ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

EVALUATION OF *PICHIA PASTORIS* AS A MICROBIAL CELL FACTORY FOR PRODUCTION OF FUNGAL ANTIMICROBIAL PROTEINS

BUSEL ÖZCAN
M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Assoc. Prof. Günseli Bayram Akçapınar

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

01.06.2023
"Busel ÖZCAN"

PREFACE AND ACKNOWLEDGEMENT

This study is a master program graduate thesis of Acıbadem Mehmet Ali Aydınlar University, Institute of Health Sciences, Department of Medical Biotechnology. The aim of the study is to obtain effective, fast and cost efficient new antimicrobial agent candidates as an alternative to the increasing antibiotics resistance by using *Pichia pastoris* recombinant expression system.

I would like to thank my esteemed thesis advisor Assoc. Prof. Günseli Bayram Akçapınar who spent time with me throughout the study and patiently shared her knowledge with me. Also, I would like to thank the valuable members of my thesis jury for their contributions, suggestions and supports.

Another thanks to Acıbadem Mehmet Ali Aydınlar University Research Project Office to financially supported the study via ABAPKO-2022-01-19 project during thesis. Also, thanks to TÜSEB not directly related thesis but financially supported me per monthly during some part of master.

I am grateful to my dear laboratory friends Betül Gündoğdu, Hande Osmanağaoğlu, Zeynep Ilgın Tozan who were with me throughout my laboratory studies for encouraging me and also I would like to thank to other members of Protein Engineering and Bioproduction Laboratory.

Lastly, I would like to express my gratitude to my unique who have not withheld their love, care and supports from me throughout my life.

Busel ÖZCAN, May 2023

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
θ	Theta
AFP	*Aspergillus gigantes*
AOX	Alcohol oxidase
AMP	Antimicrobial peptides
BMGY	Buffered glycerol complex
BMMY	Buffered methanol complex
CHO	Chinese hamster ovary
COVID-19	Coronavirus 2019
Cys	Cysteine
CSαβ	Cysteine stabilized alpha beta
DewA	*Aspergillus nidulans*
DLPs	Defensin-like peptides
FDA	Food and Drug Administration
GAP	Glyceraldehyde 3-phosphate dehydrogenase
GRAVY	Grand average of hydropathicity
HepG2	Hepatocellular liver cancer cell line
LB	Luria Bertani
MDR	Multi-drug resistant
MHB	Mueller-Hinton Broth
MIC	Minimal inhibition concentration
Mpg1	*Magnaporthe grisea*
MRSA	Methycilline resistant *S. aureus*
PAF	*Penicillium chrysogenum*
PEBL	Protein engineering and bioproduction laboratory
PVDF	Polyvinylidene fluoride

ROS	Reactive oxygen species
SDS-PAGE	Sodium-dodecyl sulphate agarose gel electrophoresis
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
TBE	Tris- borate EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TFA	Trifluoroacetic acid
U.S.	United States
VRSA	Vancomycin resistant *S. aureus*
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose agar
WST-8	Water-soluble tetrazolium-8

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ÖZET

Fungal Antimikrobiyal Proteinlerin Üretimi İçin Bir Mikrobiyal Hücre Fabrikası Olarak *Pichia pastoris*'in Değerlendirilmesi

Antimikrobiyal direnç mikroorganizmaların neden olduğu enfeksiyonların etkili bir biçimde bertaraf edilememsinin en büyük nedenidir. Patojenik mikroorganizmalar antimicrobiyal ilaçlara karşı hızla direnç geliştirmekte ve bu durum global bir kriz haline gelmektedir. Günümüzde yaygın bir şekilde kullanılan bu ilaçların fikir, keşif gibi aşamalardan üretime kadar geçen süreçleri hem uzun hem de oldukça maliyetlidir. Bu sebeple, hem yüksek etkiye sahip hem de düşük maliyetli alternatiflere ihtiyaç duyulmaktadır. Fungal defensinler günümüz konvansiyonel tedavilerine alternatif olabilme potansiyeli olan aday peptitlerdir. Sistein bakımından zengin, katyonik ve fungusların savunma mekanizmaları tarafından salgılanan bu peptitler antimikrobiyal özelliğe sahiptir. Hidrofobinler de mantarlar tarafından salgılanan, hidrofobik aminoasitlerden oluşan, korunumlu sistein bölgelerine sahip küçük aday peptitlerdendir. Bu tez çalışması kapsamında, fizikokimyasal analiz sonuçlarına göre veritabanlarından seçilen ve daha önce karakterize edilmemiş üç defensin; F2T142, F2T142Mut, D6RKI7 ve bir hidrofobin, RodA, metilotrofik bir maya olan *Pichia pastoris* KM71H suşunda recombinant olarak üretilmiştir. Vektörde bulunan alfa sekresyon sinyali ile hücre dışına salgılanan peptidler biyolojik karakterizasyonları sonrasında antimikrobiyal, antifungal ve sitotoksisite bakımından fonksiyonel olarak değerlendirilmiştir. Elde edilen sonuçlara göre, recombinant üretim başarılı bir şekilde gerçekleşmiştir. Karakterizasyon sonrasında yapılan fonksiyonel testlerde F2T142 peptidinin *Staphylococcus aureus* ATCC 29213 suşuna karşı 240 µg/ml doz seviyesinde aktivitesi görülmüştür. Yine aynı peptidin, HepG2 karaciğer kanser hücreleri üzerinde yapılan toksisite çalışmalarında diğer peptidlere göre daha az toksik olduğu belirlenmiştir. D6RKI7 adayının da *Candida albicans* ATCC 90028 suşuna karşı aktivitesi görülmüş ancak yüksek toksisite göstermiştir.

Anahtar Sözcükler: Antimikrobiyal peptitler, antimikrobiyal aktivite, fungal defensinler, hidrofobinler, *Pichia pastoris.*

ABSTRACT

Evaluation of *Pichia Pastoris* as a Microbial Cell Factory for Production of Fungal Antimicrobial Proteins

Antimicrobial resistance is the major reason why infections caused by microorganisms cannot be eliminated effectively. Pathogenic microorganisms are rapidly developing resistance to antimicrobial drugs and this is becoming a global crisis. Research and development of these drugs which are widely used today, from discovery to production are both long time and costly. Therefore, there is a need for both efficient and cost-effective alternatives. Fungal defensins are candidate peptides that have the potential to be an alternative to current conventional therapies. These peptides are cysteine rich, cationic and secreted by the defense mechanism of fungi which have antimicrobial properties. Hydrophobins are also small candidate peptides secreted by *ascomycetes* and *basidiomycetes* which consisting of hydrophobic amino acids with conserved cysteine residues. With the scope of this thesis, three defensins and one hydrophobin were selected from the databases according to the result of physicochemical analysis and which have not been characterized before; defensin F2T142, F2T142Mut, D6RKI7 and hydrophobin RodA were expressed recombinantly in *Pichia pastoris* KM71H strain, a methylotrophic yeast. The peptides were secreted out of the cell and antimicrobial, antifungal, cytotoxicity test results were evaluated after the biochemical characterization. According to results, recombinant expression has been successfully realized. In functional tests performed after the characterization, antimicrobial activity of the F2T142 against *Staphylococcus aureus* ATCC 29213 strain was observed at 240 µg/ml dose. The same peptide was found to be less toxic than the others in toxicity studies on HepG2 liver cancer cells. D6RKI7 also showed the activity against *Candida albicans* ATCC 90028 strain, but showed high toxicity.

Keywords: Antimicrobial peptides, antimicrobial activity, fungal defensins, hydrophobins, *Pichia pastoris*.

1 INTRODUCTION AND AIM

The discovery of antibiotics and antifungals increases the life quality and treatment options. However, few years after the discovery, microorganisms have resistant to treatments. Nowadays, antimicrobial resistance is one of the most serious problems all over the world which is related to 700.000 deaths per year (1). Most pathogenic microorganisms have the capability of developing resistance to least some antimicrobial agent. Generally, resistance is related to drug, patient or dosage. However, increasing antibiotics usage is a huge risk to the development of resistance. Multidrug resistant bacteria exhibiting resistance for one or more antibiotics and with related biofilm-forming capacity, mostly play role during antibiotics resistance (2). Fungal infections are a serious problem worldwide, just like bacterial infections and annually 150 million people are affected from this case (3). As a result of that, 1.7 million deaths are seen per year. Nail infections, vaginal candidiasis are the most common fungal diseases all over the world. Moreover, in the past few months, coronavirus pandemics (COVID-19) has taken the world by storm. Recent studies report the presence of secondary fungal infections during the post Covid-19 period. Pulmonary aspergillosis and Covid-19 related candidiasis are the well-known diseases at this point. Symptoms are usually seen in patient on ventilators and poor prognosis. Due to that, some deaths are related with fungal infections (4).

1.1 Antimicrobial Peptides

Antimicrobial peptides (AMP's) are novel therapeutic peptides as an alternative for conventional treatments during infectious diseases. They have short amino acid sequences between 10-60 amino acid length and part of the innate immune system which makes the primary defense for mammals, plants, fungi and other microorganisms (5). In general, AMP's have alpha (α) helical, beta (β) sheet, α-β sheet and random coil secondary structures which do not include specific motifs especially, but they have a net positive charge and contain hydrophobic residues. The biggest advantage of this peptides is that they are strong competitor candidates against conventional antibiotics. Antimicrobial activity of this peptides is regulated by

electrostatic forces, hydrophobicity, chemical and physical properties. However, amino acid composition, cationic charge and size are important factors to attach and insert into the bilayer membrane of microorganisms.

Defensins are the small peptides which are secreted from the innate immune system of mammals, fungi, plant and bacteria to eliminate against microorganisms or foreigner molecules. They are mostly amphiphilic and contain cationic residues with α-helical structure, cysteine stabilized α-β sheet structures. Nearly all defensins have a β-sheet structure with three or four cysteine (Cys) disulfide bonds.

1.1.1 Fungal defensins

Fungal defensins are the subtype of defensins that are secreted from fungi which have low molecular weight between 5.8-6.6 kDa. They have a strong potential to discover novel antimicrobial drugs and antibiotics. Mainly, they have cysteine-stabilized α-helix and β-sheet (CSαβ) motif and that regulates stability and adaptation for medical applications such as pH stability, proteolytic activity and temperature. In literature, different defensin and defensin-like peptides (DLP's) were evaluated as AMP's. First identified fungal defensin "Plectasin" is a pioneer to discover new candidates against microorganisms (6). That was highly active against gram-positive and gram- negative bacteria but real mechanism is not well-known. The general mechanism based on binding of the defensin to the cell wall precursor lipid II inhibition of cell wall biosynthesis. After the identification of plectasin, most of the potent fungal defensins were discovered and studied to their antimicrobial profiling (2,6–8).

1.1.2 Hydrophobins

Hydrophobins are secreted proteins of fungi that are found lower than 20 kDa. Mainly characterized with high hydrophobicity and eight conserved cysteine residues and both hydrophobic-hydrophilic tails. They have disulfide bond formation as a pattern (9). The first identified hydrophobin was founded during *Schizophyllum*

commune gene studies. Hydrophobins are mainly divided into two classes as a Class I and Class II according to their hydropathy and solubility (10). What they have owned biotechnologically with interesting physicochemical properties of these proteins which are used in many application areas; adhesion, surface modification, surfactant-like coating and protector. However, they might have a potential against microorganisms for antimicrobial resistance.

