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**Production and characterization of therapeutic peptides with
potential antimicrobial properties**

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Pichia pastoris

Structural characteristics



Abstract

Production and characterization of therapeutic peptides with potential antimicrobial properties

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Bacterial drug resistance is a current and growing serious health problem worldwide because of the long-term and negligent use of antibiotics. Finding new antibiotics and shepherding them through the clinical trials and approval process is becoming increasingly difficult. In addition, the declining number of new innovative antibiotic candidates has worsened this burden, necessitating the development of new classes of antimicrobial agents. Antimicrobial peptides (AMPs) are protein molecules that often form part of the innate immune system and cellular defense mechanisms found in many organisms. AMPs are promising antibiotic alternatives for the treatment of drug-resistant infections. They are, however, constrained by their high manufacturing costs.

Genetic engineering and heterologous recombinant peptide expression are promising approaches for producing such molecules at a low cost. In this study, peptide Gene 1 and peptide Gene 2 obtained from the bioinformatics analysis on marine metagenomes sampled from the TARA Oceans Project were produced using the heterologous recombinant peptide expression method. The peptide Gene 1 and peptide Gene 2 were cloned into the pPIC9 vector, transformed, and integrated into the *P. pastoris* genome. The expression was observed in BMMY medium, pH 6, 1% methanol, and 48 hr, 72 hr, 96 hr with a band at a molecular mass of 6.05 kDa for peptide Gene 1 and 11.37 kDa for peptide Gene 2 following SDS-PAGE analysis.

The recombinant peptide Gene 1 and peptide Gene 2 were produced in 100 ml culture medium using the 250 ml baffled shake flasks. The recombinant peptides were recovered in 100 ml supernatants and were characterized using antimicrobial activity testing. Then, the recombinant peptide Gene 1 and peptide Gene 2 were

tested on some gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* to investigate the antimicrobial effects and showed no activity. There is a possibility that these peptides are not antibacterial peptides as they were tested only on two bacterial strains, instead, they might be an antifungal peptide, antiviral peptide, antiparasitic peptide, or anticancer peptide. In this study, the peptide Gene 1 and peptide Gene 2 were successfully cloned into the pPIC9 vector, and we were able to produce the peptides recombinantly in *Pichia pastoris*. Production of these two peptides by this method costs very little and paves the way for further studies and therapeutic usage.



Declaration

I declare that the *Production and Characterization of Therapeutic Peptides with Potential Antimicrobial Properties* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Raphaella Ratshilume

Date 27 June 2024

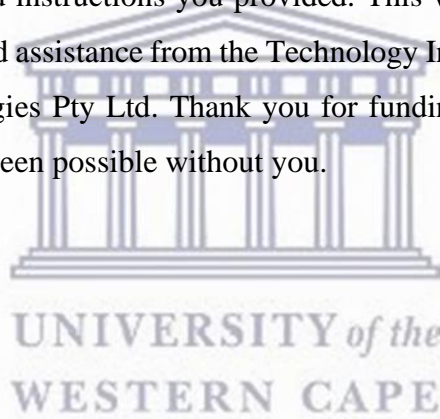
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List of Abbreviations

%	Percentage
°C	Degree Celsius
µl	Microlitres
ABPs	Antibacterial peptides
ACPs	Anticancer peptides
AFPs	Antifungal peptides
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
APPs	Antiparasitic peptides
AVPs	Antiviral peptides
BLAST	Blast Local Alignment Search Tool
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
bp	Base pair
Ca	Calcium
CaCl ₂	Calcium chloride
cm	Centimetre
ddH ₂ O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
g	Grams
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
kb	Kilobase
kDa	Kilodalton
KNN	K-nearest neighbor
LB	Luria Bertani
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
M	Molar

MD	Minimal dextrose medium
Mfa 1	Alpha mating factor
Mg	Magnesium
mg/L	Milligram per litre
MgCl ₂	Magnesium chloride
ml	Millilitres
mM	Millimolar
MOA	Mode of action
MW	Molecular Weight
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
ng/μl	Nanogram per micro litre
NN	Neural network
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PS	Phospholipid
RF	Random Forest
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl-sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sp.	Species
SVM	Support vector machine
TAE	Tris acetate-EDTA
TNF-α	Tumor necrosis factor-α
UV	Ultraviolet
V	Voltage
v/v	Volume per volume
w/v	Weight per volume
WBCs	White blood cells
WHO	World Health Organization
x g	Times Gravity

YPD	Yeast extract peptone dextrose
α	Alpha
β	Beta
Θ	Theta



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Chapter 1: Literature Review

1.1 Introduction

Antimicrobial peptides (AMPs), also known as host defense peptides, have recently sparked significant interest in the search for new antibiotics (Haney et al., 2019, Bhattacharjya and Straus, 2020, Mahlapuu et al., 2020a). These natural compounds are currently being researched as novel anti-infective drugs and immunomodulatory candidates (Luong et al., 2020, Mahlapuu et al., 2020a). AMPs are small molecular peptides that are found in nature and are mainly made up of less than 100 amino acid residues (Moretta et al., 2021). These peptides are an essential part of the innate immune system. Antimicrobial peptides play an important role in preventing the entry of foreign microbes and are a crucial part of nearly all biological innate immunity (Zhang et al., 2020).

Antimicrobial peptides exhibit strong broad-spectrum activity against bacteria, fungi, and viruses (Van Eijk et al., 2020, Buccini et al., 2021, Kurpe et al., 2020) and have a positive net charge and an amphiphilic structure that permits strong interactions with hydrophobic surfaces and membranes (Moretta et al., 2021, Van Eijk et al., 2020). Inhibiting the production of cell walls, nucleic acids, and proteins, as well as controlling the host immune response, AMPs show antimicrobial activity by targeting intracellular targets such as proteins, RNA, and DNA (Rowe-Magnus et al., 2019, Mansour et al., 2014). Antimicrobial resistance (AMR) occurs when microorganisms such as bacteria, viruses, fungi, and parasites evolve against the antimicrobial drugs such as antibiotics used to treat them (World Health Organisation, 2021). In addition, the current range of antibiotics does not provide a solution to the multidrug-resistant bacteria, also known as superbugs.

Since the number of antimicrobial resistances is rising quickly and there are not many new antimicrobial drugs being developed to address this problem, antimicrobial resistance (AMR) has become one of the biggest worldwide concerns of the twenty-first century (Prestinaci et al., 2015).

The effects of overuse or irresponsible use of antibiotics in various situations, particularly in clinical treatment along with agricultural use, animal healthcare, and the food system, might be one of the main causes of the current problem (Llor and Bjerrum, 2014). Antimicrobial resistance is commonly known as the “Silent Pandemic” and is a problem that requires immediate action, and better management, and should not be considered an issue that will arise in the future (Founou et al., 2021). According to the estimates, AMR could potentially become the main cause of death globally by 2050, if preventive measures are not taken (O'Neill, 2016).

Based on the estimates provided worldwide, the number of fatalities directly related to antimicrobial resistance (AMR) has increased to over 1.2 million in 2019 and is expected to increase to approximately 10 million deaths annually by 2050, if insufficient action is taken to manage AMR (O'Neill, 2016). As a result of the 2020 Covid -19 pandemic, more drugs, including ceftriaxone and azithromycin (Chen et al., 2020), were provided to COVID-19 patients, exacerbating antibiotic-resistant issues (Sohrabi et al., 2020, Fard et al., 2021). Therefore, the burden on global health has led to an urgent demand for the development of new classes of antibiotics. Antibiotic resistance is a problem and as a result, it has become critical to seek alternatives to conventional antibiotics that have novel modes of action and are less susceptible to bacterial resistance.

1.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are oligopeptides of low molecular weight which are found in plants, animals, and humans. They have wide-ranging antibacterial activity and are essential to the host's innate immune responses. Antimicrobial peptides are produced by animals (Zaslhoff, 2002), plants (Tam et al., 2015), fungi (Essig et al., 2014), protists (Andrä et al., 2003), archaea (Charlesworth and Burns, 2015) and bacteria (Riley and Wertz, 2002). AMPs are also produced by specific cells such as chemokines, leukocytes, phagocytic granulocytes, and mast cells and they are either constitutive or inducible and expressed by specific external factors (Tang et al., 2018a, Chang et al., 2019). Antimicrobial peptides are 5-100 amino acid oligopeptides that include a large amount (usually 50%) of hydrophobic

residues and a positive net charge (usually +2 to +11) (Pasupuleti et al., 2012, Chen and Lu, 2020). They are effective against a broad range of microorganisms in mammals, such as viruses, fungi, bacteria, and unicellular protozoa (Chromek et al., 2006, Pretzel et al., 2013, de Breij et al., 2018, Ahmed et al., 2019).

AMPs have a variety of reported modes of action, most of which result in the pathogen's direct killing; however, several AMPs can also cause indirect pathogen killing by influencing the host's immune response (Steinstraesser et al., 2011, Hancock et al., 2012). Attacking several low-affinity targets, including bacterial membranes, is a key characteristic of AMPs that distinguishes them from traditional antibiotics and is thought to slow the development of antibiotic resistance (Mahlapuu et al., 2020b). Additionally, AMPs are accessible to peptide engineering and mutagenesis, which have previously produced several molecules with reduced cytotoxicity and increased bioactivity (Wang and Craik, 2018, Troeira Henriques et al., 2017).

1.3 History of Antimicrobial Peptides

Alexander Fleming discovered lysozyme in the late 1920s, and some authors have considered that this was the first peptide to exhibit antimicrobial properties (Zhang and Gallo, 2016). Lysozyme was initially identified as a nonenzymatic antimicrobial agent, currently, we understand that it functions by enzymatically breaking down bacterial cells (Phoenix et al., 2012). AMPs were discovered in 1939 by Dubos, who isolated an antimicrobial agent from *Bacillus spp.* in soil samples. This agent protected against pneumococcal infection in mice, and they named the isolated fraction gramicidin, which was the first recognized antimicrobial peptide (AMP) (Dubos, 1939). However, an unknown kind of toxicity was noted after intraperitoneal administration of gramicidin which exhibits potential as a topical treatment of wounds and ulcers.

Another AMP called tyrocidine was discovered and proven to be effective against both gram-negative and gram-positive bacteria (Dubos and Hotchkiss, 1941). The mixture of cyclic decapeptides that *Bacillus brevis* produces forms this AMP

(Mootz and Marahiel, 1997). The first antibiotic to be commercially available was tyrocidine, however, it is harmful to human blood cells (Rammelkamp and Weinstein, 1942). Another AMP, named purothionin was isolated from the wheat plant *Triticum aestivum* in the same year. It was discovered that purothionin was effective against harmful bacteria and fungi including *Pseudomonas solanacearum* and *Xanthomonas campestris* (Balls et al., 1942, OHTANI et al., 1977). This peptide belongs to the family of AMPs known as thionin, which is found in the plant kingdom-wide group (Phoenix et al., 2012).

Phagocytin was isolated from rabbit leukocytes in 1956 and is the first reported AMP of animal origin (Hirsch, 1956). Additional AMPs were found in the following years, including lactoferrin from cow milk (Groves et al., 1965) and bombinin (1962) from the orange-speckled frog *Bombina variegata* (Kiss and Michl, 1962). Other antimicrobial peptides were discovered in the lysosomes of human leukocytes at the same time (Zeya and Spitznagel, 1963). Additionally, in the 1960s when *Pseudomonas aeruginosa* was compromised in the hemolymph of wax moth larvae, researchers discovered some small, inducible antimicrobial compounds (Phoenix et al., 2012).

Many antimicrobial peptides and antimicrobial proteins from leukocytes (Levy, 2004, Selsted et al., 1983) were mentioned in several reports in the late 1970s and 1980s, including those that are now known as α -defensins from humans and rabbits (Ganz, 2003). By injecting bacteria into the silk moth *Hyalophora cecropia* pupae, Steiner *et al.* (1981) induced the cationic antimicrobial proteins P9A and P9B from the pupae's hemolymph (Steiner et al., 1981). These peptides were reported as the first important α -helical antimicrobial peptides and renamed as the more well-known cecropins and characterized (Hultmark et al., 1980). Zasloff extracted cationic antimicrobial peptides from the African clawed frog *Xenopus laevis* in 1987 and identified them as defense antimicrobial peptides called magainins (Zasloff, 1987). A few years later, β -defensins and θ -defensins were characterized following extraction from bovine granulocytes and leukocytes, respectively, of the rhesus monkey which differs from α -defensins by their cysteine pairings and were

characterized (Ganz, 2003, Selsted et al., 1993, Ganz, 2005). In organisms lacking an adaptive immune system, antimicrobial peptides were gradually considered to have a defensive role (Phoenix et al., 2012). This role was confirmed in the middle of the 1990s for the fruit fly *Drosophila melanogaster*, where the insect became more susceptible to a massive fungal infection caused by the deletion of the gene encoding an antimicrobial peptide. Later, antimicrobial peptides' function in plants, insects, and invertebrates lacking adaptive immune systems was thoroughly studied (Phoenix et al., 2012).

Researchers have concluded that antimicrobial peptides (AMPs) are synthesized by almost all multicellular organisms, including humans, and have a function in an immune system after considering the findings of all these studies (Phoenix et al., 2012). These peptides have been found in most areas of the human body that are usually in contact with bacteria, such as the skin and mucous membranes (Wiesner and Vilcinskas, 2010).

1.4 Classification of the Antimicrobial Peptides

Classification of natural antimicrobial peptides is challenging because of their diversity. Antimicrobial peptides are categorized based on the four factors: (1) source, (2) activity, (3) structural characteristics, (4) amino acid-rich species (Figure 1).

1.4.1 Classification of Antimicrobial Peptides Based on Sources.

Antimicrobial peptides from various species, such as amphibians, insects, mammals, and fish, make up 75.65% of all AMPs. The remaining AMPs mostly come from plants and bacteria and represent 13.5% and 8.53% of the total AMPs, respectively (Hazam et al., 2019).

