptide [29].

Therefore, use of the pPIC9 vector in this study enabled us to apply the minimal culture medium of buffered minimal methanol (BMM) and to separate the secreted TP4 along with the supernatant from yeast cells. It is while most of the common shuttle vectors lack the simultaneous presence of these two features. For example, although two pPIC3.5 and pHIL-D2 contain *his4*, they lack alpha factor and so are not able to secrete the protein to the environment. Similarly, pPICZa and pAO815 contain the secretion alpha factor but lack the *his4* and cannot grow in minimal culture medium such as BMM (All sequences are available at: www.snapgene.com/resources/plasmid_files/yeast_plasmids/)

Antimicrobial activity of the crude TP4 on common bacterial pathogens (Table 2) showed that the gram-positive bacteria are more susceptible to this peptide. The TP4 MIC on *E. coli* and *S. aureus* bacteria, which are considered more in the studies of marine AMPs, were measured 4 and 2-4 µg/ml, respectively. This result is much better than the two marine AMPs SA-hepcidin1 and Sp Hyastatin, and almost equal with acipensin1 marine AMP (Table 3) [30-35].

In this study, we succeeded to produce about 71 mg crude TP4 per each liter of BMM medium. The production of this peptide, in addition to reducing the costs of future studies, can also be used for large-scale production for therapeutic applications (at least for local use). In this study, to produce 71 mg of crude TP4 using the designed and optimized *P. pastoris*, about 10$ and five days are required. However, according to the table of Peptide Synthesis Pricing [Accessed June 8, 2018] by the Bio Basic Inc. (www.biobasic.com/peptides-splash-custom-peps/), to produce the same amount of crude TP4, about 200$ (20 times more expensive) and 14-21 days are required. However, the time will also be more for the distant countries and shipping costs will also be added. It is interesting to be noted that to produce this amount of TP4 with the purity of 98%, more than 1000$ (100 times more expensive) is required.

CONFLICTS OF INTEREST

Authors declare there is no conflict.

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