1.2 Pichia pastoris

Pichia pastoris is a methylotrophic yeast that use methanol as a carbon source. That has strong potential for expression system and protein production. However, there are many advantages of to usage of *Pichia pastoris* in expression. The first advantage is that the capability of making complex proteins with post-translational modification, folding and disulfide bonds. N-Glycosylation very close to higher eukaryotes and proven system without virus and pyrogen. Soluble protein expression and purification are easy, proteins are secreted into the media. However, fast, simple and production yield is high (11).

1.3 An Overview

Under the light of all these information, the aim of this thesis is the recombinant expression, purification and characterization of a diverse set of previously unidentified fungal antimicrobial proteins in *Pichia pastoris* and analysis of their antibacterial-antifungal properties along with cytotoxicity profiles. All the candidate fungal antimicrobial proteins were expressed and characterized for the first time except RodA hydrophobin. However, hydrophobins are potent candidates against fungi and bacteria to study. Antibacterial activity was tested *Staphylooccus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 strains. However, antifungal activity tests were done against *Candida albicans* ATCC 90028 strains.

2 BACKGROUND

2.1 The History of Antibiotics

The first pre-history of antibiotics starts from ancient Greek, Egypt and China. Many types of natural products were used as an antimicrobial agent such as herbs, honey, feces (12). With the combination of science and curiosity, the first antibiotics which is called Penicillin was discovered in 1928 by Alexander Fleming. A mold that flew away while Alexander Fleming was working on *Staphylococcus* bacteria, create a new era of antimicrobial agents. That accident was influenced the discovery of new antibiotics and milestone to increase human lifespan by 23 years (13).

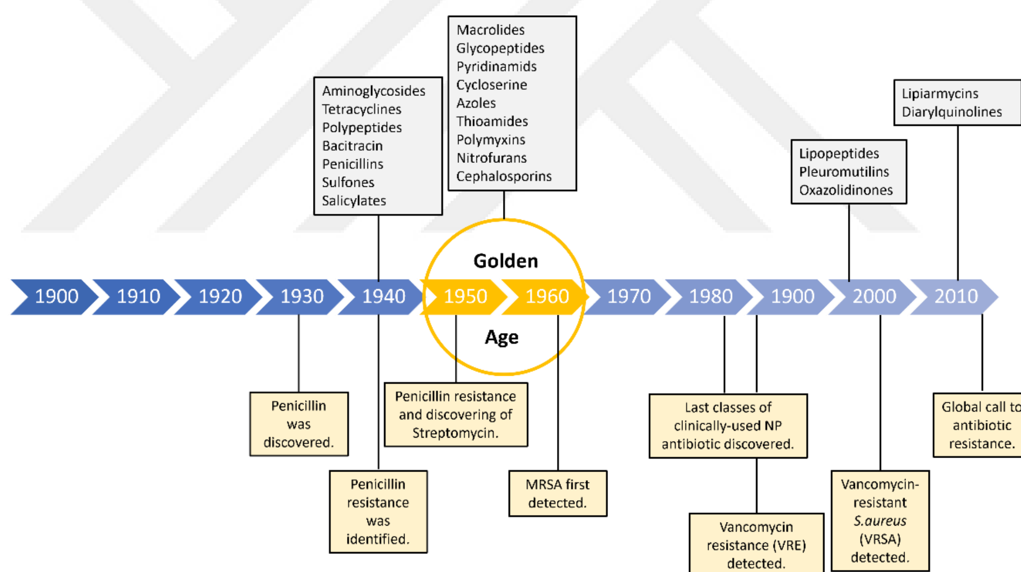


Figure 1. Developmental timeline of the antibiotics from early 1900s to 2010s.

In 1940's, penicillin resistance was identified and scientists turn to new pursuits such as Tetracyclines, polypeptides, Bacitracin and Sulfones. Between 1950's and 1960's, "The Golden Age" of the antibiotics was started (Figure 1). Macrolides, Nitrofurans, Pyridibamides, Azoles, Cycloserines were discovered in that period. When the detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria in the 1960's, new antibacterial drug studies were gained speed. The Golden Age is

over and antimicrobial resistance were seem very fast and surplus (12,13). In order to that, Ciprofloxacin, Vancomycin, Daptomycin, Tigecycline were introduced to the markets up to mid-of 2000's (14).

Nowadays; Plazomicin, Eravacycline, Sarecycline, Omadacycline, Rifamycin, Pretomonaid, Lefamulin, Cefiderocol were approved by Food And Drug Administration (FDA) as a new synthetic or semi-synthetic options (15).

2.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are novel therapeutic peptides as an alternative for conventional treatments during infectious diseases. They have short amino acid sequences between 10-60 amino acid length and part of the innate immune system which makes the primary defense for mammals, plants, fungi and other microorganisms. They include mostly positively charged amino acids such as lysine, arginine, histidine and more than 50% of them were found as a hydrophobic (16). Magnetic Resonance Spectroscopy (NMR) studies showed that, they have different structures which are predicted as 13,8% helical, 4% of β-strand and 4% of them mixed. In addition, Collection of Antimicrobial Peptide (CAMP) database brought to light 18,7% of them helical, 18,9% β-strand and 60,1% mixed and extended (17) . According to all these studies, they were classified into four different groups as α-helical, β-strand, coiled, mixed and do not contain specific motifs.

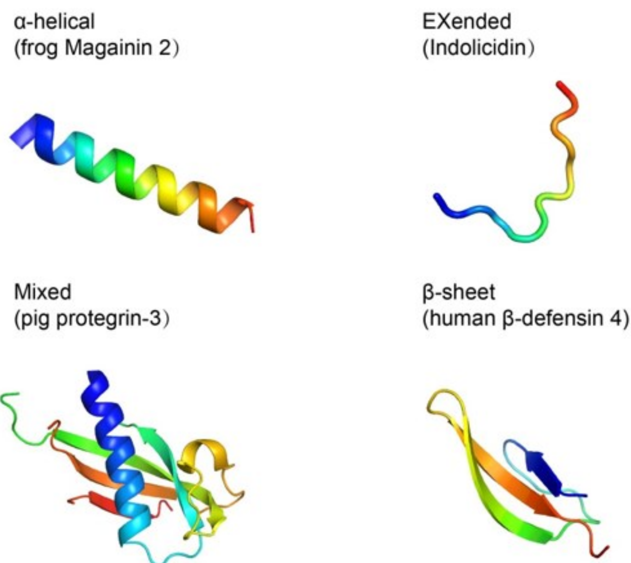


Figure 2. Different types of 3-D structures of the defensins as α-helical, β-sheet, mixed and extended (18).

AMPs can classify based on their source, activity, structural characteristics and amino acid rich species (Figure 3). Yuchen Huan et al, classify that based on amino acid rich species into four groups;

- Proline-rich peptides,
- Tryptophan-arginine rich peptides,
- Histidine-rich peptides,
- Glycine-rich peptides (19).

Proline is a cyclic and non-polar amino acid which is called imino acid. That type of AMPs show different mode of action than the other groups. SbmA is a membrane transporter and by using that, peptide enters to the cytoplasm of the bacteria. After entering, proline-rich AMPs target directly to the bacterial ribosomes and block aminoacyl-tRNA to peptidyl-transferase binding (20). Tryptophan is a non-polar amino acid and plays role in the inner lipid bilayer region of the membrane. However, arginine is an essential amino acid that has a positively charged guanidino group which regulates hydrogen bond interactions and charge. By that way, tryptophan-arginine-rich AMPs affect the lipid bilayer membrane of the microorganisms (21). Histidine is a positively charged amino acid that includes functional imidazole group. The

mechanism of this type of peptides focused on increasing of the membrane permeability. So, membrane rupture and death are occurred. Lastly, glycine-rich AMPs are mainly associated with the tertiary structure of peptide chain and activates the phagocyte-mediated microbicidal mechanism (19).

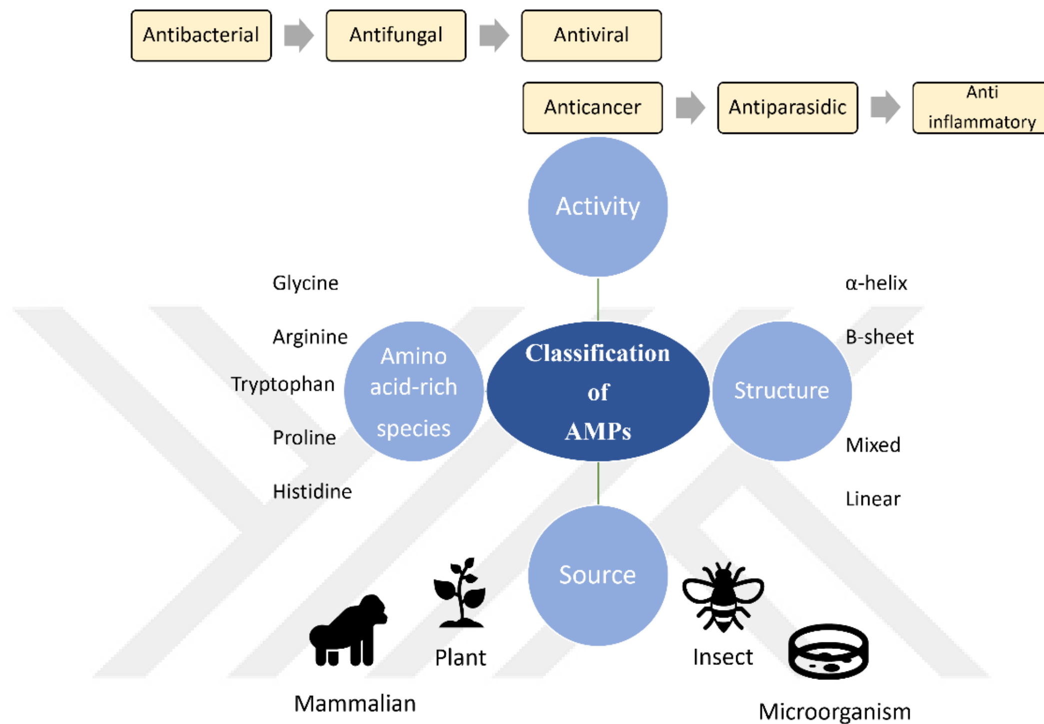


Figure 3. Classification of AMPs according to their amino acids, structures, activities and sources.

Biological activity and specificity of the AMPs are related physicochemical parameters that modulate specificity and activity. Secondary structure, charge, hydrophobicity and amphipathicity are the most significant parameters to design and select novel peptides (22). AMPs mostly have positively charge between +2 to +9. The charge affects membrane pores and channels to change membrane permeability. Positively and negatively charge meeting provide that because of the electrostatic interactions (23). That makes the translocation of the gram-negative bacteria outer membrane via Ca^{+2} and Mg^{+2} cations. The translocation changes permeability and provide to being bulky. Because of that, temporary cracks are occurred on membrane (24). The other modulator hydrophobicity effect is related with red blood cell hemolysis and antimicrobial effect. More hydrophobic AMPs are entered to

hydrophobic core of the red blood cells. That causes pore forming and high hemolytic activity (25). On the other hands, hydrophobic peptides are active against *Staphylococcus epidermis*, *Corynebacterium xerosis* and *Escherichia coli* with the non-amphipathic cores (26).

2.2.1 Action mechanisms of antimicrobial peptides

According to membrane action mechanisms AMPs can divided into two main groups as a membrane targeting mechanisms and non-targeting membrane mechanisms (19,22).

2.2.1.1 Membrane targeting mechanisms

Membrane targeting mechanisms divided into three groups; barrel-stave model, carpet-like model, the toroidal model (Figure 4).