1.4.1.1 Antimicrobial Peptides from Bacteria

Bacterial antimicrobial peptides are commonly referred to as bacteriocins. There are several differences between them, although their mode of action and other characteristics are the same as those of eukaryotic AMPs. In comparison with eukaryotic antimicrobial peptides, bacteriocins are more effective at lower concentrations. Furthermore, eukaryotic AMPs can target a greater variety of bacterial groups than bacteriocins which have limited effect on a few species or genera (Nissen-Meyer and Nes, 1997). Bacteriocins are categorized based on their size, mechanism of action, structure, and origin. Larger protein-structured colicins and /or smaller peptide-structured microcins are the two groups of bacteriocins derived from Gram-negative bacteria such as *E. coli* and /or other enterobacteria (Duquesne et al., 2007).

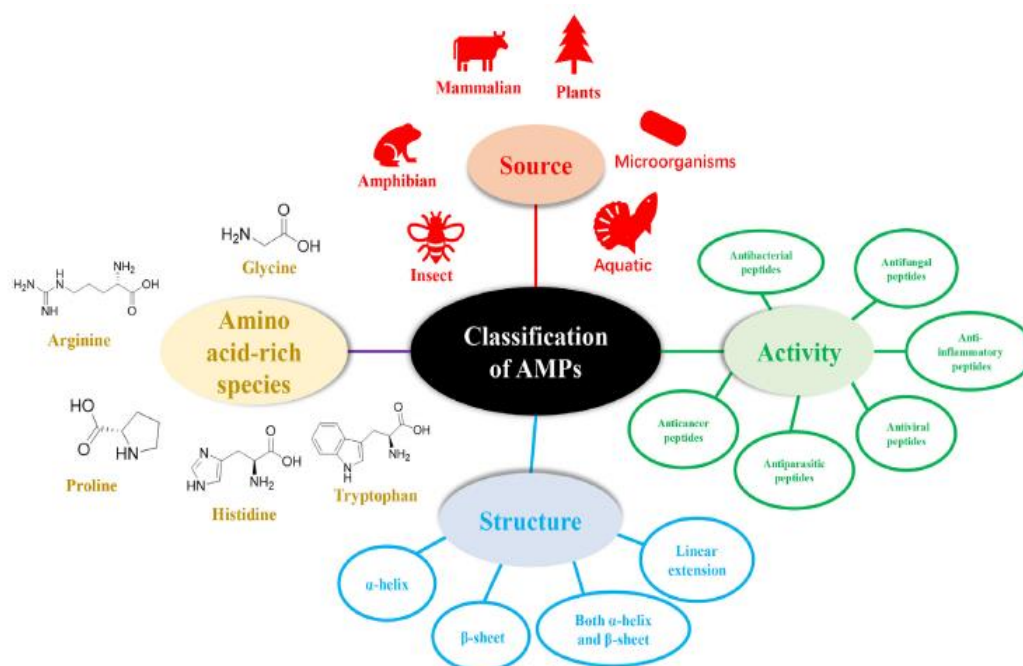


Figure 1. Classification of antimicrobial peptides based on the source, activity, structure, and amino acid-rich species.

Gram-positive bacteria produce bacteriocins that are divided into two main groups: non-lantibiotics (Class II) which contain unmodified antimicrobial peptides and

lantibiotics (Class I) which contain thioether-based ring structures called lanthionine or β -methyllanthionine (Hassan et al., 2012). According to Kalyani and Rajina (2017), *actinomycetes* are significant microbial groups that are well-adapted to the soil environment and serve as rich sources of peptide antibiotics. Novel antimicrobial peptides that are obtained from soil-derived *actinomycetes* strains include lipopeptide arylomycin A6 from *Streptomyces parvus* HCCB10043 (Rao et al., 2013), ohmyungsamycins A and B isolated from *Streptomyces sp.* (Um et al., 2013), and the well-known natural antibiotics vancomycin and daptomycin produced by different actinomycetes species, pargamicins B, C and D produced by *Amycolatopsis sp.* ML1-hF4 (Hashizume et al., 2017).

1.4.1.2 Antimicrobial Peptides from Marine Sources

One of the richest sources of antimicrobial peptides known is the marine environment. Oceans are tremendous sources for the discovery of potential antimicrobial peptides and cover just over 70% of the Earth (Charlet et al., 1996, Cheung et al., 2015). The marine environment is usually characterized by low temperatures, high pressure, complete darkness, and high salinity, unlike the terrestrial environment (Lauro and Bartlett, 2008). Marine antimicrobial peptides are structurally different from terrestrial antimicrobial peptides and are more adaptive to harsh environmental conditions like high salinity (Falanga et al., 2016).

Marine antimicrobial peptides (AMPs) are extracted from microorganisms and marine organisms. Marine antimicrobial peptides are usually categorized into four basic groups based on their structural and biochemical characteristic, without consideration of their mode of action. Within this classification, peptides in the same structural class may exhibit considerably distinct modes of action. Linear α -helical antimicrobial peptides (i) include styelins, hedistin, piscidin, myxinidin, pleurocidin, and clavanins; these marine AMPs have hydrophobic and hydrophilic regions in a linear and short-chain structure that acquire a helical conformation after interaction with the membrane (Lehrer et al., 2001, Pundir et al., 2014). Linear or helical peptides with an abundance of one amino acid (ii) proline -and arginine-rich callinectin (Khoo et al., 1999, Noga et al., 2011), histidine-rich chrysofopsin (Iijima

et al., 2003, Mason et al., 2007), and proline- and glycine-rich collagencin (Ennaas et al., 2016). Peptides that form a hairpin-like β -sheet or α -helical/ β -sheet mixed structures stabilized by intramolecular disulfide bonding are the third group (iii). Defensins are the most well-known example of this group, they are characterized by multiple disulfide bonds, that provide additional stability and compactness in high salt concentrations (Scudiero et al., 2010, Scudiero et al., 2013). Cyclic peptides (iv) generally exhibit antimycotic activity and their antibacterial activities have not been studied in detail, although they are extracted from the marine environment in large amounts (Falanga et al., 2016). The most well-known example of cyclic marine antimicrobial peptides is discodermin A, which was isolated from the sea sponge (Matsunaga et al., 1985).

1.4.1.3 Antimicrobial Peptides from Plants

Plant-derived antimicrobial peptides (AMPs) are small protein molecules found in plants that exhibit strong and broad-spectrum antimicrobial activity. Purothionin isolated from wheat flour was the first reported plant antimicrobial peptide *Triticum aestivum* (De Caley et al., 1972). Most plant antimicrobial peptides have a molecular weight of 2 to 10 kDa and are naturally basic. They also have 4-12 cysteine residues, which enhance thermodynamic and structural stability (García-Olmedo et al., 2001). Plant antimicrobial peptides (AMPs) are generally, categorized based on their peptide chain length, the number and position of cysteines that form disulfide bonds (de Souza Cândido et al., 2011, Marcus et al., 1997).

Several plant-derived antimicrobial peptides groups such as defensins, snakins, puuroindolines, glycine-rich proteins, cyclotides, hevein-type proteins, thionins, knottins, and lipid transfer proteins, have been purified, identified, and studied (Nawrot et al., 2014, Stotz et al., 2012, Tang et al., 2018b). According to Montesinos (2007), these antimicrobial peptides were isolated from various parts of plants such as flowers, seeds, roots, stems, and leaves (Montesinos, 2007). They also have anti-insect activity against oomycetes and herbivorous pests, and anticancer activity against some cancer cells, in addition to the strong microbiocidal

activity of plant antimicrobial peptides against viruses, bacteria, fungi, parasites, and protozoa (Allen et al., 2008, Koike et al., 2002, Kong et al., 2004, Nawrot et al., 2014).

1.4.1.4 Antimicrobial Peptides from Insects

The essential role of insect antimicrobial peptides is in the humoral immune system. Insect antimicrobial peptides are produced in an insect's body fat and stored in the haemolymph (Brown et al., 2009, Bulet and Stocklin, 2005). To date, more than 200 antimicrobial peptides from insects have been found. Cecropins, insect defensins, glycine-rich peptides, proline-rich peptides, and lysozymes are under the five main groups in which these peptides are classified (Hwang et al., 2009).

1.4.2 Classification of Antimicrobial Peptides Based on Activity.

The APD3 database statistics show that there are twenty-four categories into which antimicrobial peptide activity can be classified. These groups of peptides can be summarized as antifungal, antiviral, antibacterial, antiparasitic, antitumor, and anti-human immunodeficiency virus (HIV) (Figure 2).

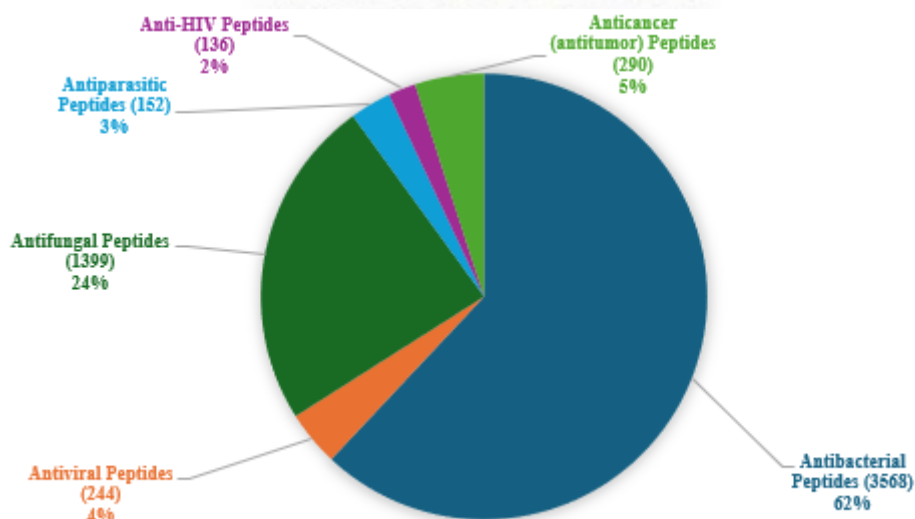


Figure 2. Statistics of the primary functions of antimicrobial peptides. Antibacterial peptides account for the largest proportion, approximately 62%, followed by antifungal peptides, which account for 24%, and antiviral, antiparasitic, anticancer, and anti-HIV peptides account for 4%, 3%, 5%, and 2% respectively (the figure is drawn based on data in APD3).

1.5 Biophysical Characteristics of Antimicrobial Peptide Activity

Despite the diversity of antimicrobial peptides found in different organisms, many of them have biophysical properties in common that give them the ability to attack the target microorganism. These properties include hydrophobicity, charge (cationicity), conformation, and amphipathicity. Despite their holistic function, these properties were discussed separately.

1.5.1 Charge

Many of the antimicrobial peptides that have been identified so far have a net positive charge, which ranges from +2 to +11, and may contain a well-defined cationic domain. The presence of residues containing lysine, arginine, and sometimes histidine is responsible for the cationic nature. Cationicity is unquestionably essential for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms (Yeaman and Yount, 2003).

1.5.2 Hydrophobicity

Peptide hydrophobicity is a percentage of hydrophobic residues in the peptide sequence. It is approximately 50% for most antimicrobial peptides. Hydrophobicity is a crucial characteristic for antimicrobial peptide membrane interactions, as it is necessary for efficient membrane permeabilization and governs how much a peptide can partition into the lipid bilayer (Yeaman and Yount, 2003, Yin et al., 2012, Chen et al., 2007).

1.5.3 Amphipathicity

Amphipathicity is the relative quantity and polarization of hydrophilic and hydrophobic domains inside the protein and is calculated using hydrophobic moment, which can be calculated as the vectorial sum of the hydrophobicities of each amino acid normalized to an ideal helix. Amphipathicity enables the permeabilization of the peptide against the microbial target (Eisenberg, 1984).

1.5.4 Conformation

Although antimicrobial peptides vary greatly in terms of their source and sequence, however, antimicrobial peptides are categorized based on several themes that are predominant in their three-dimensional topology. The majority of the remaining peptides can be categorized as those that are enriched in one or more amino acid residues, such as proline-arginine or tryptophan-rich, while the two largest groupings are the α -helical and β -sheet peptides (Hancock, 1997).

1.6 Mechanism of Action of Antimicrobial Peptides

The mechanism of action of AMPs has been thoroughly studied since they have been a promising antibacterial agent. Characterization of AMP is very essential to increase their use as therapeutic agents (Kumar et al., 2018). Antimicrobial peptides develop less resistance to microbes than traditional antibiotics and have both bacteriostatic and bactericidal properties (Li et al., 2017). These peptides have hydrophilic and hydrophobic residues and are amphiphilic molecules with a positive charge. Positively charged cationic peptides experience electrostatic interaction with negatively charged cell membranes, resulting in membrane adsorption and conformational change. Through different mechanisms, such as the barrel stave model, the carpet model, the toroidal pore model, etc., these peptides bind to the cell membrane and then complete their activity. Barrel Stave Model: AMPs insert into the membrane and form a pore by aligning perpendicularly to the membrane, much like staves of a barrel. Carpet Model: AMPs cover the surface of the membrane like carpet, leading to membrane destabilization and disintegration.

Toroidal Pore Model: AMPs induce the formation of pores where the lipid monolayers bend continuously through the pore, forming a toroidal shape (Lei et al., 2019).

Antibiotics and antimicrobial peptides have different mechanisms of action. These peptides may function through several hypothetical mechanisms, such as plasma membrane disruption, intracellular antimicrobial mechanism, the inhibition of the synthesis of macromolecules such as protein, nucleic acids, and enzyme activity, and antimicrobial effect through participating in immune regulatory effects (Pushpanathan et al., 2013, Jäkel et al., 2012).

Based on secondary structure, antimicrobial peptides are divided into four significant groups including linear α -helical peptides, β -sheet peptides, linear extended peptides, and both α -helix and β -sheet peptides (Huan et al., 2020). According to the broad studies on members of all four groups of antimicrobial peptides, the main mechanism for most AMPs appears to kill cells, through the permeabilization of microbial cytoplasmic membranes (Zhang and Gallo, 2016). Through the barrel-stave or carpet pore model, the helical peptides cause damage to the membrane. Their major function cause structural disruption to bacteria cell membranes by introducing amphipathic helices (Moravej et al., 2018).

The β -sheet peptides can function in different ways such as inhibiting the formation of the cell wall and binding to certain lipid components in membranes (Zhang and Gallo, 2016). They move across lipid bilayers, which are connected to the formation of temporary pores. The α and β structures are both members of the $\alpha\beta$ family. Antimicrobial peptides elongated are linear and abundant in one or more amino acids such as glycine, tryptophan, arginine, and histidine. When they come into touch with a membrane, the member of this group has flexible structures in the aqueous environment that enable them to change into an amphipathic structure. They penetrate pathogen membranes and interact with cytoplasmic proteins instead of acting directly on the pathogen membranes.

As illustrated in Figure 3, the mode of action (MOA) of antimicrobial peptides can be broadly divided into two categories: direct killing and immunological regulation. The two classes of direct killing modes of action are membrane targeting and non-membrane targeting. Membrane permeabilizing peptides which are mainly identified by cationic peptides, such as defensin, LL37, and melittin, can form temporary pores on the membrane. Non-membrane targeting peptides such as pleurocidin, pyrrolicidin, and mersacidin, can penetrate the cell membrane and hinder important cellular processes that eventually result in the death of cells without permeabilizing the membrane (Pushpanathan et al., 2013).

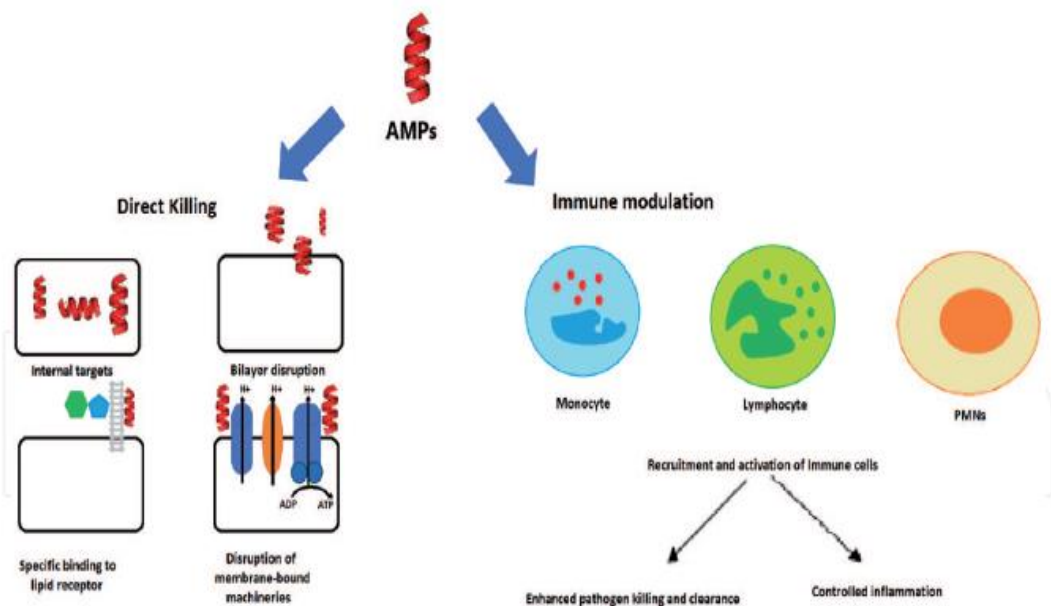


Figure 3. Mechanism of action of antimicrobial peptides

1.6.1 Direct killing: Membrane Targeting Mechanism of Action.

Antimicrobial peptides disrupt the membrane through hydrophobic and electrostatic interactions by binding to negatively charged membranes, such as bacterial outer membrane lipids with anionic head groups like phosphatidylglycerol and cardiolipin. Because of the positive charge present on their α -helix surface,

which is essential for killing microbes, AMPs can interact with negatively charged microbes membranes and show their antimicrobial activity. The zwitterionic phospholipids in mammalian membranes are only weakly interacting with the hydrophobic regions of AMPs. Since eukaryotic cell membranes are normally neutral and comprise uncharged neutral phospholipids (such as phospholipids comprising of phosphatidylcholine or phosphatidylethanolamine), sphingomyelins, and an enormous concentration of cholesterol, these peptides exhibit less cytotoxicity towards eukaryotic cells (Figure 4).

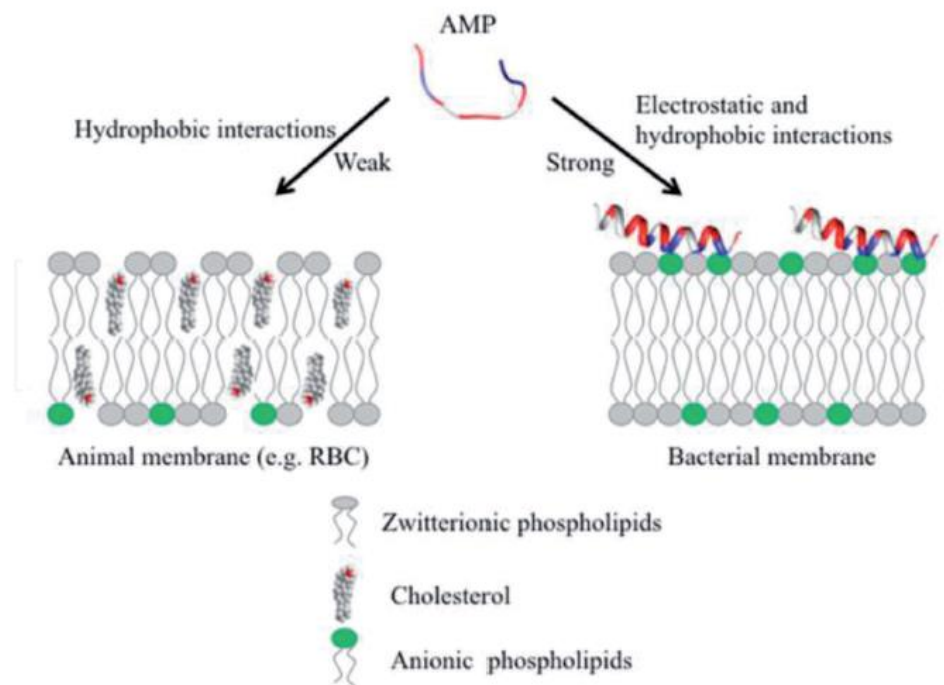


Figure 4. AMP interaction with mammalian membrane or bacterial membrane (Kumar et al., 2018)

The binding of AMPs to mammalian cells is reduced by cholesterol. Amphiphilic characteristics, net charges, and hydrophobicity of AMPs are determined by their amino acid composition, which is responsible for their essential effects on the selective action of microbes (Jäkel et al., 2012). The electrostatic interactions of negatively charged bacterial membranes with cationic AMPs and anionic phospholipids are essential for cellular communication. The outer surface of the

eukaryotic cell membrane is composed of phospholipids with phosphatidylcholine head groups and sphingomyelin with a small part of some ganglioside; as a result, the hydrophobic contact between cationic AMPs and the mammalian membrane is rather weak. The hydrophobic part of AMPs and the outer surface of the bacterium membrane come into significant contact because of the presence of negatively charged phospholipids (Seyfi et al., 2020). Receptor-mediated and non-receptor-mediated mechanisms are the two ways in which membrane-targeting AMPs interact.

1.6.2 Receptor-Mediated Mechanism

A small group of AMPs, or receptor-mediated peptides which consist of receptor-binding and pore-forming domains, mediate this (Zhang and Gallo, 2016). They normally function by interacting with membrane components to resist bacteria in vitro at micromolar or nanomolar concentrations. Most AMPs produced by bacteria, viruses, and tumor cells, such as nisin, lactococcin, and mesentericin, have this mechanism (Zhang and Gallo, 2016). As a membrane-bound element involved in the synthesis of peptidoglycan, lipid II interacts with nisin through a specific receptor-like interaction, which supports nisin's antimicrobial activity at low concentrations. Therefore, nisin is considered more effective against those gram-positive organisms rich in peptidoglycan than others (Kumar et al., 2018). It consists mostly of two domains: the first has a high affinity for the lipid II molecule, a cell wall precursor found in the membrane, and the second is a membrane-anchored pore-forming domain. Mersacidin is another AMP produced by *Bacillus* species that belongs to the lantibiotics group, much like nisin.

According to previous studies, mersacidin directly targets lipid II and interferes with the production of transglycosylation and peptidoglycan in gram-positive bacteria. Another example that exhibits a receptor-mediated mechanism to the membrane receptor SbmA is PR-39. Proline-arginine-rich PR-39 is a linear cathelicidin AMP in nature (Moravej et al., 2018). Although this AMP is unable to form pores in the bacterial membrane, it is known to have multi-functional activities including wound healing by inhibiting the expression of syndecan, anti-

inflammation through inhibiting NADPH oxidases, chemoattraction for neutrophil leucocyte and intervening protein and DNA production by quickly inducing proteolytic activity, which causes some proteins involved in DNA replication to degenerate (Moravej et al., 2018, Huang et al., 2010).

1.6.3 Non-Receptor Mediated Mechanism

Most vertebrate and invertebrate AMPs that exert their activity by interacting with membrane components are mostly part of the non-receptor-mediated action mechanism (Kumar et al., 2018). Gram-positive bacteria have teichoic in the outer membrane, while Gram-negative bacteria have lipopolysaccharide and each causes a net negative charge on the membrane surface and binds with cationic AMPs through electrostatic attraction (de Leeuw et al., 2010, Brogden, 2005). The most studied mechanism to understand the mode of action of antimicrobial peptides is membrane permeability. The membrane structure of bacteria and cancer cells is destroyed by antimicrobial peptides once they bind to microbial membranes causing the release of the cell contents and resulting in cell death (Moravej et al., 2018, Som et al., 2008, Wang GuangShun, 2017, Zasloff, 2019).

The extra-cellular membrane of Gram-negative bacteria is made up of negatively charged lipopolysaccharide (LPS). By substituting ions such as Mg^{2+} and Ca^{2+} bound to lipopolysaccharide (LPS), the cationic antimicrobial peptides cause a break or create a hole in the outer membranes of bacteria permitting them to translocate through extracellular membranes. On the other hand, the cell wall of Gram-positive bacteria is made up of a thick layer of lipoteichoic acid (LTA) and peptidoglycan, which together form a thick matrix that keeps the bacterial cell rigid. The peptidoglycan layers are permeable with nanopores, through which AMPs may diffuse (Meroueh et al., 2006). Lipoteichoic acid is an essential part of the Gram-positive bacteria's cell wall. It is a negatively charged molecule attached to the peptidoglycan through a diacylglycerol moiety. Gram-positive bacteria's cell walls that contain anionic teichoic acids may facilitate AMP penetration by giving AMPs another location to interact with (Zhang and Gallo, 2016).

AMPs bind to the phospholipids present on the inner cellular membranes of bacteria after penetrating through the outer membrane, the single layer of peptidoglycan in Gram-negative bacteria, and the thick layers of peptidoglycan in Gram-positive bacteria. This causes the cell membranes to break down or become permeable, which ultimately releases the contents of the bacteria, causing more bacterial cell lysis and death (Lei et al., 2019). There are two processes in the mechanism of cell membrane damage. First, the negatively charged bacterial cell membranes are selectively bound by the cationic AMPs, which then destroy bacterial membranes through perforation or non-perforation mode. Four distinct models- a barrel-stave, carpet, toroidal-pore, and aggregated channel models- have been proposed for the antibacterial activity of AMPs that result in membrane disruption by permeabilization (Figure 5) (Huan et al., 2020).

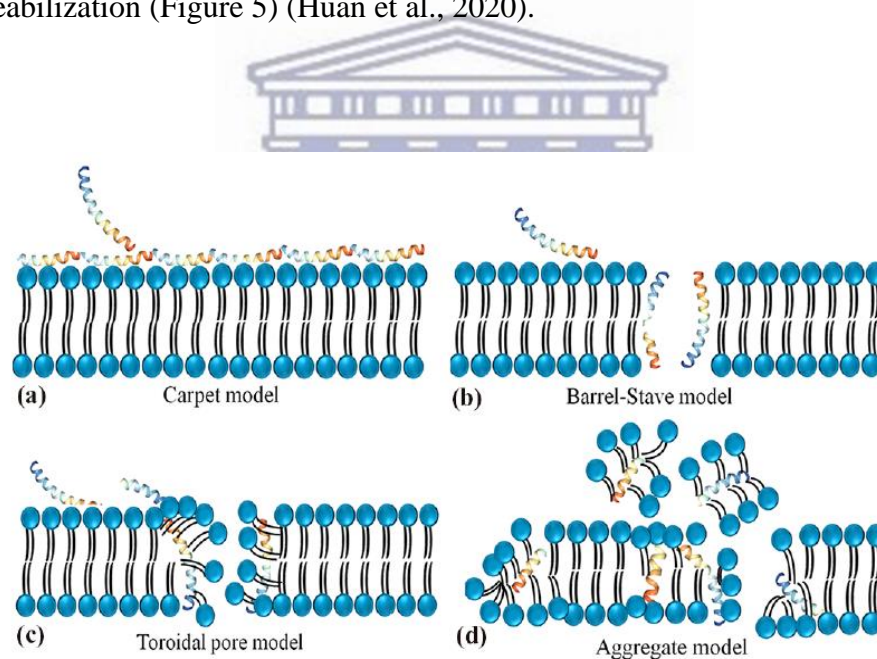


Figure 5. A schematic illustration of the potential mechanism by which antimicrobial peptides break membrane and /or translocate. **(a) Carpet model:** In this model, AMPs cover another membrane face, creating a “carpet,” and the membrane experiences some deformation and disturbance. **(b) Barrel-Stave model:** AMPs interact laterally and generate transmembrane pores. **(c) Toroidal pore model:** AMPs pierce the bilayer membrane and create a highly curved toroid. **(d) The aggregated model**

In the barrel-stave model, AMPs create a pore in the membrane bilayer by being inserted perpendicularly. The hydrophobic sides of this pore interact with the membrane's lipid to create the channel's interior side. Thus, they function as pore formers or as inhibitors of bacterial metabolism (Brogden, 2005). Protegrins (Brogden, 2005), alamethicin (Wimley, 2010), and pardaxin (Shai et al., 1990) formed barrel-stave channels, according to compelling evidence. In the toroidal pore model, peptides are introduced vertically into the membrane, where they create a pore that passes through both the peptides and head phosphates of phospholipids. Using this model, certain peptides are allowed to reach the cytoplasm and target intracellular components (Uematsu and Matsuzaki, 2000), such as melittin (Lee et al., 2016), lactacin Q (Lee et al., 2016), aurein 2.2 (Cheng et al., 2009) and magainin 2 (Lee et al., 2016). In the carpet model, The AMPs cover the membrane's surface and create tension, which causes the membrane to break down and micelles to form. Certain AMPs, including LL-37 (Shai, 2002) and cecropin (Sitaram and Nagaraj, 1999), indolicidin (Rozek et al., 2000), and aurein 1.2 (Rozek et al., 2000) create carpet models. According to these three hypothesized theories, membrane integrity can be compromised, leading to membrane malfunction metabolites, and ion leakage (Mahlapuu et al., 2016).