The toroidal pore model: Some human AMPs such as magainins, mellitin make some changes on lipid bilayer membranes and creates toroidal pores. In that case, head-groups of the lipids align the pores and peptides. Collection of the mellitin-like peptides at the same location disrupt the membrane such as detergent-like collapsing (27). In there, AMPs are buried into the bilayer and form ring-like structures between 1-2 nm (28). Molecular dynamics simulations prove that magainin family of peptides such as MG-H2 provokes toroidal pore formation (29).

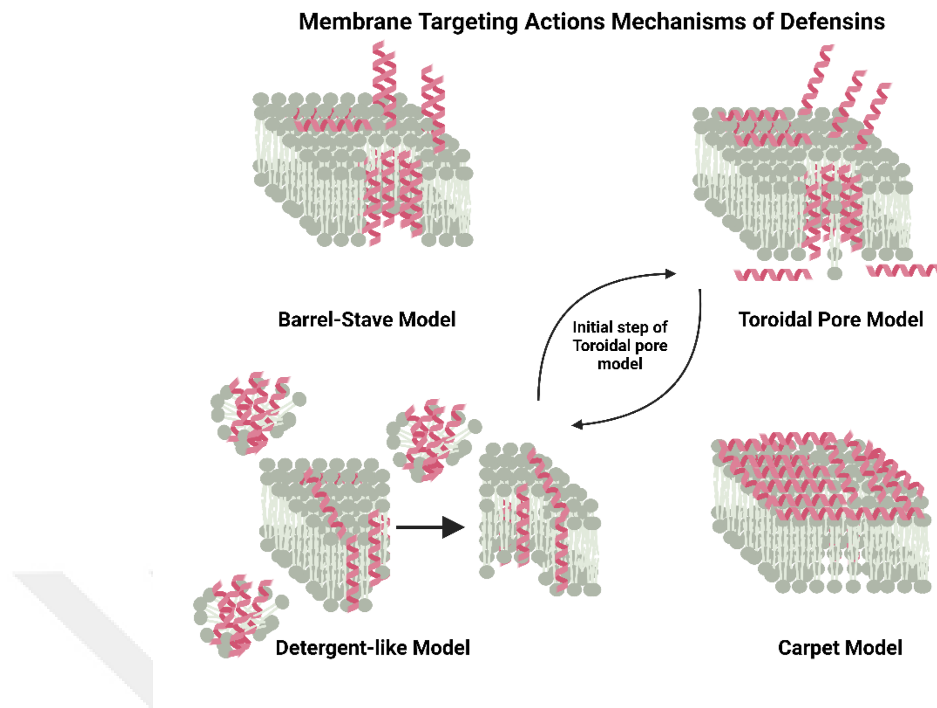


Figure 4. Membrane targeting action mechanisms of the AMPs. ("Created with BioRender.com").

Barrel-stave model: In barrel-stave model, peptides make interaction to each other and multimerize. The multimerization situation acts like channel forming formation. As a results of that, cell membrane collapse and cell death is occurred. The main difference between barrel-stave and toroidal models is that, there is no specific interactions at toroidal model instead barrel-stave and alignment of the peptides is possible with toroidal cooperativity unlike specificity (19,30).

Carpet-like model: AMPs do not always make directly pore formation to disrupt or collapse lipid bilayer membrane. In carpet-like model, peptides come together and accumulate as a parallel to membrane surface. Hydrophilic parts take place across the solution, hydrophobic parts take place across the phospholipid bilayer membrane. In this instance, peptides coated all the membrane surface like carpet (31). The carpet-like model is accepted as an initiative step of the toroidal pore model at some studies such as indolicin (32) and cecropin (33).

2.2.1.2 Non-membrane targeting mechanisms

The non-membrane targeting mechanisms are; inhibition of protein synthesis, inhibition of nucleic acid synthesis, inhibition of protease activity and inhibition of cell division.

Some AMPs target the inhibition of transcription and translation. Mardirossian et. al 2018 , showed that the Tur1A peptide makes the inhibition of protein synthesis in *Escherichia coli* and Bac7 translation (34,35). However, proline-rich peptides disrupt the bacterial membrane via inhibiting protein synthesis (36). Nucleic acid synthesis is the one of key point to cell survival and during that essential enzymes take place. AMPs can affect the enzymes and nucleic acid degradation is occurred (37). As an example indolicidin inhibits DNA topoisomerase I enzyme (38). Different from them, some AMPs might affect metabolism and metabolic activity. Indolicidin, eNAP2, histatin-5 inhibits the proteases as an example (39). Lastly in some cases, DNA replication and damage is promoted by AMPs. As a result of that, inhibition of cell division is occurred. APP peptide affects the *Candida albicans* (40) and MciZ blocks Z-ring structure during division (41).

2.3 Defensins

Defensins are the small and biologically active peptides which are secreted from innate immune system of mammals, fungi, plant and bacteria. The main goals are that to eliminate microorganisms or foreigner molecules. They are mostly amphiphilic and include cationic residues α-helical, cysteine stabilized a β-sheet structures. These peptides have 12 to 50 amino acid length which contain mostly β-sheet structure with three or four cysteine disulfide bonds (42). Defensins are categorized as into three groups; alpha, beta and theta defensins according to their disulfide bonds, cysteines and structures (43). That peptides have a good potential to deal with antibiotic resistance due to their antimicrobial activities. They kill gram-negative, gram-positive bacteria, fungi and viruses (6,44). In addition to that, some α-defensins like HNP1-4

(45) and β-defensins like hBD1-6 (46) activates epithelial cells, mast cells to provoke proinflammatory factors.

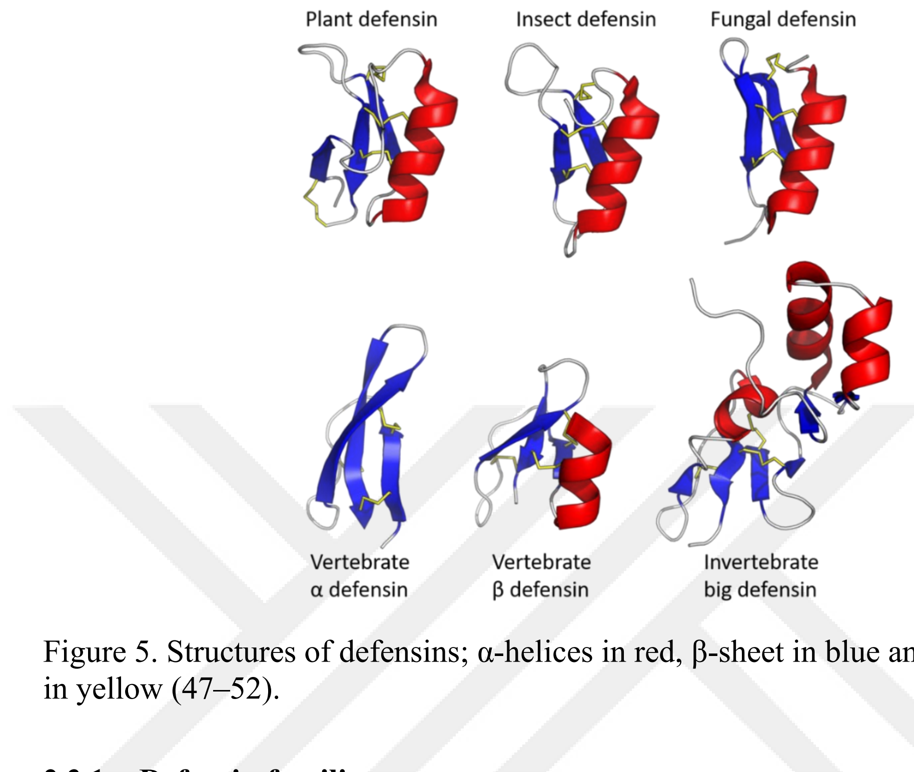


Figure 5. Structures of defensins; α-helices in red, β-sheet in blue and disulfide bonds in yellow (47–52).

2.3.1 Defensin families

Defensins families known as an alpha, beta and theta defensins (43) (Figure 6).

Alpha (α) defensins: Alpha defensins are known as arginine rich which have different disulfide bond modeling such as 1-6, 2-4 and 3-5. Human, monkey, rats, rabbits contain that type of defensins in their neutrophiles and macrophages (53). In mammals, they called as “cryptdins” which is discovered first. Three disulfide bonds are made up six cysteine residue. A three stranded β-sheet coordinates secondary and tertiary structures (54). Alpha defensins are defined in broad-spectrum category about their antimicrobial activities. As an example, cryptdin-4 is one of the most bactericidal defensin between different isotypes (55).

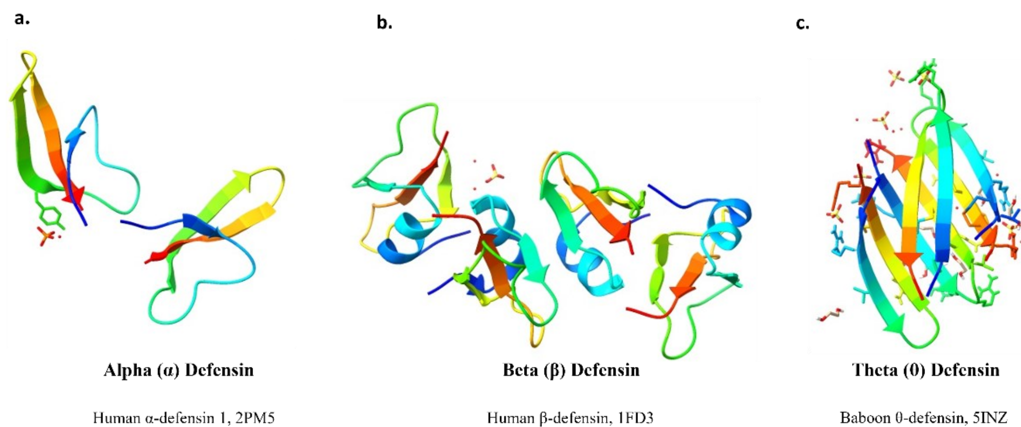


Figure 6. α, β and θ defensin families (a. Human α-defensin 2PM5, b. Human β-defensin 1FD3, c. Baboon θ-defensin 5INZ).

Beta (β) defensins: Beta defensins are categorized a family of vertebrate defensins. The first β-defensin was discovered in bovine airway in 1991 (56). However, every mammalian species which discovered until now have β-defensins. In example, cows have 13 β-defensins (57). On the other hand, epidermal cells, bronchial cells which have epithelial origin contains that (58). Although, most of the human epithelial cells express β-defensins like HBD-1 gene (59). Structure of them are consisted from cysteine-stabilized α-β motif via antiparallel β-sheet and α-helix. Amino acid number changes between 36 to 42 amino acid length with 1-5, 2-4, 3-6 disulfide bond alignment (43).

Theta (θ) defensins: Theta defensins are small circular defensins which are originated from precursor α-defensins. They mostly found in rhesus monkeys and consisted from 18 amino acids length a pair of antiparallel β-sheets (60,61). Theta defensin coding genes are take place in human but not expressed present because of the premature stop codon (43). In *Macaca mulatta* and *Papio Anubis*, RTD1-2 genes form mature θ-defensins (62). BTD genes have extra importance during construction of that in baboons (63).

2.3.2 Antimicrobial activity of defensins

Past to present rapid and widespread rises of antibiotic resistance directs the science to find new searches (64). In 2016, the international call of World Health

Organization (WHO) to deal with resistance had great repercussions (65). From 2013 to now, just several antimicrobial agents took place at markets. Plazamicin, eravacycline, sarecycline, omadocycline, imipenem and lefamulin are might be an example about that (15). Unfortunately, growing antimicrobial resistance leads to decrease effectiveness of treatment. On the other hand, research and development period takes long time and has high cost. That situation distressingly correlated via long-time treatment duration and mortality (66). United States (U.S.) reported that 19,000 people die annually because of Methicillin-resistance. In the future, world economy will lose 100 trillion dollars because of this problem (67). At this point, defensins are emerging as antimicrobial candidates with their strong potential. They are categorized as broad-spectrum microbicides (68) fungal and bacterial infections. In 1980s, magainins and cecropins were isolated and the story of the peptide based drugs was started (69,70).