The membrane permeabilization prevents critical cellular processes like protein/nucleic acid synthesis, enzymatic/protein activity, protein folding, intracellular pathways, and/or cell wall synthesis. It also plays a role in the subsequent translocation of AMPs into the intracellular region (Mahlapuu et al., 2016). Additionally, AMPs are mostly produced by some immune cells such as neutrophils and macrophages, and they have an array of immunomodulatory functions, including immune cell recruitment and activation, the start of adaptive immunity, inflammation reduction (Diamond et al., 2009), immune cell chemoattraction, the induction of chemokine, cytokine, and histamine production and secretion, stimulation of wound healing, angiogenesis, and adjuvant city (Mousavizadegan and Mohabatkar, 2018).

1.7 Direct killing: Non-Membrane Targeting Mechanism of Action.

The non-membrane targeting MOA is mainly divided into two groups: AMPs that target the bacteria cell wall and those that target the intracellular components of bacteria (Kumar et al., 2018).

1.7.1 Peptides that Target Cell Wall

Similarly to conventional antibiotics, AMPs prevent the development of cell walls by interacting with a range of precursor molecules that are necessary for the formation of the cell walls. Lipid II is one example of a precursor molecule that AMPs primarily target (Lohner, 2017). For instance, the anionic pyrophosphate sugar moiety of lipid II molecules is bound by peptides such as defensins (Münch and Sahl, 2015). This binding has the potential to cause pores to develop, which in turn causes membrane disruption (Lohner, 2017). Human β -defensin 1 (de Leeuw et al., 2010) and human- β defensin 3 (Münch and Sahl, 2015) are antimicrobial peptides (AMPs) that exhibit their bactericidal action mechanism through binding to lipid II.

1.7.2 Peptides that Target Intracellular Components

AMPs have been shown in many research studies to be able to cross bacterial cell membranes and interact with intracellular targets such as DNA and RNA, distracting the physiological activities of the bacterium. Protein and cell wall production may be disrupted as a result (Brogden, 2005). Antimicrobial peptides block essential cellular functions by interacting first with the cytoplasmic membrane and then targeting intracellular components. Inhibit the formation of the cell walls, preventing the production of macromolecules like proteins or nucleic acids, or preventing enzymatic activity are examples of mechanisms that include intracellular targets. Certain AMPs, such as burofin II and indolicidin can pass through and penetrate the bacterial membrane. Once inside, they attach to nucleic acids (DNA or RNA) and prevent the synthesis of nucleic acids (Kumar et al.,

2018). It is assumed that the cationic amino acids in the peptides interact electrostatically with the negatively charged phosphate groups in the nucleic acids or other synthesized proteins, however, the exact mechanism of action is yet unknown (Wang GuangShun, 2017). Certain AMPs now target intracellular components because they do not cause membrane permeabilization even at the minimal optimal dose and nonetheless cause bacterial death (Brogden, 2005).

1.8 Immunological Regulation Mechanism of Action

In addition to specifically targeting and destroying bacteria, AMPs have the potential to exert their antimicrobial activity through immune modulatory mechanisms (Zasloff, 2019). Different mechanisms are used by AMPs to show immune-modulatory effects, including the reduction of endotoxin-induced inflammatory response, the induction of pro-inflammatory proteins and cytokines, the regulation of adaptive immunity, and the recruitment of macrophages (Niyonsaba et al., 2002, Condé et al., 2012, Mojsoska et al., 2015). Rather than killing microbes directly, these peptides strengthen the body's ability to fight microbes (Lei et al., 2019).

Antimicrobial peptides are secreted by epithelial surfaces from glandular structures and barrier epithelial (Zasloff, 1987, Diamond et al., 1991, Ouellette and Selsted, 1996, Jones and Bevins, 1992). Different kinds of microbial and digesting enzyme storage organelles, or granules, are seen in phagocytic cells (Levy, 1996, Ganz and Lehrer, 1997). Granules fuse to phagocytic vacuoles containing ingested microorganisms during the phagocytosis process, exposing the bacteria to extremely high concentrations of digestive and microbicidal chemicals. Some granules are released into the extracellular fluid, where the microorganisms they contain are either killed or prevented from multiplying. Antimicrobial peptides are present in large quantities in both types of granules (Ganz et al., 1985, Selsted et al., 1984, Cowland et al., 1995). Certain AMPs show a range of immunological responses, including the differentiation and stimulation of white blood cells (WBCs), and management of chemokine and reactive nitrogen/oxygen species expression, and the decrease in the expression of inflammatory chemokines (Juretić

et al., 2017, Lee et al., 2016, Harder and Schröder, 2015, He et al., 1996, Yeaman and Yount, 2003). In mammals, AMPs activate the immune system through different methods: (i) by activating T cells; (ii) by stimulating Toll-like receptors; (iii) by an increase in phagocytosis; (iv) by activating dendritic cells; and (v) by neutrophil chemoattraction (Figure 6) (Matejuk et al., 2010).

Different cells in the body such as epithelial cells, lymphocytes, phagocytes, neutrophils, and keratinocytes, produce AMPs. These cells can be found in the lymphatic system, genitourinary tract, gastrointestinal tract, and immune system, among other areas. As AMPs have been studied more thoroughly, it has become clear that AMPs are either constitutively (often) generated or activated by inflammation (Stevenson et al., 2006). Some immune cells, such as macrophages and neutrophils, make AMPs constitutively, while other immune cells, such as epithelial cells, produce them in response to stimulation of the mucosal surface (Stevenson et al., 2006). Induction is responsible for the production of the majority of β -defensin, and α -defensins are among the most frequently produced AMPs (Ganz, 2003). Human AMPs such as LL-37 and β -defensin can attract immune cells, such as mast cells (Niyonsaba et al., 2002), dendritic cells (Liu, 2001), and leukocytes (García et al., 2001).

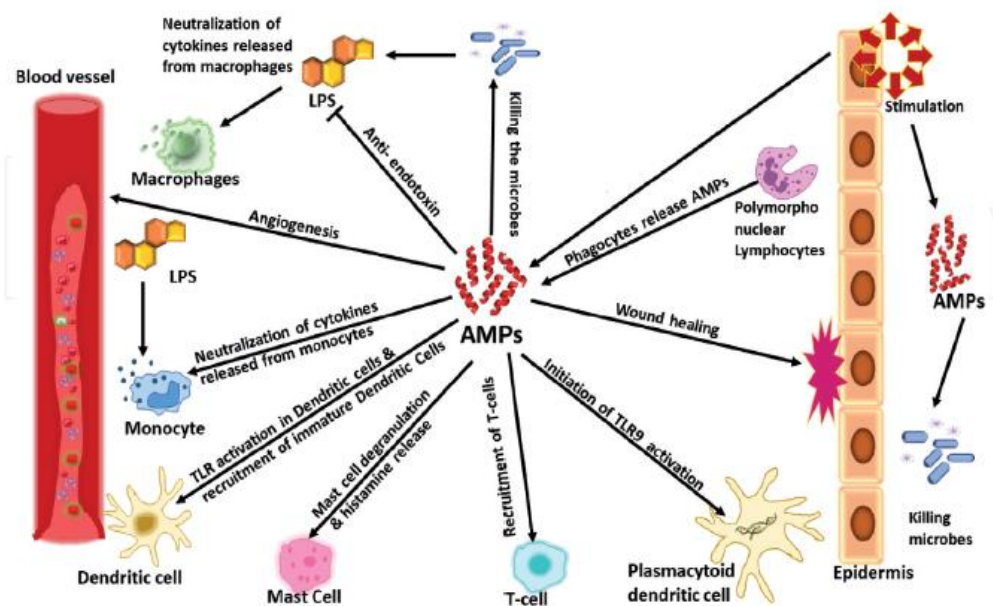


Figure 6. AMPs influence the expression of genes in various cell types, such as macrophages, neutrophils, monocytes, and epithelial cells. These cell types then release chemokines and cytokines, which cause leukocytes to return to the infection site, induce cell differentiation, activate specific cells, and block or activate the Toll-like receptor signalling cascade. AMPs have a variety of beneficial effects including infection prevention, wound healing, inflammation management, and inducing adaptive immunity (Moravej et al., 2018).

1.9 Identification of Antimicrobial Peptides Using Deep Learning

In antimicrobial peptide research, computational techniques are becoming more and more significant. Traditional methods of machine learning and deep learning are considered efficient techniques for recognizing patterns from sequences that were previously unknown and aid in the prediction of the antimicrobial potential of new sequences (Yan et al., 2022). Traditional machine learning includes all machine learning techniques such as random forest (RF), k-nearest neighbor (KNN), support vector machine (SVM), and single-layer neural network (NN) (Wittek, 2014), but excludes deep learning techniques, which often involve many layers of NN. The time and cost in the search for new active antimicrobial peptides can be greatly reduced by prioritizing and selecting candidate sequences for experimental validation based on the prediction results.

A computational study's main advantage is that investigation is not restricted to known peptides. While antimicrobial potency is maximized by performing mutations or alterations based on template sequence, random libraries containing an almost infinite number of sequences can also be searched on the prediction for novel AMP patterns (Nakatsuji and Gallo, 2012, Gan et al., 2021, Mookherjee et al., 2020, Yan et al., 2022). The putative antimicrobial genes used in this study were obtained from bioinformatics analysis performed on the marine metagenomes sampled from the TARA Oceans Project. Metagenome co-assemblies from the TARA oceans generated in a previous study were used to mine for AMPs being investigated (Delmont et al., 2018).

The 12 metagenome assemblies of >2.5 kilobases are stored in the NCBI Bioproject PRJNA326480. As described by (Ma et al., 2022), a set of neural network models was utilized to learn antimicrobial sequence features in the metagenomics data. The PALADIN software (version 1.4.0) and SAM tools (version 1.7) used to predict the antimicrobial peptides can be found at https://github.com/mayuefine/c_AMPs-prediction. The bioinformatics department provides several peptide hints with potential antimicrobial properties. Only two antimicrobial genes were selected for this project as the gene of interest from the AMP open reading frames predicted through deep learning. The selected antimicrobial genes were named peptide Gene 1 and peptide Gene 2 as they are unknown genes.

1.10 Heterologous Expression of Antimicrobial Peptide

The most effective method for enhancing the production of proteins, peptides, or enzymes is often considered to be recombinant DNA technology. The advantage of the technology is not limited to shorter turnaround times and well-established procedures, it also includes lower manufacturing cost, simple purification, simple scalability, and the ability to perform post-translational modification (Cereghino et al., 2002). Cloning target genes encoding the desired protein or peptide into a specific vector allows the gene to be expressed in the host cellular expression system. The most widely used host systems for the expression of recombinant products are bacteria and yeast (Gupta and Shukla, 2017). These two expression hosts have been reported to produce more than 95% of the heterologous expressed antimicrobial peptides in the case of AMPs (Li and Chen, 2008). In the production of eukaryotic heterologous proteins, *Pichia pastoris* (*Komagataella phaffii*) is the most widely used and studied yeast expression system (Balamurugan et al., 2007).

In the *P. pastoris* expression system, AMPs such as cecropins (Jin et al., 2006, Wang et al., 2011), defensins (Hsu et al., 2009), ABC-CM4 peptide (Zhang et al., 2006), and human CAP18/LL37 AMP (Kim et al., 2009) were successfully expressed. *Pichia pastoris* has exhibited success in the expression of hybrid AMPs (Jin et al., 2009). Because it allows numerous eukaryotic post-translational modifications, including glycosylation, signal sequencing processing, and disulfide

bond formation, which are required for cysteine-rich cationic AMPs, the *P. pastoris* expression system was considered an ideal heterologous host (Cereghino and Cregg, 2000). For example, HD5, a cationic peptide containing six cysteine residues forming three intramolecular disulfide bonds, was expressed using this method (Hsu et al., 2009).

Pichia pastoris expressed most of the heterologous AMPs that were produced in yeast. The levels of expressed AMPs varied from 55 to 748 mg/L of culture media. The expression of scygnodin isolated from the crayfish *Cherax quadricarinatus* was initially attempted in *E. coli* (Peng et al., 2010). Research has exhibited that *P. pastoris* expresses scygnodin, and in comparison, to *E. coli*, this strain produced 1.3 times more recombinant AMPs (Peng et al., 2012). Another example was using the pET22b vector to try and express the plant defensin SPE10 from *Pachyrrhizus erosus* seeds in *E. coli* AD494, a thioredoxin reductase mutant strain, but the experiment failed to produce any detectable amounts of protein (Song et al., 2005). After purification, 7.0 mg/L of recombinant peptides was recovered when this peptide was produced in *P. pastoris*. The antifungal activity of the defensin-encoding gene PDC1 from corn against the phytopathogen *Fusarium graminearum* was evaluated by comparing the expression of this gene in *E. coli* and *P. pastoris* (Kant et al., 2009).