Human defensins HNP-1, hBD-1 and hBD-3 showed activity against *Staphylococcus aureus* and *Escherichia coli* strains. Application of rifampicin and defensins as a synergistically together indicated the high potential (44). Another human defensin which is known HBD-2 showed the significant effect on *E.coli* ATCC 25922 strain (71). Mathew et al. 2015, designed an active peptide from human α-defensin 5 with the addition of fatty acid chain to enhance the activity by interacting arginine residue (72). Yang et al. 2018, indicated that human cationic defensins kill the *Pseudomonas aeruginosa* and MRSA *Staphylococcus aureus* (73). Plant defensins are also an important reservoir for antimicrobial activity. Many of firstly characterized defensins from seeds such as Ah-AMP1, Ct-AMP1, Dm-AMP1-2 were evaluated against bacteria (74). In addition to that, MtDef4 defensin disrupted the lipid outer membrane of *Pseudomonas* species and has activity. Also, inhibit the translation in the scope of mode of action. Velivelli et al. 2018 evaluated the antifungal activity of defensin MtDef5 (75).

Fungal defensins are a kingdom for promising new drug candidates. The first identified fungal defensin “plectasin” showed high antibacterial and antifungal activity (6) (Figure 7). The variants of plectasin, Py4 exhibited activity against *Staphylococcus*

species with low toxicity (76). Blapersin which is described as fungal defensin-like peptide and it's mutants W2F, W2Y enhanced the bactericidal effect on gram-positive bacteria with low toxicity and high serum stability (77). Furthermore, fungal defensins have antifungal activity. As an example, PAF (*Penicillium chrysogenum*), AFP (*Aspergillus gigantes*) showed the activity 1 to 200 µg/ml minimal inhibition concentration (MIC). PAF interacted with G protein signal pathways and promote to releasing reactive oxygen species (ROS) to induction of apoptosis (78). AFP blocked cell wall biosynthesis to exhibit activity (79).

2.3.3 Fungal defensins

Fungal defensins are the subtype of defensins that are secreted from fungi. They have low molecular weight between 5.8-6.6 kDa with 4-10 cysteine residues (42) which have potential source of antimicrobial drugs. Mainly, fungal defensins have a cysteine-stabilized α-helix and β-sheet (CSαβ) motif. This motif is consists of one α-helix, two parallel β-sheets, six or eight cysteines (2). According to recent studies, cysteine residues increase the pH stability and provide the adaptation of proteolytic activity, extreme temperature (80). However, CSαβ defensins have similar mechanisms for bacteria during the action of mechanism. The mechanism based on binding of the defensin to the cell wall precursor lipid II and inhibition of the cell wall biosynthesis (7).

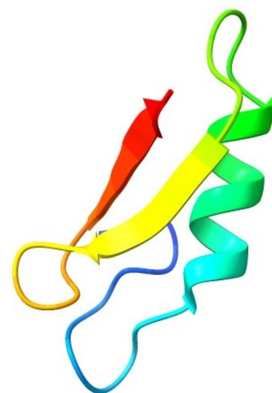


Figure 7: Structure of the first identified defensin plectasin.

In literature, different defensins different fungal defensins and defensin-like peptides (DLPs) were evaluated as AMPs (6). Yang et al. 2019, showed novel fungal DLPs from UniProt then they expressed that in *Pichia pastoris* expression system by recombinantly and characterized to that their antibacterial, antibiofilm properties against multi-drug resistance (MDR) *S.aureus* (2). Ten different synthetic DLPs from P1 to P10, micasin, plectasin and vancomycin as positive controls were evaluated. Most of the peptides were effective to kill bacteria but especially P2 is too because of the lower MIC level. However, hemolytic activity was very low. The point is that have enormous importance to being as a candidate for antimicrobial drugs. Because plectasin has high antimicrobial activity but showed high hemolytic activity. Hemolytic activity is correlated high toxicity. Low toxicity level carries great importance to delivering of the drug to patients during treatment. Then they checked the protease and pH stability of the candidate peptides to the suitability of the oral administration. Meantime; biofilm formation ability, mRNA expression of biofilm-related genes were tested. All these results indicated that P2 is the most potential candidate. Recently, the anti-infective effects of the dermatophytic defensins were studied about their therapeutic potentials by Zhu et al. 2012. They reported 17 new fungal DLPs from *Microsporum canis* and characterized. The studies showed the fungal DLP micasin was active on both gram-negative and gram-positive bacteria by killing them within three hours approximately 100% with low hemolytic activity (8).

The action mechanisms of defensins are not fully known. However, studies showed that defensins bind to lipid II and inhibit cell wall biosynthesis (7). Qi et al. 2022, demonstrate that the cationic fungal DLPs pyronesin4 has antimicrobial activity against *Staphylococcus* species by binding lipid II and inhibit that cell wall biosynthesis precursor. Pyronesin4 changed the bacterial morphology and made cytoplasmic stacking (76). Likewise, fungal defensin eurocin acts on both gram-positive and gram-negative bacteria with a similar mechanism like detergent micelles and inhibit lipid II (81). In this way, that showed fast activity on *Streptococci* species. AfusinC which is *Aspergillus fumigatus* defensin with the high potential against microorganisms. Contreras et al. 2018, reported that the defensin showed lower MIC levels against *S.aureus*, *Bacillus megaterium*, *Micrococcus luteus* and *Bacillus subtilis*

(82). While there are cases where a single defensin is effective more than one defensin working synergistically has also been reported (83). According to that, synergism caused to first step weaking of bacterial lipid outer membrane (84).

2.4 Hydrophobins

Hydrophobins are small proteins within amphipathic biofilms which have self-assembling ability. They are produced by filamentous fungi and consisting from nearly 100 amino acids. The first hydrophobin was discovered during *Schizophyllum commune* studies. Wessel et al., named that because of the high hydrophobic amino acids it contains (85). Generally they are lower than 20 kDa, mainly characterized with eight conserved cysteine residues which have disulfide bond as a pattern.

Table 1. Difference between class I and class II hydrophobins.

Class I Hydrophobins	Class II Hydrophobins
Soluble only strong acids like formic acid, Trifluoroacetic acid (TFA) and insoluble in SDS.	Soluble in ethanol and SDS.
Consisting from 100-250 amino acids.	Consisting from 50-100 amino acids.
Identified from *Basidiomycetes* and *Ascomycetes*.	Identified from only *Ascomycetes*.

According to studies, hydrophobins are generally found as small gene families with two or ten members but there is an exception for fungi *Coprinus cinereus* with the twenty-three genes (80). Hydrophobins are mainly divided into two classes as Class I and Class II according to their hydropathy and solubility (Table 1). Class I hydrophobins form highly insoluble polymers. There is an important variation in the inter-Cys spacing. That type of hydrophobins are composed of fibrillar structures which are known as rodlets and assemble into highly insoluble polymeric monolayers. The rodlets are very stable and harsh acid treatments are needed to make them soluble. Although, soluble forms polymerize again into the rodlets. However, they have low sequence similarity. In class II, sequence similarity and inter-Cys spacing are very conserved according to class I. Fibrillar rodlet structure is easily eliminated and solubilized by an organic solvent or detergent.

Fungi shows two types of growth morphologies which are known unicellular and multicellular filamentous. Yeasts are hydrophilic and due to that case they lost their hydrophobins. The vegetative hyphae of filamentous fungi is grown on a moist area and known as hydrophilic. So, they do not have rodlets. However, asexual spores are hydrophobic and contain many hydrophobins. The functions of hydrophobins are associated with surfactant activity and self-assembly. Due to that they can form an amphipathic monolayer (86). Structural studies indicate that all hydrophobins have a similar β-structure with four disulfide bonds and great hydrophobic areas which are related to surface activity. However, there are some studies that are associated with class I and class II. As a class I, DewA (*Aspergillus nidulans*), Mpg1 (*Magnaporthe grisea*) and class II from *Neurospora crassa*. For all these monolayer formation does not related with major conformational changes. In contrast to that, rodlet formation is related to that changes. *Splitgill commune* studies have shown that Cys-3 and Cys-4 loops are important to adhesion (87).

Recent studies evaluate the relation between antimicrobial activity properties of hydrophobins to open new perspectives to that field (88). Soorentino et al., evaluated that class I chimeric hydrophobin Vmh2 had the antimicrobial activity against gram-positive and gram-negative bacteria (89). On the other hand, HYTLO1 hydrophobin which is secreted from *Trichoderma longibrachiatum* had antifungal property against *Botrytis cinerea* (90). Artini et al., indicated the reducing of *Staphylococcus epidermis* biofilm formation (91). Anastasia et al., showed that the hydrophobin Sa-HFB1 which is secreted from *Sodiomyces alkalinus* has antifungal activity against pathogenic clinical fungi and yeast (92). All these studies showed that hydrophobins have important potentials to be strong candidates against increasing antibiotic resistance. Future researches will shed light on some questions that have not yet been answered at this point.

2.5 Recombinant Technologies And Protein Production

A recombinant protein is an expression host organism which transfected with a recombinant target gene isolated from another organism, a protein produced artificially

by the expression system. That is used to produce large amounts of recombinant gene encoded protein for medical and academic research. Nowadays, there are many recombinant expression system such as mammalian, yeast, bacterial, insects and algal. The most populars are to produce therapeutic proteins; *Pichia pastoris*, *Escherichia coli*, *Saccharomyces cerevisiae* and Chinese Hamster Ovary cells (CHO) (93). Today, there are many recombinantly produced drugs from different expression systems approved by FDA. Insulin, glucagon, HbsAg vaccine, anticoagulants, clotting factors, proteolytics, cytokines and enzymes are the recombinant drugs which are which produced by yeast expression system (94). Insulin, long-acting insulin, glucagon, human parathyroid hormone, recombinant calcitonin hormone and interferon are produced by bacterial expression system (95). Monoclonal antibodies, cytokines, enzymes, fusion proteins, hormones and clotting factors are produced from mammalian expression system. Lastly, cervical cancer vaccine is produced by insect expression system (96).

Table 2. Recombinantly produced peptide based drugs in different expression systems which are approved by FDA (96).

Biopharmaceutical	**Expression Sytem**	**Target**
Cervical cancer vaccine	Insect	Cervical cancer
rFVIIIFc	Mammalian- HEK293 cells	Hemophilia A
Dulaglutide	Mammalian- HEK293 cells	Type 2 diabetes
Agalsidase alfa	Mammalian- HT1080 cells	Fabry Disease
CL184	Mammalian- PER.C6 cells	Rabies Virus Infection
MOR103	Mammalian- PER.C6 cells	Rheumatoid arthritis
Adalimumab	Mammalian- CHO cells	Autoimmune diseases
Epoetin alfa	Mammalian- CHO cells	Anemia and HIV
Laronidase	Mammalian- CHO cells	Hurler-Schele Syndrome
Certolizumab pegol	Bacteria- *E.coli*	Autoimmune diseases
Insulin	Bacteria- *E.coli*	Diabetes
IntronA	Bacteria- *E.coli*	Genital warts, cancer
Roferon A	Bacteria- *E.coli*	Hairy cell leukemia
Interleukin-2	Bacteria- *E.coli*	Metastatic melanoma

Table 3. FDA approved biopharmaceuticals which are produced by yeast expression system (94).

Biopharmaceutical	**Expression System**	**Target**
Insulin	Yeast- *S.cerevisiae*	Diabetes
Glucagon	Yeast- *S.cerevisiae*	Hypoglycemia
HbsAg	Yeast- *S.cerevisiae*	Hepatitis B
Hirudin	Yeast- *S.cerevisiae*	Venous thrombosis
Factor XIII A-subunit	Yeast- *S.cerevisiae*	Congenital Factor XIII
Combination vaccine	Yeast- *S.cerevisiae*	Multiple
HPV capsid proteins	Yeast- *S.cerevisiae*	Human papillomavirus
Colony-stimulating factors	Yeast- *S.cerevisiae*	Neutrophenia
Truncated plasmin	Yeast- *P. pastoris*	Vitreomacular
Insulin glargine	Yeast- *P. pastoris*	Diabetes
Plasma kallikrein inhibitor	Yeast- *P. pastoris*	Hereditary angioedema

2.5.1 Recombinant expression systems

Biopharmaceuticals are the main drugs which are produced by different recombinant expression systems. From 2014 to 2018; 68 monoclonal antibodies, enzymes, factors were approved by FDA (97). The fact that the research and development processes take a long time and are high cost has increased the interest in different recombinant expression systems. Although, the most commonly used systems are bacterial, yeast and mammalian; algae and insect systems can also be used (98). These systems have different advantages and challenges according to their usage area. A s an example, mammalian systems are the well optimized systems to produce bioactive drugs nevertheless production time is long, needs to high cost and protein yields are lower. Bacterial expression systems are fast and inexpensive way to produce drugs but unfortunately there are many challenges about protein yield, solubility and non-efficient post-translational modifications. However, yeast-based expression systems are the kingdom of post-translational modifications via high protein yield up to gram per liter. Moreover, that is easy and low-cost according to other expression systems. Fermentation needs and glycosylation problems might be challenges sometimes but can be overcome. Lastly, insect and algal systems are a way to

production but take long time and less developed technologies according to other systems (99).

Table 4. Recombinant expression systems, advantages and challenges.

Expression system	Advantages	Challenges
Mammalian	• Best for the bioactive proteins • High level protein processing • Comprehensive post-translational modifications	• Production time is long • Expensive • Protein yields are lower • Takes long time
Yeast	• Diverse post-translational modifications • High yield protein production (up to grams per liter) • Eukaryotic processing • Fast • Cost-effective	• Glycosylation problem • Fermentation required to obtain high yields • Needs to optimization
Bacterial	• Relatively inexpensive • Simple genetics • Fast • Easy to scale up	• Protein solubility problem might occur. • Hard to express some mammalian proteins. • Low yield • Not efficient post-translational modification
Insect	• Good secretion • Prove the toxic gene products	• Long time • Expensive
Algal	• Good way to biofuel production	• Less developed

2.5.1.1 Bacterial expression system

For the production of eukaryotic and prokaryotic proteins, especially *E.coli* has been an important expression system for over 20 years. Its low cost, rapid growth and easy cultivation have contributed to this development. In addition, the well-known genetic structure and the fact that there are many studies on it also support the development. The first recombinant human insulin was produced in *E.coli* in 1978 (95). After this date, many biopharmaceuticals have been produced recombinantly into *E.coli*. Todays, there are many different types of drugs which are produced by that system for autoimmune diseases, diabetes, cancer and rare diseases (95,100).

In addition to all these advantages, there are also many limiting challenges. In this system, produced protein cannot be secreted from the cell into the medium. For this reason, protein is obtained by distrupting the cell while this may be a problem in obtaining the protein, it also requires an extra step. Post-translational modifications are

not possible in *E.coli.* As a result, protein stability decreases and the risk of immunogenicity increases. However, protein inactivation and toxicity may occur. Proteins can be misfolded and aggregated. Since they cannot form disulfide bonds effectively, they cannot produce proteins with many disulfide bonds. To reduce these risks, the medium conditions such as pH, temperature, CO_2, O_2 can be re-optimized by using different strains and promoters.

2.5.1.2 Yeast expression system

Yeast expression system is an advantageous system to produce eukaryotic proteins. That is cost-effective, rapid growth and easily cultivated system which is used. The most common yeast expression hosts are *Saccharomyces cerevisiae (C. cerevisiae)*, *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Kluyveramyces lactis* and *Schizosaccharomyces pombe* (101). This system has many advantages over the *E. coli* expression system; yield is very high compared to other expression systems and can reach gram per liter levels. Generally safe and non-toxic. Since it can make post-translational modifications seen in mammalian cells, it produces more yield and stable proteins when compared to *E. coli* system. In this way, proteins are secreted to the media because of that purer proteins were obtained. However, it is very fast and cost-efficient in terms of production time.

Yeast expression systems are classified into methylotrophic and non-methylotrophic. *S. cerevisiae* is non-methylotrophic and *P. pastoris* is methylotrophic which were used as a most frequently hosts. With the sequencing of *S. cerevisiae* S288C strain for the first time in 1996, eukaryotic biology studies had shifted in this direction. After this point, it started to be preferred with pH, ethanol, glucose, and osmotic pressure tolerance (102). *S. cerevisiae* also known as safe which is called “generally regarded as safe” (GRAS). Because it has been used safely in the pharmaceutical and food industry for many times from past to present (103).

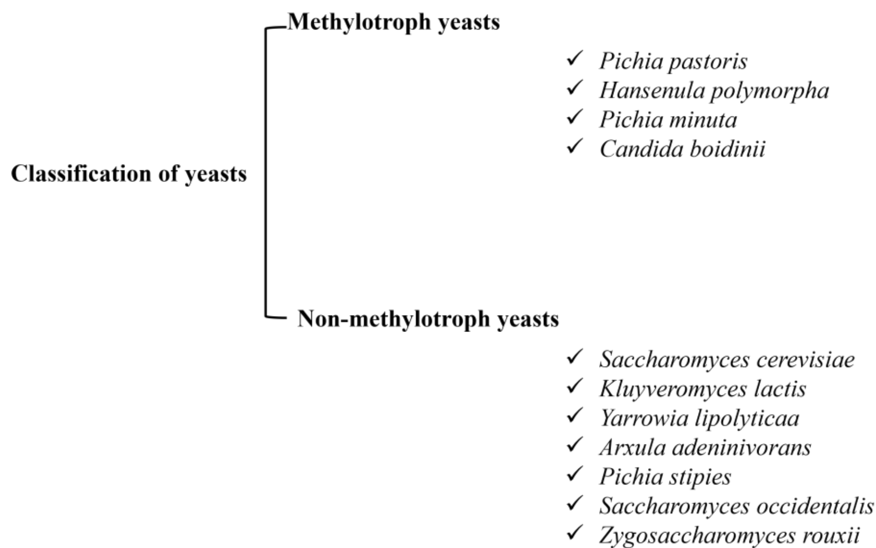


Figure 8. Classification of yeasts.

There are many strains which were used in industry and research such as 288C, A634A, BY4716, CEN.PK, Σ1278b and BJ5464. 288C is the first isolated strain which have some limitations like low rate sporulation and inefficient growing on maltose (104). A634A strain is used to cell genetic studies (105). BY4716 is used as a control strain (105). CEN.PK strain have the growing ability on different carbon sources (106). Σ1278b is essential to nitrogen metabolism (107). Lastly, BJ5464 strain is frequently used for recombinant expression (108). Selection marker can be Ampicillin, Neomycin or Kanamycin which depends their plasmid types (109,110).

However, problems such as protein hyperglycosylation, low yield and plasmid instability have paved the way for new alternatives. Development of *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Kluyveramyces lactis* based on these limitations as a solution.

2.5.2 *Pichia pastoris*

Pichia pastoris is a kind of methylotrophic yeast which uses methanol as a carbon source to provide energy. It is an eukaryotic organism with the oval-shaped, single celled and 5 µm diameter. Generally known as belonging to the *Komagataella* species from the fungi kingdom. *Komagataella* species are divided into three as a

Komagataella pastoris, *Komagataella phaffii* and *Komagataella pseudopastoris* and they were the most used strains during biotechnological applications. With the discovery of *P. pastoris* in 1960s, it has become one of the important elements of biochemical researches and industry. It has capable of both sexual and asexual reproduction. For this reason, two types of cells emerge as a haploid and diploid. In the asexual cycle, haploid cells reproduce by mitosis. In the sexual cycle, diploid cell undergoes sporulation by meiosis. *Pichia pastoris* is generally found in nature in trees and can use methanol, glycerol and glucose as a carbon sources. On the other hand, cannot use lactose.

2.5.2.1 A recombinant expression cell factory

P. pastoris is a major expression host to express proteins such as biopharmaceutical and industrial enzymes (111). This system has been successfully produced human erythroprotein, phospholipase C, superoxide dismutase, trypsin, albumin, monoclonal antibodies and collagen (112).

Table 5. Industrial products and biopharmaceuticals which are produced by *P. pastoris* expression system.

Strain	**Products**	**Usage**
Pichia pastoris	Insulin glargine	Biopharmaceutical
	Phytase	Agricultural supplement
	Trypsin	Protease to research
	Jetrea	Biopharmaceutical
	Phospholipase C	Vegetable oil degumming
	Collagen	Biopharmaceutical
	Proteinase K	Molecular Biology
	Ecallantide	Biopharmaceutical

The main advantages of *P. pastoris* recombinant expression system;

- *P. pastoris* can grow into simple and cost-effective medium with high cell density (113).
- *P. pastoris* has two alcohol oxidase (AOX) genes such as AOX1 and AOX2 which are known strong promoters to induction. Because of that, methanol is used as a carbon source to during expression. This both reduces the cost and ensures fast production (114).
- *P. pastoris* provide high yield production up to gram/liter levels (101).
- It is a familiar organism because genetically well-known and used as a model organism (101).
- *P. pastoris* can make post-translational modifications. Because of that, immunogenity risk decreases and protein stability increases (99).
- *P. pastoris* can make the disulfide bond formation (99).

On the other hand, there are some disadvantages of this system. For example, chaperonin proteins are needed to folding of many mammalian protein and *P. pastoris* does not contain this suitable chaperons. This case still required some improvements.

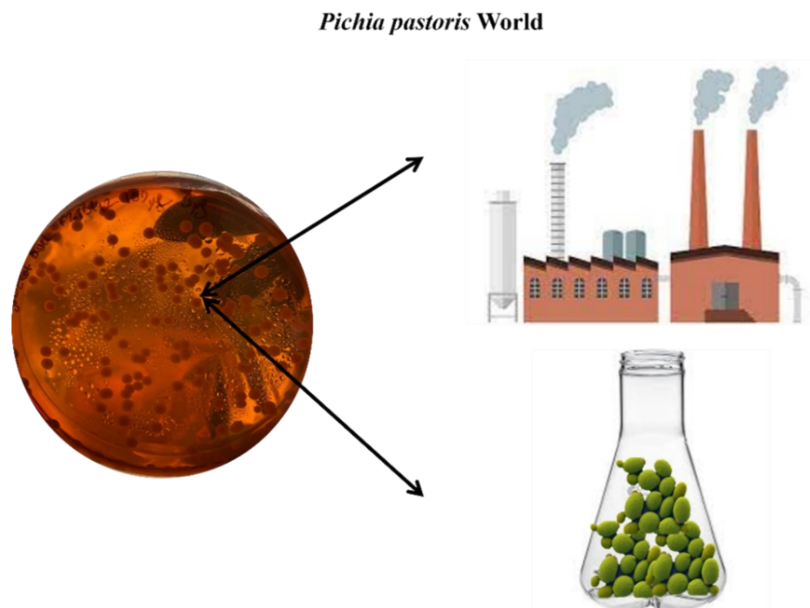


Figure 9. *P. pastoris* as a recombinant production factory.