The proteins generated by *P. pastoris*, and *E. coli* are antifungal, according to the author. Nevertheless, the protein produced in yeast exhibits a two-fold higher inhibition of the growth activity of *Fusarium graminearum*'s. *Pichia pastoris* produced recombinant peptides with more β -sheets and less random unordered structure than when it was produced in *E. coli*, according to the structural analysis of the recombinant protein expressed in both systems (Kant et al., 2009). The production of recombinant AMPs in most studies using *P. pastoris* is still using shake flasks. A process to produce porcine interferon has recently been developed (Yu et al., 2010). A methanol feed was optimized for maximum recombinant protein production without cell inhibition after a high cell density of 110-130 GI-1 was initially achieved (Yu et al., 2010). Therefore, *Pichia pastoris* is the best yeast

for host expression, even though it still requires the establishment of a more suitable promoter (constitutive or inductive) concomitant to process development to achieve the desired yield of recombinant AMPs. In this study, we aim to express and study putative novel antimicrobial peptides detected from metagenomes sampled from marine microbiomes using deep learning models.

1.11 Aim

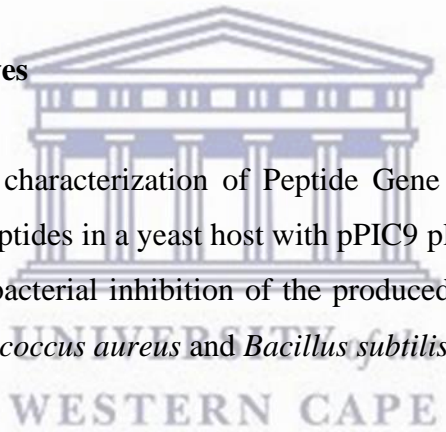
To express and produce novel antimicrobial peptides to characterize and assess their antimicrobial properties.

1.12 Hypothesis

Antimicrobial peptides from pristine marine microbiomes have a potential for antimicrobial activity against terrestrial pathogens and microbes.

1.13 Objectives

- i. Expression and characterization of Peptide Gene 1 and Peptide Gene 2 antimicrobial peptides in a yeast host with pPIC9 plasmids.
- ii. Assessment of bacterial inhibition of the produced antimicrobial peptides against *Staphylococcus aureus* and *Bacillus subtilis*



Chapter 2: Material and Methods

2.1 Materials

2.1.1 Bioinformatic Analysis of Gene 1 and Gene 2 DNA Sequences

The bioinformatics department at Capebio Technologies Pty Ltd provided the recombinant peptide coding sequences of Gene 1 and Gene 2. The peptide sequence of genes of interest was sent to gene universal for DNA synthesis and cloned into the pPIC9 vector.

2.1.2 Chemicals

Unless otherwise stated, suppliers of chemicals and reagents used for this project are as follows: Thermo Fisher Scientific, Inqaba Biotech, Sigma Aldrich, Merck Millipore, Biolab, Glentham Life Sciences, Associated Chemical Enterprises, Cleaver Scientific, Bio-Rad Laboratories, Radchem, and Reflecta Laboratory Supplies.

2.1.3 Enzymes and Kits

Table 1: List of enzymes and kits

Restriction enzyme	Description	Supplier
XhoI	R0146S	New England Biolabs
NotI	R0189S	(Ipswich, Massachusetts,
SacI	R3156S	USA)
Kits		
GeneJET Plasmid Miniprep Kit	K0502	Thermo Fisher Scientific (Waltham, Massachusetts, USA)

Molecular weight standards		
1 kb DNA Ladder	DNA marker 0.5-10.0 kb	New England Biolabs (Ipswich, Massachusetts, USA)
PageRuler™ Prestained Protein Ladder	Protein Marker 10–180 kDa	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
Bacterial Strains		
Microswab aureus	Staphylococcus WDCM00032, WDCM00193 ATCC6538	Mecconti (Warsaw, Mazovia, Poland)
Kwik Stik Bacillus subtilis	Derived from ATCC19659	Kwik Stik (Saint Cloud, Minnesota, USA)
Others		
Ampicillin Sodium Salt	GA7355	Glenthams Life Sciences (Corsham, Wiltshire, UK)
DNA Gel Loading Dye	Purple (6X)	New England Biolabs (Ipswich, Massachusetts, USA)
Zymolyse™	E1006	Zymo Research (Irvine, California, USA)
Mast Discs® CIPROFLOXACIN 5µg	AST CIPROFLOXACIN 5µg Cartridge discs	Mast Group (Liverpool, Lancashire, UK)
Pichia pastoris GS115 Stab		Thermo Fisher Scientific (Waltham, Massachusetts, USA)

2.1.4 Equipment

Table 2: Equipment used in the study.

Equipment	Model	Supplier
AccuBlock™ Digital Dry Bath	D1100	Labnet International (Edison, New Jersey, USA)
Analytical Weighing Balance	PB3001	Mettler Toledo (Columbus, Ohio, USA)
Autoclave Hirayama	HV-50	Hirayama Holdings (Kounan, Minato ward, Tokyo, Japan)
Heraeus Centrifuge	Pico 17	Thermo Scientific (Waltham, Massachusetts, USA)
Beckman Centrifuge	GS-15R	Beckman (Brea, California, USA)
E-Box Gel Documentation Imaging System	Doc-Print-CX3	Vilber (Lemont, Illinois, USA)
Electrophoresis Chamber and Casting trays		Cleaver Scientific (Rugby, Warwickshire, UK)
SDS-Page Electrophoresis Chamber and Casting trays		Bio-Rad (Hercules, California, USA)
Electrophoresis Power Pac Basic (SDS-Page gel)	Power Pac™ Basic	
Electrophoresis Power Pro (Agarose gel)	Power Pro-300	Cleaver Scientific (Rugby, Warwickshire, UK)

Forma Reach-in CO2 Incubator	311	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
Gyro-rocker	SSL3	Stuart Scientific (Stone, UK)
Laminar Flow hood bench	4006H	Vivid Air (Durban, KwaZulu Natal, SA)
Orbi Shake platform shaker	262	Labotec (Midrand, Gauteng, SA)
pH Meter	Orion 2 Star	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
Thermal Cycler	T100	Bio-Rad (Hercules, California, USA)
UV Platform	ECX-F20.M- V1 Table 312nm	Vilber (Lemont, Illinois, USA)
Vortex Mixer	Wizard Advanced IR Sensor	Velp Scientifica (Usmate Vellate MB, Italy, NY)
Qubit Fluorometer		Thermo Fisher Scientific (Waltham, Massachusetts, USA)

2.1.5 Plasmid Vector

pPIC9

is a yeast expression vector that contains a signal sequence of α -mating factor (MF α 1) for the secretory expression of a target protein. Expression is under the control of the AOX1 promoter induced by the alcohol oxidase 1 and the single restriction endonucleases site available for construction is Xho I, SnaB I, EcoR I, Avr II, Not I (Invitrogen, CA USA).

Table 3: Recombinant plasmid used

Plasmid	Size (bp)	Selectable marker	Inducer	Supplier
pPIC9	8029 bp	AmpR	Methanol	Gene Universal

2.1.6 Buffers

Unless otherwise stated, buffers used for this study are listed below.

Table 4: Buffers used

Buffers/Solutions	Constituents
TAE buffer	40 mM Tris-HCl pH 7.5, 20 mM sodium acetate, 1 mM EDTA adjusted pH to 8.2 with acetic acid
SDS-PAGE lower buffer (4 x)	1.5 M Tris-HCl pH 8.8
SDS-PAGE upper buffer (4 x)	1 M Tris-HCl pH 6.8
SDS-PAGE running buffer	25 mM Tris-HCl pH 7.9, 192 mM glycine, 0.1% (w/v) SDS
SDS-PAGE sample buffer (8 x)	16 ml 10% SDS, 4 ml glycerol, 2.2 ml Tris-HCl pH 6.8, 800 μ l β -mercaptoethanol, 1 spatula tip bromophenol blue
SDS-PAGE Resolving gel (15%) (Separating gel)	7.5 ml acrylamide 40% (w/v), 5 ml 1.5 M Tris-HCl pH 8.8, 7.1 ml ddH ₂ O, 200 μ l 10% SDS solution (w/v), 200 μ l 10% APS, 20 μ l TEMED
SDS-PAGE Stacking gel (15%)	1.25 ml acrylamide 40% (w/v), 5 ml 1.0 M Tris-HCl pH 6.8, 6.3 ml ddH ₂ O, 100 μ l 10% SDS solution (w/v), 100 μ l 10% APS, 10 μ l TEMED
SDS-PAGE Staining solution	0.25% (w/v) Coomassie Brilliant Blue R-250, 30 % (v/v) ethanol, 10% (v/v) acetic acid, fill up to with ddH ₂ O
SDS-PAGE destaining solution	40% (v/v) ethanol, 10% (v/v) acetic acid, fill up to with ddH ₂ O
Buffer A	1.0 M Sorbitol, 10mM Bicine, pH 8.35, 3% (v/v) ethylene glycol
Buffer B	40 % (w/v) Polyethylene glycol 1000, 0.2 M Bicine, pH 8.35
Buffer C	0.15 M NaCl, 10 mM Bicine, pH 8.35
Ampicillin	100 μ g/ml Ampicillin

2.1.7 Culture Media

The following culture media were used in this study and were purchased from a reputable supplier.

Table 5: Culture media used

Culture media	Constituents
Yeast extract peptone dextrose medium (YPD)	1% yeast extract, 2% peptone, 2% dextrose (glucose)
Buffered minimal methanol (BMMY)	100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acid, $4 \times 10^{-5}\%$ biotin, 0.5% methanol
Buffered minimal glycerol (BMGY)	100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base with ammonium sulfate without amino acid, $4 \times 10^{-5}\%$ biotin, 1% glycerol
Minimal dextrose medium (MD)	1.34% yeast nitrogen base without amino acid, $4 \times 10^{-5}\%$ biotin, 2% dextrose
Mueller Hinton agar (MH)	2.0 g beef extract, 17.5 g acid hydrolysate of casein, 1.5 g starch, 17.0 g bacteriological agar, 1 liter of distilled water
Luria Bertani agar (LB)	20.0 g bacteriological agar, 10.0 g sodium chloride, 10.0 g tryptone, 5.0 g yeast extract
Luria Bertani medium (LB)	10.0 g sodium chloride, 10.0 g tryptone, 5.0 g yeast extract

All media were autoclaved for 20 minutes at 121°C before use.

2.2 Methods

2.2.1 Construction of Recombinant Plasmid

The bioinformatics department at Capebio Technologies Pty Ltd provided several peptide hints with potential antimicrobial properties and two peptides were selected for this project as the gene of interest from the AMP open reading frames predicted through deep learning. The construction of Gene 1 and Gene 2 into the pPIC9 vector to produce recombinant peptides was generated by inserting Gene 1 and Gene 2 DNA sequences between XhoI and NotI restriction sites respectively. The putative antimicrobial peptides gene was synthesized and cloned into the *Pichia pastoris* expression vector pPIC9 (Table 3) by Gene Universal Inc (Newark DE, USA, <https://www.geneuniversal.com/>).

2.2.2 Preparation of Competent *E. coli* DH5 α Cells

The *E. coli* DH5 α cells glycerol stock was obtained from Capebio Technologies laboratory. All chemicals were prepared before and stored at 4°C until use. Frozen glycerol stock of bacterial strains (DH5 α) was streaked onto the LB agar plate in the absence of antibiotic and allowed to grow overnight at 37°C. A single colony was picked and inoculated into 5 LB media and allowed to grow overnight in the absence of antibiotics at 37°C with shaking. The overnight 200 μ l cell culture was inoculated into the 50 ml of LB media and was allowed to grow until the optical density OD₆₀₀ was in the range of 0.35-0.4 at 37°C with shaking. The culture was aliquoted into five 15-ml pre-chilled conical tubes and the tubes were incubated on ice for 10 minutes. The cells were harvested by centrifugation at 1,600 x g for 7 minutes at 4°C. Supernatants were discarded and pellets were gently re-suspended in 10 ml of ice-cold MgCl₂ solution (100 mM MgCl₂). The cells were harvested by centrifugation at 1,100 x g for 5 minutes at 4°C. Supernatants were discarded and pellets were re-suspended in about 10 ml of ice-cold CaCl₂ solution (100 mM CaCl₂). The suspension was incubated on ice for 30 minutes and after incubation, the cells were harvested by centrifugation at 1.100 x g for 5 minutes at 4°C. Supernatants were discarded and pellets were resuspended in 10 ml of ice-cold CaCl₂ solution (85 mM CaCl₂, 15% glycerol). The suspension was transferred into

the sterile 15 ml ice-cold conical tube. The cells were harvested by centrifugation at 1,100 x g for 5 minutes at 4°C. Supernatants were discarded and pellets were resuspended in 5 ml ice-cold CaCl₂ solution (85 mM CaCl₂, 15% glycerol). The 50 µl of cells were aliquoted into ice-cold sterile 1.5 ml microfuge tubes and immediately stored at the -80°C freezer.

2.2.3 Bacterial Transformations with Plasmid DNA

Transformations were carried out using the competent bacterial strains described in Section 2.2.2. Generally, 50 µl of the competent *E. coli* DH5α cells were transformed with 25-50 ng of DNA. Firstly, the cells were allowed to thaw on ice and then allowed to incubate with Gene 1, Gene 2 and pPIC9 construct for 30 minutes. After that the cells were heat shocked at 42°C for 90 seconds, followed by incubation on ice for 2 minutes. Pre-warmed LB media was added and transformed cells were incubated with shaking at 37°C for 60 minutes and plated onto LB agar plates with the appropriate antibiotic. After drying, the plates were incubated at 37°C overnight.