P. pastoris has many promoters. AOX and GAP (glyceraldehyde 3-phosphate dehydrogenase) are the well-known promoters. GAP provides protein production with the highest efficiency in shortest time but this cause a toxic effect because it is a burden for the yeast cells (115). Therefore, toxic proteins cannot be produced. GAP promoter also uses methanol as a carbon source just like AOX, which causes heating in expression and therefore high oxygen consumption. AOX promoter is tightly regulated from AOX gene uses methanol as a carbon source. Totally, there are two AOX genes in *P. pastoris* as a AOX1 and AOX2. AOX1 promoter is responsible from more than 90 enzymes, AOX2 less than 10 (115). On the other hand, AOX1 has the capacity to continue production during expression of toxic proteins without harming the cell.

pPICZ and pGAPZ vectors are suitable for the recombinant expression in *P. pastoris*. pPICZαA, pPICZαB and pPICZαC are both highly productive and easy to select because they contain Zeocin resistance gene.

Table 6. *P. pastoris* promoters and encoded genes.

Strain	**Promoter**	**Gene**
Pichia pastoris	AOX	Alcohol oxidase
	GAP	Gleyceraldehyde 3-phosphate dehydrogenase
	ICL-1	Isocitrate lyase
	TEF1	Translation elongation factor I
	ADH1	Alcohol dehydrogenase
	PGK1	3-Phosphoglycerate kinase
	DAS	Dihydroxyacetone synthase
	FLD1	Formaldehyde dehydrogenase

P. pastoris is divided into three according to their methanol usage; Mut^+, Mut^S and Mut^-. These strains grown in methanol and are suitable for production in fermentation. Since the Mut^S strain does not have the AOX1 gene, it continues with the AOX2 pathway. Mut^+ strains always have a higher growth rate than Mut^S strains.

Table 7. *P. pastoris* strains and subtypes.

Strains	Subtypes
Wild-type	• Y-11430 • X-33
Different methanol usage ability	• Mut^{+} (AOX1^{+}, AOX2^{+}) • Mut^{S} (AOX1^{-}, AOX2^{+}) • Mut^{-} (AOX1^{-}, AOX2)
Protease activity deficient	• SMD1163 strain • SMD1165 strain • SMD1168 strain
Histidine dehydrogenase deficient	• GS115 (His4) • KM71 • SMD1168

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Devices

In this thesis study, Acibadem Mehmet Ali Aydinlar University Research and Development Laboratory devices were used as they listed below.

Table 8. Used devices and modals.

Name	Modal
Power supply	Biorad Universal
Shaker incubator	New Brunswick Innova 44
Hotplate stirrer	Witeg SMHS-3
Autoclave	Systec
Microbiology cabinet	Safe-2020
Fluorescent microscope	Zeiss AX10
Precision weigher	Shimadzu-AUW220D
Speed-Vac concentrator	Savant-SC250EXP
Centrifuge	Thermo SL16
Angled centrifuge	Beckman Coulter-Allegra 64R
Mini centrifuge	Thermo MicroCL17
Gel imaging system	Biorad ChemiDoc MP
Thermal cycler	Biorad T100
Electroporator	Biorad Gene Pulser Xcell
Double distilled water device	Mili-Q
Nanodrop	Thermo Nanodrop One C
Multi rotator	Biosan MultiBioRS-24
Large rotator	Thermo 3D Rotator
Microwave	Samsung TDS
Dry heater	Nüve FN120
Thermomixer heater	Eppendorf Thermomixer Comfort
Ice maker	Brema Ice Makers

Table 8. Used devices and modals. (Continued)

Name	Modal
Western blot semi-dry system	Biorad TransBlot Turbo
Elisa reader	Thermo Varioskan Flash

3.1.2 Strains and plasmid vectors

Escherichia coli DH5α, *Pichia pastoris* KM71H, *Staphylococcus aureus* ATCC 29213*, Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 cells, HepG2-HB8065 hepatocellular carcinoma cell line and plasmid vector pPICZαA were used.

3.1.3 Stock solutions, buffers and media

Stock solutions, buffers and media were prepared by following to *the Pichia pastoris* manual (Table 9) (116).

Table 9. Used stock solutions, buffers, media and their recipes.

Stock Solutions, Buffers and Media	Recipes
Super Optimal Broth (SOB) (1000 ml)	28 grams of SOB was dissolved in 1000 ml of ddH_2O and then autoclaved for 15 minutes at 121°C.
Super Optimal Broth with Catabolite repression (SOC) (1000 ml)	28 grams of SOB was dissolved in 1000 ml of ddH_20 and then autoclaved for 15 minutes at 121°C. After the cooling, 20 ml of 1 M glucose was added and sterilized by 0,22 µm filter.
Inoue Transformation Buffer (1000 ml)	55 mM $MnCl_2$ 4 H_20, 15 mM $CaCl_2$ H_20, 250 mM KCl, 10mM PIPES (pH:6,7) were dissolved in 1000 ml of ddH_20 and chilled to 0°C before use.

Table 9. Used stock solutions, buffers, media and their recipes. (Continued)

Stock Solutions, Buffers and Media	Recipes
Low Salt Luria Bertani Broth (LB) (1000 ml)	20 grams of LB broth was dissolved in 1000 ml ddH_20 and then autoclaved for 15 minutes at 121°C.
Low Salt Luria Bertani Agar with Zeocin (Invitrogen) (1000 ml)	35 grams of LB agar was dissolved in 1000 ml ddH_20 and then autoclaved for 15 minutes at 121°C. Allowed the cool to 55°C, 25 µg/ml Zeocin (Invitrogen) was added and plated to the petri dishes. Protected from the light.
Yeast Extract Peptone Dextrose Broth (YEPD or YPD) (1000 ml)	50 grams of YPD agar was dissolved in 1000 ml ddH_20 and then autoclaved for 15 minutes at 121°C.
Mueller-Hinton Broth (MHB) (1000 ml):	22 grams of MHB was dissolved in 1000 ml ddH_20 and then autoclaved for 15 minutes at 121°C.
Yeast Extract Peptone Dextrose Agar (YPD) with Zeocin (Invitrogen) (1000 ml):	65 grams of YPD agar was dissolved in 1000 ml ddH_20 and then autoclaved for 15 minutes at 121°C. Allowed the cool to 55°C, 100 µg/ml Zeocin (Invitrogen) was added and plated to the petri dishes. Protected from the light.
10X Yeast Nitrogen Base (YNB) (without Ammonium sulphate and amino acids) (1000) ml	34 grams of YNB and 100 grams of ammonium sulphate was dissolved in 1000 ml ddH_20. Filter sterilized and stored at 4°C.
BMM (Buffered Methanol Complex) Agar	4 grams of agar and 138 ml of ddH_20 were autoclaved for 15 minutes at 121°C. Then, 20 ml of potassium phosphate buffer pH:6, 20 ml of 10X YNB, 20 ml of 10X methanol, 400 µl of 500X Biotin were added before cooling and poured to the petri dishes.
500X Biotin (0,02% Biotin)	20 miligrams of biotin was dissolved in 100 ml of ddH_20 and filter sterilized. Stored at 4°C.
10X Methanol (5% Methanol)	5 ml of methanol was mixed with 95 ml of ddH_20. Filter sterilized and stored at 4°C.

Table 9. Used stock solutions, buffers, media and their recipes. (Continued)

Stock Solutions, Buffers and Media	Recipes
10X Glycerol	10 ml of glycerol was mixed with 90 ml of ddH_20 and then autoclaved for 15 minutes at 121°C.
1000X $CuSO_4$ (0,5 M $CuSO_4$)	7,98 grams of $CuSO_4$ was dissolved in 100 ml of ddH_20. Filter sterilized and stored at 4°C.
1 M Potassium Phosphate Buffer, (pH: 6) (1000 ml)	132 ml of 1 M K_2HPO_4 and 868 ml of 1 M KH_2PO_4 were combined and pH was adjusted via 1 M KOH.
1X Buffered Glycerol Complex Media (BMGY) (1000 ml)	100 ml of 1 M Potassium phosphate buffer (pH: 6), 100 ml of 10X YNB, 100 ml of 10X Glycerol, 2 ml of 500X Biotin and 698 ml of sterile ddH_20 were combined under the microbiology cabinet stored at 4°C.
1X Buffered Methanol Complex Media (BMMY) (1000 ml)	100 ml of 1 M Potassium phosphate buffer (pH:6), 100 ml of 10X YNB, 100 ml of 10X Methanol, 2 ml of 500X Biotin and 698 ml of sterile ddH_20 were combined under the microbiology cabinet stored at 4°C.
10X Tris- Borate-EDTA (TBE) Buffer (1000 ml)	108 grams of Tris base and 55 grams of Boric acid were dissolved in 900 ml of ddH_20. 40 ml of 0,5 M Na_2EDTA (pH:8) was added and volume was adjusted to 1000 ml. Stored at room temperature.
10X SDS-PAGE Running Buffer (1000 ml)	30 grams of Tris base (0,25 M), 144 grams of glycine (1,92 M) and 10 grams of Sodium dodecyl sulphate (SDS) (0,03 M) were prepared in 800 ml of ddH_20. Mixed very well and volume was adjusted to 1000 ml.
Tris-HCL 0,5 M (pH:6.8)	6 grams of Tris base was dissolved in 70 ml of ddH_20. pH was adjusted to 6,8 via HCl and volume was made up to 100 ml.

Table 9. Used stock solutions, buffers, media and their recipes. (Continued)

Stock Solutions, Buffers and Media	Recipes
Tris-HCL 1,5 M (pH:8.8)	18 grams of Tris base was dissolved in 70 ml of ddH_20. pH was adjusted to 8,8 via HCl and volume was made up to 100 ml.
2X Urea Sample Buffer (120 mM Tris-HCl, pH:6.8, 4% SDS, 4 M Urea, 20% Glycerol)	4,8 ml of Tris-HCl, 16 ml of 10% SDS, 9,6 grams of urea, 8 ml of glycerol and 40 mg of bromophenol blue was combined into the 40 ml of ddH_20.
60% Glycerol	60 ml of glycerol was mixed with 40 ml of ddH_20 and autoclaved autoclaved for 15 minutes at 121°C.
SDS-PAGE Fixing Solution (6 water: 3 ethanol: 1 acetic acid ratio)	60 ml of ddH_20, 30 ml of ethanol and 10 ml of acetic acid were mixed and stored at room temperature.
SDS-PAGE Wash Solution	10 ml of ethanol was mixed with 90 ml of ddH_20 and stored at room temperature.
SDS-PAGE Stop Solution	5 ml of acetic acid was mixed with 95 ml of ddH_20 and stored at room temperature.
10% SDS	10 grams of SDS was dissolved in 100 ml of ddH_20 and filter sterilized.
Towbin Transfer Buffer	3 grams (25 mM) of Tris base and 14 grams (192 mM) of glycine were mixed and stored at room temperature.
10X Tris-Buffered Saline (TBS) Solution:	24 grams of Tris base and 88 grams of NaCl were dissolved in 900 ml of ddH_20. pH was adjusted to 7,6 via 12N HCl.
10X Tris-Buffered Saline with Tween-20 (TBS-T)	400 µl (0,5%) of Tween-20 was added into the TBS and mixed.