2.2.4 Isolation of Plasmid DNA

A single freshly transformed *E. coli* DH5α colony containing construct plasmid pPIC9-Gene 1, pPIC9-Gene 2, and pPIC9 was picked (Section 2.2.3) and transferred to 5ml LB media with the 100 mg/ml of ampicillin antibiotic. The culture was allowed to grow at 37°C overnight with shaking. A GeneJet plasmid miniprep kit was used to isolate and purify construct plasmid DNA according to the manufacturer's instructions. The construct plasmid of pPIC9-Gene 1 and pPIC9-Gene 2 were confirmed by sequencing at Inqaba Biotech, South Africa.

2.2.5 Preparation of Glycerol Stock

Glycerol is added to bacterial cell culture containing a plasmid of interest to prevent the cells from lysing during the freezing process allowing their long-term storage (Kurachi et al., 2014). The glycerol stocks of pPIC9-Gene 1, pPIC9-Gene 2, and pPIC9 clones were separately prepared by inoculating a single colony of bacteria containing the recombinant plasmid into a 5 ml LB medium containing 100 mg/ml

of ampicillin antibiotic. The culture was incubated at 37°C overnight with shaking. In a 1.5 ml labelled cryovial storage tube, 850 µl of overnight bacterial culture and 150 µl of the sterile glycerol were mixed by vortexing and stored at -80°C freezer for longer storage.

2.2.6 Construct Plasmid DNA Linearization using Restriction Digestion for Efficacious Integration into the *P. pastoris* Genome.

The integration of the recombinant vector into *P. pastoris* is facilitated by vector linearization employing restriction enzymes such as SacI (Vogl et al., 2018). Restriction enzymes recognize specific nucleotide sequences in double-stranded DNA and specifically cleave these. Most enzymes recognize palindromic sequences and produce either blunt or sticky ends (Roberts, 2003). Ten µl of 105 ng/µl pPIC9-Gene 1 or (110 ng/µl pPIC9-Gene 2 and 119 ng/µl pPIC9) construct plasmid DNA were transferred to a PCR tube on ice. Similarly, 5 µl restriction buffer (10 x) and 1 µl of SacI was added. The final volume was made up to 50 µl with ddH₂O. The tubes were incubated at 37°C for 1 hour. The small portion of digested construct plasmids DNA was analyzed by agarose gel electrophoresis to confirm the complete digestion of the fragments. The digested construct plasmids DNA were extracted with 3M Sodium acetate, pH 5.2:100% ethanol, and 70% ethanol and ethanol precipitated the digested DNA. The Air-dry DNA pellet was resuspended in 20 µl of nuclease-free water and stored at -20 until ready to transform.

2.3 Transformation into *Pichia Pastoris* by PEG 1000 Method

2.3.1 Preparation of Competent Cells *Pichia pastoris* GS511

The *Pichia pastoris* GS511 strain was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All chemicals were prepared before and stored at 4°C until use (Table 4). *Pichia pastoris* strains (GS115) were streaked onto the YPD agar plate in the absence of antibiotics and incubated at 30°C for two days. A single colony was picked from the plate inoculated into YPD media and allowed to grow overnight at 30°C with shaking. The overnight 10 ml cell culture was

inoculated into the 100 ml of YPD culture media to a starting optical density OD₆₀₀ of 0.1 and was allowed to grow at 30°C until the optical density OD₆₀₀ was in the range of 0.5-0.8. Cells were harvested by centrifugation at 3000 x g for 5 minutes at room temperature. Supernatants were discarded and pellets were washed once in 50 ml of Buffer A (Table 4). The cells were resuspended in 4 ml of Buffer A and distributed in 0.2 ml aliquots to sterile 1.5 microcentrifuge tubes. 11 µl of dimethyl sulfoxide (DMSO) was added to each tube and mixed. Once mixed tubes were immediately stored in the -80°C freezer.

2.3.2 Transformation of pPIC9-Gene 1, pPIC9-Gene 2 and pPIC9 in *P. pastoris*

The 20µl of purified linearized construct plasmid DNA from section 2.2.6 was added directly into a still-frozen tube of competent cells labeled as pPIC9-Gene 1, pPIC9-Gene 2, and pPIC9. The sample tubes were incubated at 37°C on a heating block for five minutes and the samples were mixed twice by vortexing during the incubation period. After incubation on a heating block 1.5 ml of Buffer B (Table 4) was added to each sample tube. The contents were mixed thoroughly, and the sample tubes were incubated at a 30°C heating block for 1 hour. After incubation, the sample tubes were centrifuged at 2,000 x g for 10 minutes at room temperature. Supernatants were discarded and pellets were resuspended in 1.5 ml of Buffer C (Table 4). The samples were centrifuged a second time, and the cell pellets were resuspended gently in 0.2 ml of Buffer C. The entire contents of each tube were spread on a minimal dextrose medium (MD), and the plates were incubated at 30°C for 2-3 days (Pichia Expression Kit, 2014).

2.3.3 PCR Screening of *P. pastoris* Clones to Confirm Successful Insertion of the pPIC9-Gene 1, pPIC9-Gene 2, and pPIC9 genes.

Polymerase chain reaction (PCR) is based on using the ability of DNA polymerase to synthesize new strands of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. To test the integration of pPIC9-Gene 1, pPIC9-Gene 2, and pPIC9 into *P. pastoris* genome,

the DNA isolation from transformants was carried out as described in the Pichia expression manual version I (Invitrogen, 1996). The primers, AXO1 forward and AXO1 reverse were used for amplification of the fragments. Generally, the PCR reaction consists of 50 ng of template DNA, 50 ng of each primer, 1 U of Taq DNA polymerase, and 200 μ M of dNTPs in a total volume of 25 μ l. Below is a table that shows all primer sequences used for the constructs for this project.

The PCR reaction was allowed to proceed under the following general parameters:

Initial denaturation 95°C for 5 minutes

Denaturation 95°C for 1 minute

Annealing 54°C for 1 minute

Extension 72°C for 1 minute

Final extension 72°C for 7 minutes

30 cycles

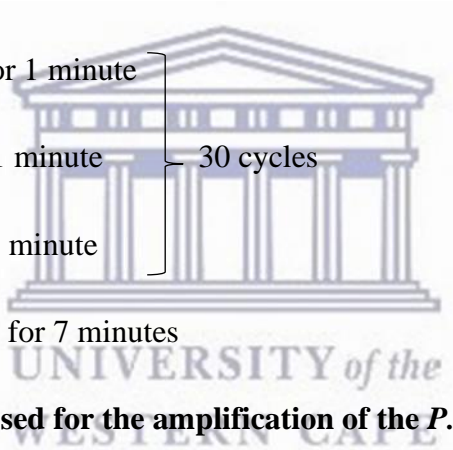


Table 6: Primers used for the amplification of the *P. pastoris* genomic DNA

Primer name	Primer sequence
5`AOX1	GACTGGTTCCAATTGACAAGC
3`AOX1	GCAAATGGCATTCTGACATCC

2.3.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a laboratory technique used to separate large molecules, such as DNA, based on their size by using electricity to carry them through a gel. The sizes of the amplified *P. pastoris* genomic DNA fragments were assessed by electrophoresis using 1% agarose gel in 1x TAE buffer. Ethidium

bromide for visualization at 300 nm was added to the gel to a final concentration of 0.5 µg/ml. DNA molecular weight marker (New England Biolabs) was loaded alongside samples. Electrophoresis was carried out at 100 V for 90 minutes unless otherwise stated. Gel was visualized and imaged using the transilluminator connected to an E-box gel documentation imaging system UV camera.

2.4 Expression of the Recombinant Peptide

A single colony of positive transformant pPIC9-Gene 1 or pPIC9-Gene 2 was inoculated into a 25 ml buffered glycerol-complex medium (BMGY) in a 250 ml baffled flask. The culture was incubated at 30°C in a shaking incubator (250 rpm) and allowed to grow until the optical density OD₆₀₀ was in the range 2-6. The cells were harvested by centrifugation at 3,000 x g for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 100 ml buffered methanol-complex medium (BMMY) to an OD₆₀₀ of 1.0 to induce expression. Then the baffled flasks were incubated at 30°C in a shaking incubator (250 rpm). To maintain induction, 100% methanol (HPLC grade) was added to a final concentration of 1% methanol every 24 hours for 4 days. At 48 hrs, 72 hrs, and 96 hrs 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube. These samples were used to analyze expression levels and to determine the optimal time post-induction to harvest. The samples were centrifuged at maximum speed in a tabletop microcentrifuge at room temperature for 3 minutes. The supernatant was stored at -80°C until ready to assay. The supernatants were analyzed for protein expression by Coomassie-stained SDS-PAGE polyacrylamide gel electrophoresis.

2.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample fractions containing pPIC9-Gene 1 and pPIC9-Gene 2 proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins based on their molecular weight. SDS is a powerful detergent that, when combined with certain reducing chemicals, breaks the disulfide bonds in proteins, disrupting the tertiary structure of proteins. In an electric field, a charge molecule migrates to the electrode with the opposite sign

(Gallagher, 2001). Fifteen μl protein samples were mixed with 5 μl of 8 x sample buffer and heated at 95°C for 5 minutes to denature the protein. The samples were loaded onto the gel and run at 100 V for 60 minutes. The gel was stained overnight in a Coomassie brilliant blue R-250 solution. The gel was destained to remove excess stain.

2.6 Antimicrobial Activity of Recombinant Peptides

To evaluate the antimicrobial activity of the recombinant peptides supernatant zone of inhibition was investigated on two gram-positive bacteria species. The 100 μl of recombinant peptide Gene 1 and peptide Gene 2 supernatant were evaluated on *Staphylococcus aureus* ATCC6538, *Bacillus subtilis* ATCC19659 after 48 hours using the agar well diffusion method. These two bacteria strains were selected as the form resistance to antibiotics. The agar well diffusion procedure is commonly used to evaluate the antimicrobial activity of plants or microbial extracts (Magaldi et al., 2004, Valgas et al., 2007). Similarly to the method used in the disk-diffusion procedure, the Muller-Hinton agar plate surfaces were inoculated by spreading a volume of the microbial inoculum over the entire agar surface. The wells were created in Muller-Hinton agar (0.2 cm diameter) by the bottom of a 1 ml blue pipette tip. 100 μl of recombinant peptide supernatant of the BMMY medium was loaded into the wells (Andrews, 2001). The Chloramphenicol 30 μg (C30) and Ciprofloxacin 5 μg (CIP5) were used as positive controls and nuclease free water was used as a negative control. The agar plates were incubated at 37°C for 48 hours and the plates were evaluated for the zone inhibition.

Chapter 3: Results

3.1 APD3 Antimicrobial Peptides Database for Peptide Gene 1 and Peptide Gene 2

The sequences were provided by the bioinformatics department and the sequences were screened using APD3 antimicrobial peptides database to check if it has any chance of being an antimicrobial peptide. The APD3 platform uses peptide properties such as hydrophobicity to classify antimicrobial peptides. Overall, this online platform indicated that indeed these peptides have potential antimicrobial properties.

```
MQSLKKFSKKIWKAESGATAIEYALIAALIAVVIIAAVTLVG
IDLGNMFNQLFNNL

MQSLKKFSKKIWKAESGATAIEYALIAALIAVVIIAAVTLVG
IDLGNMFNQLFNNL

MQSLKKFSKKIWKAESGATAIEYALIAALIAVVIIAAVTLVG
IDLGNMFNQLFNNL
```

Figure 7. Graphic representation of amino acids composition of Peptide Gene 1 using APD3 platform.

Using the APD3 platform, the prediction of the antimicrobial peptide was done using the percentage hydrophobicity of the peptide. The hydrophobic residues are in red, and the hydrophobic residues on the same surface are underlined. The total hydrophobic residues on the same surface are 28, and it has been indicated that this peptide may form alpha helices. With a hydrophobic ratio of 59%, it is indicated that this peptide has potential antimicrobial properties. The APD3 platform also indicated that this peptide has a chance to be an antimicrobial peptide and may interact with membranes Figure 7.

```

M E T S Y H F Q N I D S S D A L K E Y A D K V V D K L S S H F S N L Q N A T V H F K
V E K I H Q I A E I T I N G D S G Q F V A E E K A E D M Y A A L D L V E K K L E K Q
I R K H K E K H L G K N Q R G

M E T S Y H F Q N I D S S D A L K E Y A D K V V D K L S S H F S N L Q N A T V H F K
V E K I H Q I A E I T I N G D S G Q F V A E E K A E D M Y A A L D L V E K K L E K Q
I R K H K E K H L G K N Q R G

M E T S Y H F Q N I D S S D A L K E Y A D K V V D K L S S H F S N L Q N A T V H F K
V E K I H Q I A E I T I N G D S G Q F V A E E K A E D M Y A A L D L V E K K L E K Q
I R K H K E K H L G K N Q R G

```

Figure 8. Graphic representation of amino acids composition of Peptide Gene 2 using APD3 platform.

Using the APD3 platform, the percentage hydrophobicity of the peptides was used to predict their antimicrobial activity. Hydrophobic residues are highlighted in red, while hydrophobic residues on the same surface are underlined. The total number of hydrophobic residues on the same surface is 24, and it has been suggested that this peptide may form alpha helices. This peptide's hydrophobic ratio of 33% suggests that it may have antimicrobial effects. The APD3 platform also revealed that this peptide has the potential to be an antimicrobial peptide and interact with membranes Figure 8.

3.2 Potential Homology of Peptide Gene 1 and Peptide Gene 2 using the BLAST Program

The Basic Local Alignment Search Tool (BLAST) finds regions of similarity between sequences. The program compares nucleotide, or protein sequences and calculates the statistical significance of matches. Blast can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. Using the BLAST program (Altschul et al., 1997), these fragments of peptide Gene 1 and peptide Gene 2 revealed several homologies, and out of all the homologies only the first top was selected for each peptide based on the per identity in this study.

Table 7: Homology of peptide Gene 1 and peptide Gene 2 from the BLAST program

Peptide Gene 1					
Homology	Organism	Potential function	Per. identity	Accession	
Flp family type IVb pillin	<i>Rhodospirillales bacterium</i>	Bacteria-bacteria interactions, biofilm formation, twitching motility, bacteriophage binding, DNA uptake, and cell adherence (Craig et al., 2004).	66.04%	NQU60202.1	
Peptide Gene 2					
Ribosomal subunit interface protein	<i>Leptospiraceae bacterium</i>	Play critical roles in interacting with tRNA substrates and the large subunit (Southworth et al., 2002)	100.00%	MBI41542.1	

3.3 Cartoon representation of Peptide Gene 1 and Peptide Gene 2

The cartoon representation visualizes the secondary structure of a protein. There are special graphical representations for the different kinds of secondary structure elements. As can be seen in Figure 9 and Figure 10 the random coil and turns are visualized as tubes, the α -helices are depicted using broad ribbons that coil following the turns of helices, and the β -sheets are drawn as flat, band-shaped arrows that point in the direction of the carboxyl group.



Figure 9. Cartoon representation of Peptide Gene 1. The structure was generated using the AlphaFold modeling tool. Structure Peptide Gene 1 with α -helices (<https://alphafold.ebi.ac.uk>).

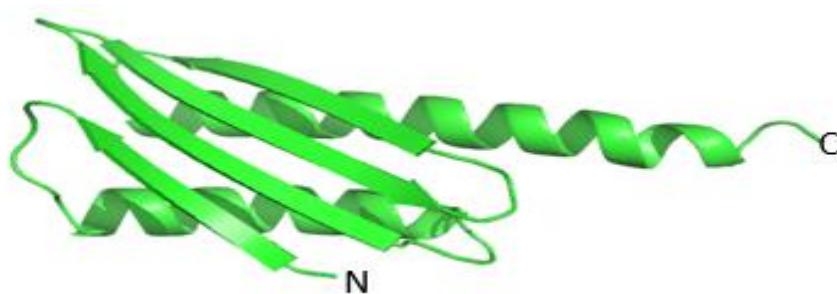
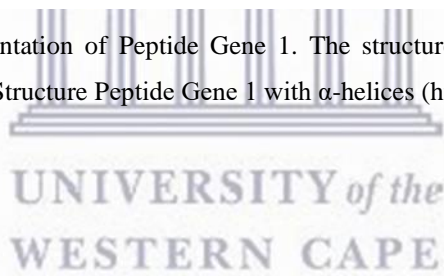


Figure 10. Cartoon representation of Peptide Gene 2. The structure was generated using the AlphaFold modeling tool. Structure peptide Gene 2 with α/β sheet (<https://alphafold.ebi.ac.uk>).

3.4 Isolation and sequencing of peptide Gene 1 and peptide Gene 2 using the AOX1 forward and reverse primers.

These figures illustrate the successful transformation of the construct plasmid DNA of pPIC9-Gene 1 and pPIC9- Gene 2 into the *E. coli* DH5 α cells and confirms that the plasmids are correctly assembled for subsequent expression studies figures below:

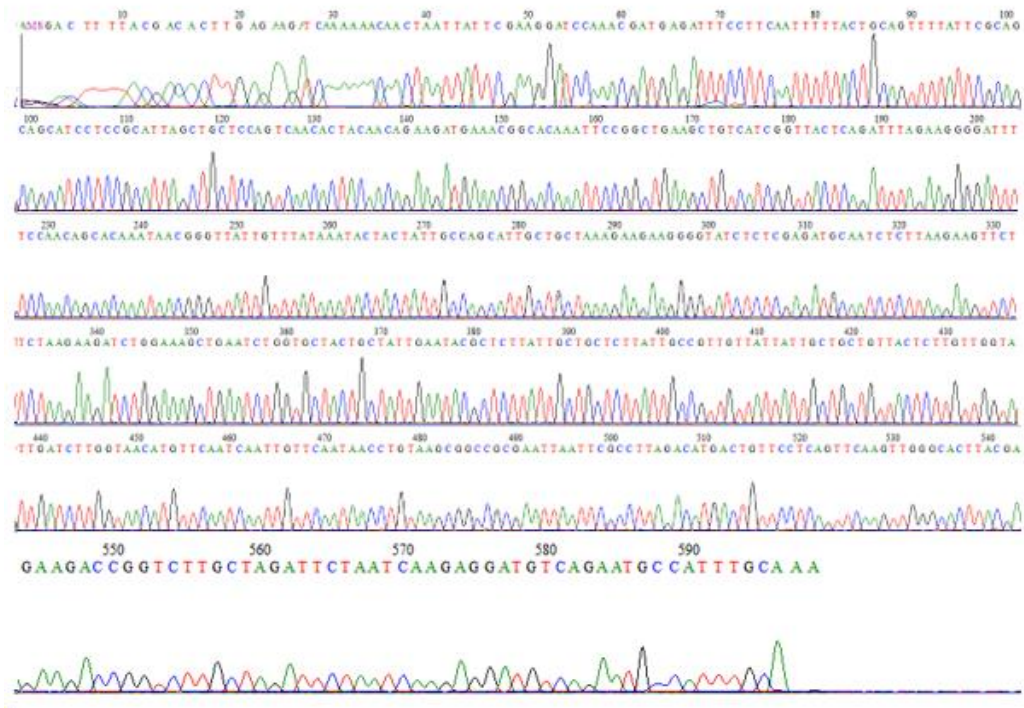


Figure 11. Chromatogram of sequencing results of pPIC9-Gene 1 with AOX1 forward primer.

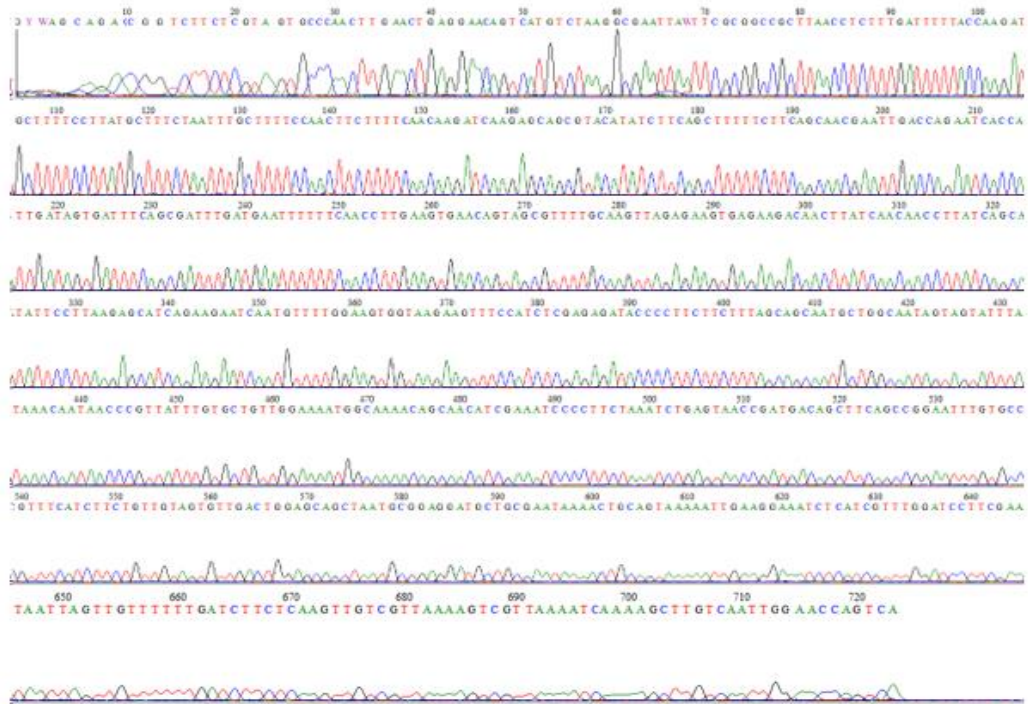
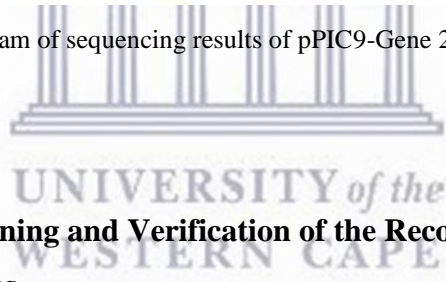


Figure 14. Chromatogram of sequencing results of pPIC9-Gene 2 with AOX1 reverse primer.



3.5 Screening and Verification of the Recombinant Producing Clones

3.5.1 Recombinant plasmid DNA linearization for efficacious integration into the *P. pastoris* genome

The recombinant plasmids of pPIC9, pPIC9-Gene 1, and pPIC9- Gene 2 were used to transform *P. pastoris* through the integration of expression tapes into chromosomes at specific loci to produce genetically stable transformants. Integration of recombinant plasmids into the *P. pastoris* genome was obtained by linearising the plasmid at the specific site of the AOX1 gene (Li et al., 2007a). In this study, the recombinant plasmids were linearized using the SacI restriction enzyme (Figure 15).

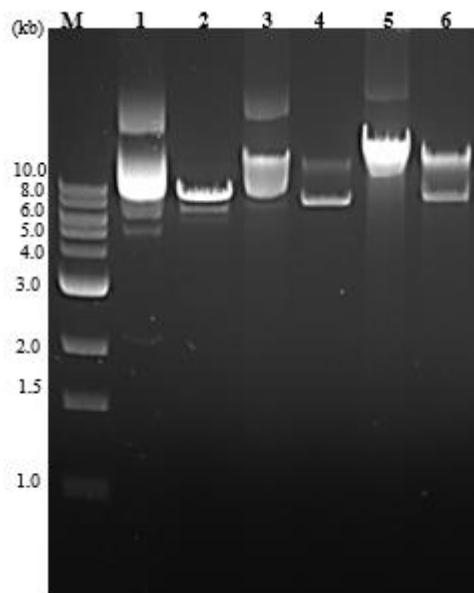
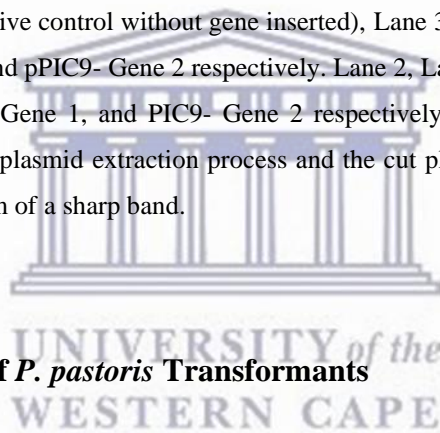


Figure 15. Linearization of the recombinant plasmids by SacI restriction enzyme. M: DNA ladder 1 kb, marker; Lane 1(negative control without gene inserted), Lane 3 and Lane 5, uncut plasmid of pPIC9, pPIC9-Gene 1 and pPIC9- Gene 2 respectively. Lane 2, Lane 4, and Lane 6 linearized plasmid of pPIC9, pPIC9-Gene 1, and PIC9- Gene 2 respectively. The uncut plasmid has a different isoform after the plasmid extraction process and the cut plasmid has a linear plasmid normally visible in the form of a sharp band.



3.5.2 PCR analysis of *P. pastoris* Transformants

The recombinant plasmid of pPIC9-Gene 1 and pPIC9-Gene 2 were linearized with SacI and transformed into the genome of *P. pastoris* GS115 cells. Transformants were selected on MD plates and incubated at 30°C for 2-3 days. PCR amplification of genomic DNA confirmed the pPIC9-Gene 1 and pPIC9-Gene 2 gene integrated into the transformants and a transformant with the pPIC9 vector was used as a negative control Figure 16.

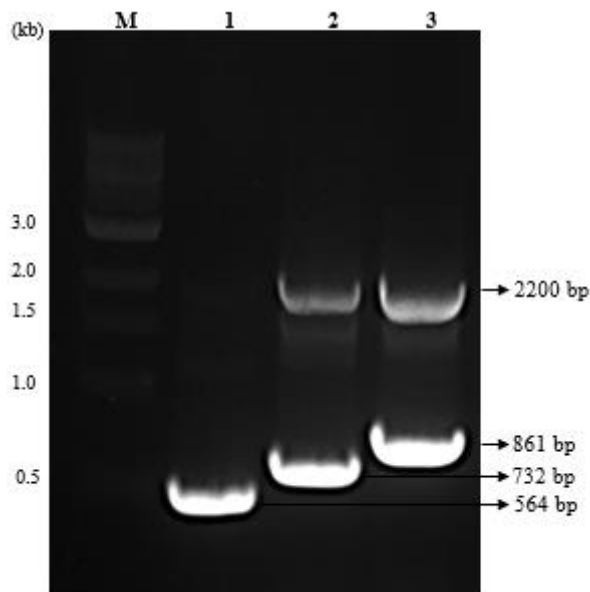


Figure 16. 1 % (w/v) agarose gel electrophoresis of the PCR analysis of clones GS115/pPIC9, GS115/pPIC9-Gene 1 and GS115/pPIC9- Gene 2. M: DNA ladder 1 kb, marker. Lane 1: Negative control of GS115/pPIC9 PCR product, Lane 2: GS115/pPIC9-Gene 1, Lane 3: GS115/pPIC9-Gene 2 PCR product.

The *Pichia pastoris* transformant carrying recombinant pPIC9-Gene 1 and pPIC9-Gene 2 plasmids with the phenotypic of Mut⁺ showed by 2 bands at 732 bp (168 bp is pPIC9-Gene 1 gene size that encoded 56 amino acids; 564 is signal α -factor gene size), 861 bp (297 bp is pPIC9-Gene 2 gene size that encoded 99 amino acid; 564 is signal α -factor gene size), 2200 bp (1636 bp is AOX1 gene size; 564 is signal α -factor gene size). The *P. pastoris* transformant carrying recombinant pPIC9 vector plasmid with the phenotypic of Mut⁻ showed by 1 band at 564 bp (564 is signal α -factor gene size) on electrophoregram's following PCR colonies with AOX1 primers. The appearance of these 2 bands (Figure 16) indicated that the AOX1 gene was not deletion (Li et al., 2007a).