Table 9. Used stock solutions, buffers, media and their recipes. (Continued)

His-Tag Purification Equilibration Buffer (pH:7.4)	20 mM of sodium phosphate, 300 mM of sodium chloride and 10 mM of imidazole were mixed and stored at room temperature.
His-Tag Purification Wash Buffer (pH:7.4)	20 mM of sodium phosphate, 300 mM of sodium chloride and 25 mM of imidazole were mixed and stored at room temperature.
His-Tag Purification Elution Buffer (pH:7.4)	20 mM of sodium phosphate, 300 mM of sodium chloride and 250 mM of imidazole were mixed and stored at room temperature.
Coomassie Colloidal Staining (For overnight stains)	120 grams of (10%) citric acid, 80 grams of (8%) ammonium sulphate, 0,8 grams of (0,08% Coomassie Brilliant Blue G250) were dissolved into the 800 ml of ddH_20. 200 ml of 20% methanol was added before use. For 1 gel; 10 ml of methanol, 40 ml of colloidal stain were used.

3.2 Methods

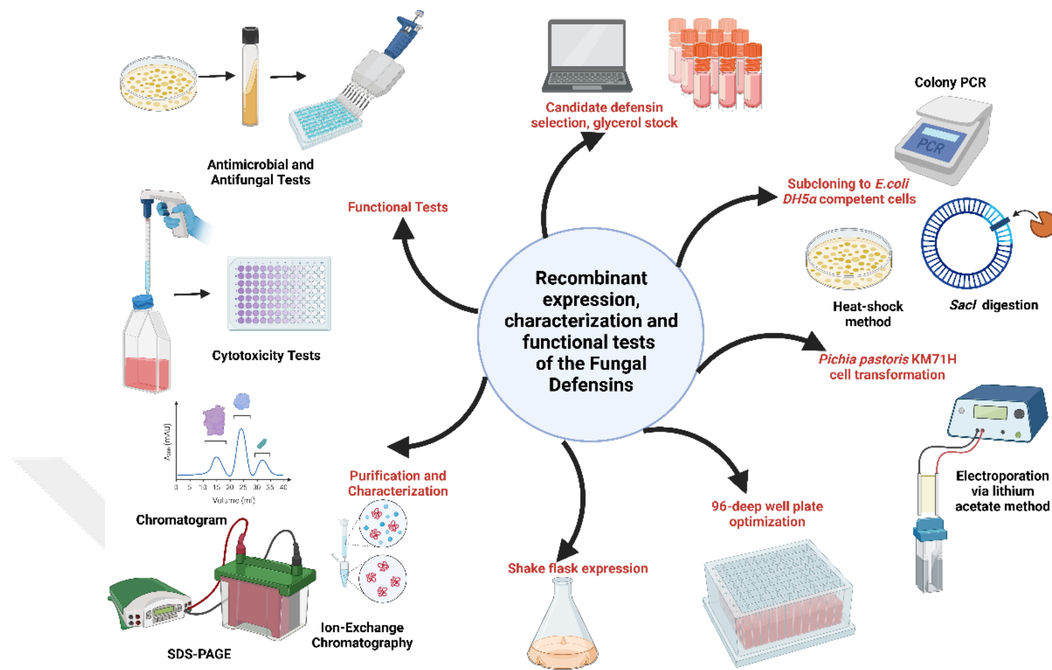


Figure 10. Experimental set-up from cloning to functional tests. ("Created with BioRender.com").

3.2.1 Candidate peptide selection, cloning and transformation

3.2.1.1 Candidate selection and gene synthesis

Candidate peptide selection and physicochemical analysis were done with BLAST (117), UniProt (118), ExPASy ProtParam (119) and Protscale (120) tools. There was some parameters to selection; instability index, grand average of hydropathicity (GRAVY) index, aliphatic index, theoretical PI, estimated PI and net charge respectively. Lower instability index was a critical point to peptide stability. However, homolog modelling was done with I-TASSER (121) which is known as Threading Assembly Refinement to predict 3-D model of the peptides.

Gene synthesis of peptides were provided by Gene Universal which is selected with codon optimization. pPICZαA expression vector was used with His-Tag which is a commonly used vector for *Pichia pastoris*. Synthetic genes were came into pPICZαA

vector and cloning was done between *EcoRI* and *NotI* regions. α-factor was selected as a secretion signal under the Alcohol oxidase promoter (AOX1 TT).

3.2.1.2 *E.coli DH5α* competent cell preparation

Single bacterial colony was collected from plate and incubated at 37°C for 16 hours. Then transferred into 25 ml of SOB medium in 250 ml of flask. Culture was incubated for 6 hours at 37°C 180 rpm. After the incubation, cells were taken to 250 ml of SOB media containing flasks as a 10 ml, 4 ml and 2 ml to incubation. The next day, OD_{600} values were measured for every 45 minutes. When OD_{600} close to 0,55 culture was taken to ice. Culture was centrifugated at 2,500 x g for 10 minutes at +4 °C. Supernatant was discarded and cells were resuspended gently in 80 ml of pre-chilled Inoue transformation buffer. Cells were centrifugated at 2,500 x g for 10 minutes at +4 °C again. Then pellets were resuspended in 20 ml of pre-chilled Inoue transformation buffer.1,5 ml of DMSO was added and kept on ice for 10 minutes. Finally they were aliquots into 1,5 ml of eppendorfs and stored at -80°C freezer.

3.2.1.3 *E.coli DH5α* transformation via heat shock method

Synthetic F2T142, F2T142Mut, D6RKI7 and RodA genes containing lyophilized vectors were dissolved into 50 µl of sterile ddH_20 as a 100 ng/µl. For plasmid propagation, heat shock method was used. 1 µl of plasmid DNA samples were put into the *E.coli DH5α* competent cells as a 5% volume of the competent cell and eppendorfs were incubated on ice for 30 minutes. Tubes were transferred to preheated 42°C heat block for 90 seconds and did not shaked. After the heat shock step, samples were transferred to ice rapidly for 1-2 minutes. 800 µl of SOC (20mM glucose containing) media was added and incubated at shaker incubator at 37°C for 2 hours to allow recovery of bacteria and express antibiotics resistant genes. Then, transformed competent cells were spreaded to LB Zeocin (25 µg/ml) containing agar plates by using 5 mm glass beads. Plates were protected from light and incubated at 37°C for 12-16 hours (122).

3.2.1.4 Colony PCR verification

E.coli DH5α positive clones were verified by colony PCR. Small parts of colonies from each sample were taken from LB Zeocin agar plates (25 µg/ml) and spreaded to eppendorfs. Then, PCR mixture was prepared and portioned as a 10 µl. 5'AOX (5'AOX: 5'- GACTGGTTCCAATTGACAAGC-3'; 3' AOX: 5'- GCAAATGGCATTCTGACATCC-3' Ta: 54°C) primers were used at PCR reaction. pPICZαA (empty vector) was used as a positive control, ddH$_2$0 was negative control. After PCR, visualization were provided by agarose gel electrophoresis. 50 ml of 1.2% agarose gel was prepared with 1µl of 10.000X Invitrogen SYBR Green Safe dye. Running conditions were 1 hour at 100V. Image was obtained with ChemiDoc MP (123).

Table 10. PCR reaction ingredients and used volumes.

PCR Mixture Ingredients	**Volume (µl)**
F primer (5'AOX) (10mM)	0,5
R primer (3'AOX) (10 mM)	0,5
2X Taq PCR Master Mix	5
ddH$_2$0	3
Template DNA	Whole cell
Total Volume	**9**

Table 11. Optimized PCR conditions.

Steps	**Time**	**Degree (°C)**	**Number of Cycles**
Initial denaturation	5 minutes	94	1
Denaturation	30 seconds	95	
Annealing	1 minute	54	34
Extension	1 minute	72	
Final extension	10 minutes	72	1

Table 12. *SacI* digested plasmid agarose gel verification ingredients and volumes.

Sample	Volume (µl)
1 kb GeneRuler DNA Ladder	3,5
Sample mixture	
6X Loading Dye	2
SacI digested plasmid	5

3.2.1.5 MiniPrep plasmid isolation

To make MiniPrep Plasmid Isolation, 1 colony from each LB Zeocin containing plates were taken into 5 ml of LB broth. Cultures were incubated into 180 rpm shaker incubator at 37°C for 18-24 hours. The next day, Qiagen MiniPrep Isolation Kit was followed. According to protocol, cultures were centrifugated at 10,000 rpm for 5 minutes and pellets were resuspended with 250 µl P1 buffer. 250 µl of P2 buffer was added and tubes gently inverted 4-6 times until solution becomes clear. 350 µl of N3 buffer was added and gently inverted again 4-6 times. After this step, samples were centifugated at 13,200 rpm for 10 minutes. Supernatants were transferred to QiaPrep Spin Colums. 500 µl of PB buffer, 750 µl of PE buffer were added and centrifugated at 13,200 rpm for 1 minutes. Lastly, columns were transferred to 1.5 ml of eppendorfs and plasmid DNA were eluted via 20 µl of sterile ddH_20 with two steps (124).

3.2.1.6 Plasmid linearization via *SacI* and agarose gel imaging

Plasmids which are obtained from MiniPrep were linearized via *SacI* restriction enzyme (*SacI* 10U/µl) and incubated at 37°C as overnight at heat block for 16 hours. Then, linearization was detected with agarose gel electrophoresis. Gel was prepared as a 1,2% and from 1X TBE buffer and 10.000X Invitrogen SYBR Green Safe dye. Ingredients and volumes were shown at (Table 12).Samples were run for 45 minutes at 100V. Visualization was done with ChemiDoc MP (125).

Table 13. *SacI* digestion ingredients and calculations.

Ingredients	F2T142	F2T142	F2T142 Mut	F2T142 Mut	D6RKI7	D6RKI7	RodA	RodA
SacI (10U/µl)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
10X *SacI* Buffer	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl
ddH_20	19,82 µl	20,98 µl	18,35 µl	19,82 µl	19,40 µl	17,73 µl	22,03 µl	12,52 µl
Plasmid DNA	15,18 µl	14,02 µl	16,65 µl	15,18 µl	15,60 µl	17,27 µl	12,97 µl	22,48 µl
Total Volume	40 µl	40 µl	40 µl	40 µl	40 µl	40 µl	40 µl	40 µl

SacI digested plasmids were purified with Qiagen "Qiaquick PCR Purification using a Microcentrifuge" protocol. First of all, plasmids were washed with 1/5 PB buffer, mixed and transferred to QiaQuick spin colums. There, samples were centrifugated for 1 minute at 13,200 rpm and 750 µl PE buffer was added. Centrifuge step was repeated and lastly, an extra centrifuge step was done to remove remains. Elution was done with two step with 20 µl of sterile ddH_20. Concentrations were measured by using NanoDrop OneC (126).

3.2.1.7 *Pichia pastoris KM71H* transformation via electroporation and lithium acetate method

Competent *Pichia pastoris* KM71H cells were placed on ice and thawed. 3 µg of plasmids were put and incubated for 5 minutes on ice. Meanwhile, 2 mm electroporation cuvettes were placed to ice for cooling. Then, samples were taken to electroporation cuvettes and incubated on ice for 3-4 minutes. Electroporation parameters were set as a 1,5 kV, 25 µF and 25 Ohm. After the electroporation, 1 ml of 1 M ice-cold sorbitol was immediately added. Cells which were taken to eppendorfs were incubated for 2 hours at 30°C incubator. Lastly, cells were spreaded to YPD Zeocin agar plates (100 µg/ml) with 5 mm 2 times autoclaved glass beads as three different concentrations; 100 µl original, 1/10 diluted and 1/100 diluted. Plates were incubated at 30 °C for 72 hours (127).