3.6 Expression of Peptide Gene 1 and Peptide Gene 2 Protein

The expression of recombinant plasmid in *P. pastoris* was carried out using Mut⁺ transformant with 1% induction of methanol for 4 days at 30°C and 250 rpm. Following SDS analysis of the supernatant showed that the selected colony

produced the isolated peptide, not excluding the possibility of also containing peptides in the truncated form. At times 48 hr, 72 hr, and 96 hr, it is possible to visualize the 6.05 kDa for peptide Gene 1 (Figure 17) and 11.37 kDa for peptide Gene 2 (Figure 18). Induction with methanol was performed since the AOX1 promoter is regulated by the presence of methanol in the medium. The presence of methanol expressing Prm1 and Mit1 activators in AOX1 (De Schutter et al., 2009).

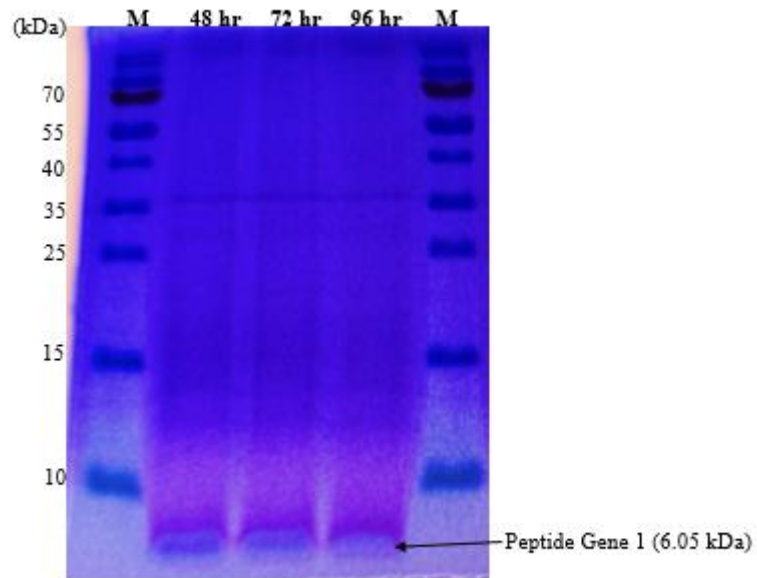


Figure 17. SDS-PAGE analysis of the effect of induction time on the expression of Peptide Gene 1 induced with 1% methanol. Samples were taken from cultures of GS115/pPIC9-Gene 1 at 48,72 and 96 hr of induction respectively. The supernatant was separated by 15% (w/v) SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular weight marker (Thermo Fisher Scientific)

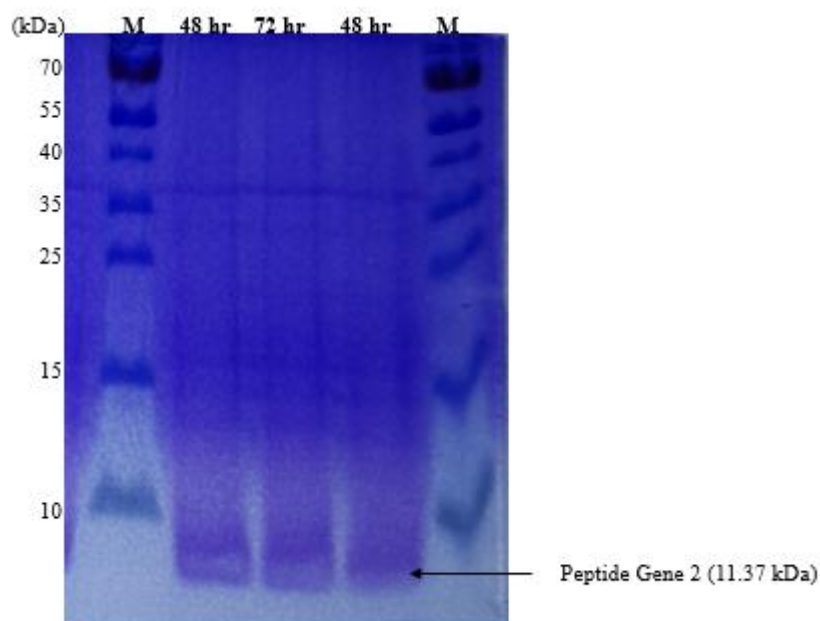


Figure 18. SDS-PAGE analysis of the effect of induction time on the expression of Peptide Gene 1 induced with 1% methanol. Samples were taken from cultures of GS115/pPIC9-Gene 2 at 48,72 and 96 hr of induction respectively. The supernatant was separated by 15% (w/v) SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular weight marker (Thermo Fisher Scientific)

3.7 Antimicrobial activity assays

The antimicrobial activity of the recombinant peptide Gene 1 and peptide Gene 2 were determined against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* using a zone of inhibition test. The supernatant of recombinant peptide Gene 1 and peptide Gene 2 expressed in *P. pastoris* taken at different times 48 hr, 72 hr, and 96 hr showed no activity against the two Gram-positive bacteria. Antibiotics Chloramphenicol (C30) and Ciprofloxacin (CIP5) were used as positive control and showed potent activity against the two Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*.

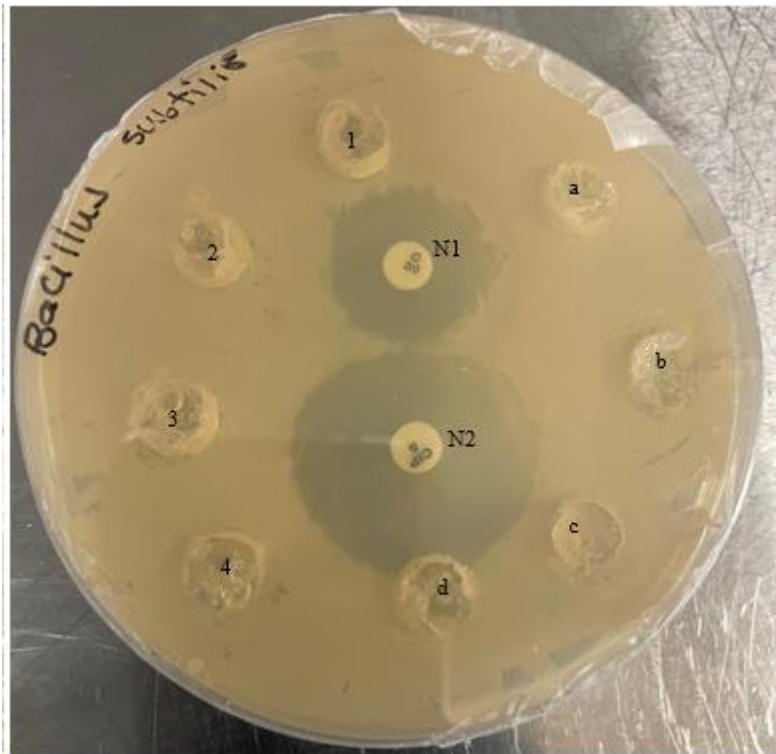


Figure 19. Well diffusion test to investigate the effect of supernatant recombinant peptide Gene 1 and peptide Gene 2 taken at different times 48 hr, 72 hr, and 96 hr against *Bacillus Subtilis*. The expression supernatant containing Peptide Gene 1 taken at 48 hr, 72 hr, and 96 hr were loaded into wells 1-3 respectively. The expression supernatant containing Peptide Gene 2 taken at 48 hr, 72 hr, and 96 hr were loaded into wells a-c respectively. Wells 4 and d were loaded with water as a negative control and wells N1 and N2 were loaded with antibiotics Chloramphenicol (C30) and Ciprofloxacin (CIP5) respectively as a positive control.



Figure 20. Well diffusion test to investigate the effect of supernatant recombinant peptide Gene 1 and peptide Gene 2 taken at different times 48 hr, 72 hr, and 96 hr against *Staphylococcus aureus*. The expression supernatant containing Peptide Gene 1 taken at 48 hr, 72 hr, and 96 hr were loaded into wells 1-3 respectively. The expression supernatant containing Peptide Gene 2 taken at 48 hr, 72 hr, and 96 hr were loaded into wells a-c respectively. Wells 4 and d were loaded with water as a negative control and wells N1 and N2 were loaded with antibiotics Chloramphenicol (C30) and Ciprofloxacin (CIP5) respectively as a positive control.

Chapter 4: Discussion, Conclusion, and Future Prospects

4.1 Discussions

The discovery of new medications has long been an attractive topic. Antimicrobial peptides are a fascinating topic in this field that has lately received attention due to the rise of antibiotic resistance (Neshani et al., 2019b, Wang et al., 2016, Pan et al., 2017). These compounds are naturally produced by living organisms and play a function in innate immunity. Antimicrobial peptides have multiple functions including wound healing, immunological modulation, and anti-cancer properties (Neshani et al., 2019c). The purpose of this study was to produce peptide Gene 1 and peptide Gene 2 antimicrobial peptides using heterologous expression. Peptide Gene 1 and peptide Gene 2 were produced as recombinant peptides.

This study intended to develop a low-cost peptide Gene 1 and peptide Gene 2 production process for research and therapeutic applications. The study used the *Pichia pastoris* expression system to produce peptide Gene 1 and peptide Gene 2. The lack of LPS allows the production of a non-toxic product. Peptide Gene 1 and peptide Gene 2 were transferred into *P. pastoris* genome using the pPIC9 vector. The successful integration of pPIC9, pPIC9-Gene 1, and pPIC9- Gene 2 plasmids into the *P. pastoris* genome was confirmed by PCR as indicated in Figure 16. The PCR product with 2 bands showed the successful integration of selected colonies which contained the Mut⁺ phenotypic when amplified with the AOX1 primers.

The PCR product of pPIC9-Gene 1 showed a band at 732 bp (168 bp is pPIC9-Gene 1 gene size that encoded 56 amino acids; 564 is signal α -factor gene size), pPIC9-Gene 2 showed a band at 861 bp (297 bp is pPIC9-Gene 2 gene size that encoded 99 amino acids; 564 is signal α -factor gene size), AOX1 gene showed a band at 2200 bp (1636 bp is AOX1 gene size; 564 is signal α -factor gene size) which was observed in the Mut⁺ colonies as a second band. The PCR product of the empty pPIC9 vector plasmid with Mut⁻ phenotypic showed 1 band at 564 bp (564 is signal α -factor gene size) when amplified with the AOX1 primers. The appearance of

these 2 bands on pPIC9-Gene 1 and pPIC9-Gene 2 colonies (Figure 16) indicated that the AOX1 gene was not deletion (Li et al., 2007b).

This shuttle vector differs from others since it contains both the HIS4 gene and the alpha factor. Alpha-factor signals the secretion and directs the recombinant peptides to be processed and released effectively into the media. Crucial for histidine auxotroph selection, which ensures that only transformants with the correct plasmid can grow in the absence of histidine. *Pichia pastoris* lacks secreted proteins (Cregg et al., 2009), hence using a protein-free culture medium correlates with the amount of recombinant peptide in the supernatant. Another sequence, His4, causes the production of an enzyme that serves as the primary growth factor in histidine biosynthesis. HIS4 allows *P. pastoris* to grow in peptide-free culture media (Neshani et al., 2019a, Lopes et al., 2014).

Using the pPIC9 vector, a buffered complex methanol medium (BMMY) was applied to produce and acquire the secreted form of peptide Gene1 and peptide Gene 2. The pPIC3.5 and pHIL-D2 vectors contain HIS4 but lack alpha-factor, hence the secreted form of protein is not obtained. pPICZa and pAO815 cannot grow in the minimum medium due to a lack of HIS4 (Cregg et al., 2009, Neshani et al., 2018). Following SDS-PAGE analysis of the supernatant of peptide Gene 1 and peptide Gene 2 showed a band at different times 48 hr, 72 hr, and 96 hr of 6.05 kDa and 11.37 kDa respectively after Coomassie staining Figure 17 and Figure 18. The SDS-PAGE results proved that the peptides were successfully expressed in the *P. pastoris* expression system. The antimicrobial activity of the expressed peptides supernatant tested against *S. aureus*, and *B. subtilis* showed no activity at all, whereas there was a zone of inhibition observed on the positive control of antibiotics disk. There is a possibility that these peptides are not antibacterial peptides as they were tested only on bacterial strains, instead, they might be antifungal peptides, antiviral peptides, antiparasitic peptides, or anticancer peptides.

According to the results obtained from the APD3 database, it indicated that peptide Gene 1 and peptide Gene 2 are antimicrobial peptides with a percentage hydrophobicity of 59% and 33 % respectively. APD3 database also stated that they may have an α - helices structure, which was supported by the cartoon structure of

this peptide obtained using the AlphaFold modeling tool. The peptide Gene 1 showed to have the α -helical structure Figure 9, and the peptide Gene 2 showed to have mixed α -helical and β -sheet peptides Figure 10. The gene construct of pPIC9-Gene 1 and pPIC9-Gene 2 synthesized by Gene Universal was confirmed by sequencing with the AOX1 forward and reverse primers at Inqaba Biotech as indicated in Figure 11 and Figure 12 for pPIC9-Gene 1, Figure 13, and Figure 14 for pPIC9-Gene 2.

4.2 Conclusion and Future Prospects

AMPs play a crucial role in innate immunity and protect disease-causing microorganisms. With the rise of multidrug-resistant bacteria, alternative antibiotics (AMPs) are becoming increasingly essential. In this study, we set out to investigate antimicrobial peptides predicted by bioinformatics resources and computational tools such as deep learning. It indicates that these techniques are critical for the analysis of AMP data and function across all species. Using deep learning has shown proof of the concept by expressing these genes in a microbial host. This study has served as a validation process to show that deep learning can predict antimicrobial peptides and is used as a starting point for further functional characterization. The peptide Gene 1 and peptide Gene 2 were successfully integrated into the chromosomal of *P. pastoris* GS115. The peptide Gene 1 and peptide Gene 2 were expressed with the size of 6.05 kDa and 11.37 kDa respectively. The method used to produce these peptides reduces the costs and can be used for large-scale production in therapeutic and local uses. More importantly, the findings provide strong evidence in support of the concept of developing human-use peptide-based medicines. Future work will focus on the expression optimization and characterization of these peptides to find out if they possess other antimicrobial characteristics.

Chapter 5: List of References

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