3.2.1.8 Microwave colony PCR verification and replatings

Microwave Colony PCR verification was done to verify *Pichia pastoris* KM71H positive transformants. This method was aimed to burst yeast and spread to DNA by the help of temperature changing. 8 colonies parts of each samples were taken from YPD Zeocin agar plates (100 µg/ml) and spreaded to 0,2 ml of eppendorfs. Then, samples were baked at microwave oven at maximum degrees for 2 minutes. Samples were taken immediately on ice and 50 µl of sterile ddH_20 was added to each tubes. That were used as a DNA template. 5'AOX (5'AOX: 5'-GACTGGTTCCAATTGACAAGC-3'; 3' AOX: 5'-GCAAATGGCATTCTGACATCC-3' Ta: 54°C) primers were used at PCR reaction. pPICZαA (empty vector) was used as a positive control, $ddH_20$was negative control. PCR conditions and ingredients were listed at Table 10 and Table 11. Only template DNA was changed as different from *E. coli* colony PCR verification by cooking at maximum degree at microwave. After PCR, visualization was provided by agarose gel electrophoresis. 150 ml of 1,2% agarose gel was prepared with 2,5 µl of 10.000X Invitrogen SYBR Green Safe dye. Running conditions were 1 hour at 100V. Image was obtained with ChemiDoc MP (123).

Table 14. Sample mixture volume and ingredients to agarose gel electrophoresis.

Sample	**Volume (µl)**
1 kb GeneRuler DNA Ladder	3,5
Sample mixture	
6X Loading Dye	2
Sample	5

When colonies were verified, each of them were streaked to the YPD Zeocin (100 µg/ml) agar plates every three days to obtain best single colony continuously.

3.2.2 Expression

Samples were expressed firstly 96-deep well plates to select the best productive transformants, production capacity. Then, they were expressed into baffled-shake flasks.

3.2.2.1 96-deep well plates expression

8 colonies of F2T142, F2T142Mut, D6RKI7 and RodA were put into 5 ml of sterile YPD broth to start 96-deep well expression and samples were incubated at 30°C, 180 rpm shaker incubator as an overnight. The next day, contamination was checked under the light microscope and then samples were centrifugated for 5 minutes at 5,000 rpm. Supernatants were discarded and 5 ml of 2X BMG media was added on pellet to dissolve. Finally, cells were vortexed and incubated again at 30°C, 180 rpm shaker incubator as an overnight. The third day, samples were diluted as a 1/50 and OD_{600} values were recorded to make 60 OD (Final 30 OD/ml). Cells were centrifugated at 5000 rpm for 10 minutes and supernatants were discarded. Then, pellets were dissolved into 4 ml of 2X BMM media. Calculated amount of cells were put into sterile glass beads containing 96-deep well plate and final volume was fixed to 1 ml. To obtain T=0 (0 hour) samples, plate was centrifugated at 5000 rpm for 10 minutes and then 50 µl of supernatants were taken from each of them to store -20°C. In that point, expression was started and culture was continued to 48 hours. The next day, T=1 (24 hours) samples were collected by centrifuge and 20,8 µl of 20% filtered methanol was added to each wells induce expression. At the last day of expression, samples were centrifugated at 5,000 rpm for 10 minutes. T=2 (48 hours) samples were collected and all supernatants were taken to eppendorfs to keep -20°C (116).

Table 15. 96-Deep well expression ingredients and used volumes for BMG and BMM.

Materials	**2X BMG (ml)**	**2X BMM (ml)**
1M Potassium phosphate buffer, pH: 6	100	100
10X YNB	100	100
10X Glycerol	100	-
10X Methanol	-	100
500X Biotin	2	2
Sterile ddH_20	198	198
$CuSO_4$	-	0,5
Total Volume	500	500

3.2.2.2 Baffled shake flask expression

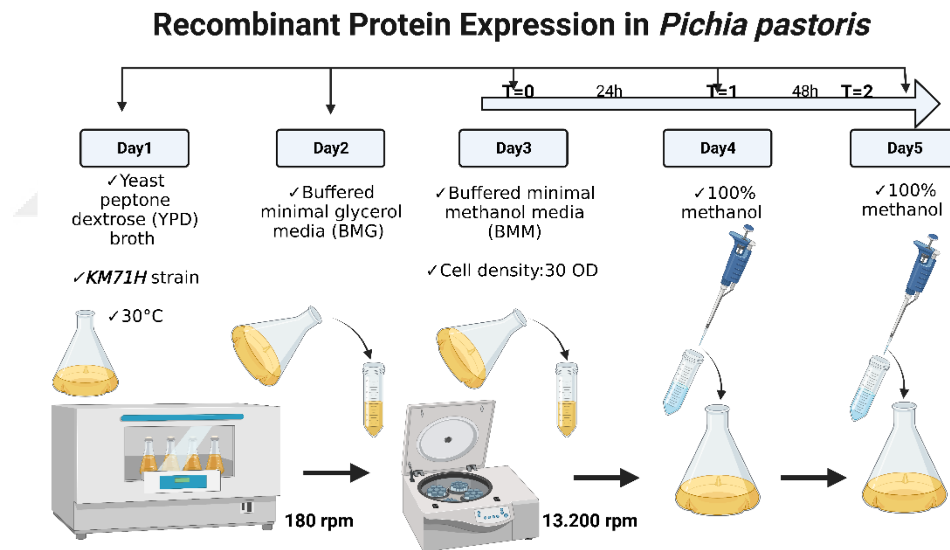


Figure 11. Recombinant protein expression timeline in *Pichia pastoris*. ("Created with BioRender.com").

According to 96-deep well plate expression results which have high production capacity colonies were put 50 ml of YPD broth into baffled flask and samples were incubated at 30°C, 180 rpm shaker incubator as an overnight. The next day, contamination was checked under the light microscope and then samples were centrifugated for 5 minutes at 5,000 rpm. Supernatants were discarded and 50 ml of 1X BMG media was added on pellet to dissolve. Finally, cells were vortexed and

incubated again at 30°C, 180 rpm shaker incubator as an overnight. The third day, samples were diluted as a 1/50 and OD_{600} values were recorded to make 60 OD (Final 30 OD/ml). Cells were centrifugated at 5,000 rpm for 10 minutes and supernatants were discarded. Then, calculated amounts of pellets were dissolved into 50 ml of 1X BMM media. To obtain T=0 (0 hour) samples, plate was centrifugated at 5,000 rpm for 10 minutes and then 1 ml of supernatants were taken from each of them to store -20°C. In that point, expression was started and culture was continued to 48 hours. The next day, T=1 (24 hours) samples were collected by centrifuge and 1 ml of 100% filtered methanol was added to each wells induce expression. At the last day of expression, samples were centrifugated at 5000 rpm for 10 minutes. T=2 (48 hours) samples were collected and all supernatants were taken to eppendorfs to keep -20°C (116).

Table 16. Shake flask expression ingredients and used volumes for BMG and BMM.

Materials	2X BMG (ml)	2X BMM (ml)
1M Potassium phosphate buffer, pH: 6	50	50
10X YNB	50	50
10X Glycerol	50	-
10X Methanol	-	50
500X Biotin	1	1
Sterile ddH_20	349	349
Total Volume	500	500

3.2.3 Biochemical characterization

3.2.3.1 BCA assay

BCA assay was done to detect total protein amounts. Standards were prepared according to Thermo Pierce Protein BCA Assay Kit manual (2,000 µg/ml, 1,500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 25 µg/ml, 0 µg/ml). 25 µl of each standards and samples were pipetted and 200 µl of working solution was added into 96 well plate. 1X BMM and ddH_20 were used as a blank. Then, plate was

incubated for 30 minutes at 37°C at dark. Scanning was done with VarioSkan at 562 nm (128).

Table 17. BCA assay test tube protocol and volumes.

Dilutions for Standard Test Tube Protocol (Working Range = 20-2,000 µg/ml)			
Vial	**Diluent Volume (µl)**	**Volume of BCA (µl)**	**Final BCA Conc. (µg/ml)**
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of stock	750
E	325	325 of stock	500
F	325	325 of stock	250
G	325	325 of stock	125
H	400	100 of stock	25
I	400	0 of stock	0 (Blank)

3.2.3.2 Tris-glycine SDS-PAGE

Peptides were run with Tris-Glycine SDS-PAGE protocol to detect biochemical characterization. SDS-PAGE is an electrophoretic method to separate proteins according to their molecular weights under the electrical field. SDS is a kind of detergent and denature the proteins via negative net charge. Stacking gel provides the entrance of the proteins to the resolving gel with the same line. Resolving gel separates the proteins according to molecular weights. Because of the lower concentration of the stacking gel (4%), pores are bigger than resolving gel so, separating effect is little.

Gels were prepared according to Table and poured 1 mm thick SDS-PAGE glasses. When the gels were polymerized, samples were prepared. 2X urea sample buffer was contained 1/3 amounts of B-mercaptoethanol. Samples were mixed as a 1:1 with that buffer and boiled at 95°C for 5 minutes at heat block. Then, tubes were immediately taken and samples were loaded to wells as a 20 µl of total. 3,5 µl of unstained protein ladder (Fermentas- Thermo Fischer) were used. Running conditions were 100 volt for 2 hours.

Table 18. SDS-PAGE gel ingredients and volumes.

Ingredients	Stacking Gel (4%)	Resolving Gel (16%)
30% Acrylamide-Bisacrylamide (29:1)	1,98 ml	7,5 ml
0,5 M Tris-HCl pH: 6,8	3,78 ml	-
1,5 M Tris-HCl pH: 8,8	-	3,75 ml
10% SDS (w/v)	150 µl	150 µl
Sterile ddH_20	9 ml	3,53 ml
TEMED	15 µl	7,5 µl
10% APS (w/v)	75 µl	75 µl
Total Volume	15 ml	15 ml

Visualization was done with silver staining method by following Thermo Pierce Silver Stain Kit. Gels were washed for two times with ddH_20 and fixed by using 6 ddH_20: 3 ethanol: 1 acetic acid ratio for 15 minutes. Then, 10% ethanol, water washings were done. Sensitizer working solution (25 ml of ddH_20, 50 µl of silver stain sensitizer), stain working solution (25 ml of silver stain, 0,5 ml of silver stain enhancer) were added step by step. Lastly, developer working solution (25 ml of developer, 0,5 ml of enhancer) was added and two or three minutes shaked until bands were appeared. 5% acetic acid was used to stop reaction for two times 15 minutes. Visualization was done via ChemiDoc MP (129).

3.2.3.3 Yeastern blot

BMM agar plates were cut into equal squares, 8 small dots were taken from third replating colonies is to 30°C. pPICZαA was negative control. After 24 hours, PVDF membrane and filter papers were cut the similar size of agar plate square. Membrane was activated for 15 seconds with the 15 ml of 100% isopropanol. Then, filter papers and membrane were soaked into transfer buffer for 5 minutes. The membrane and filter papers were neatly placed over the colonies by using sterile tweezers. Half full 500 ml of bottle was also placed on them and 4 hours incubated at room temperature to manual transfer. After the transfer, membrane was washed with 15 ml of 1X TBS for 5 minute and twice. To membrane blocking, 20 ml of 5% skim milk was added on membrane and incubated for 1 hour. Before antibody treatment, membrane was washed with 15

ml of 1X TBS-T for 5 minutes and antibody incubation were done for overnight at +4°C (1:2000 Novex Anti-His C Term AP/Ab antibody into TBS containing 0,1% (v/v) Tween-20 and 1% (w/v) non-fat skim milk). The next day, membrane was washed with 15 ml of 1X TBS-T for four times and 1 ml of 1-Step NBT/BCIP substrate solution was added on membrane gently at dark and incubated for 10 minutes. Visualization was done via ChemiDoc MP (130).

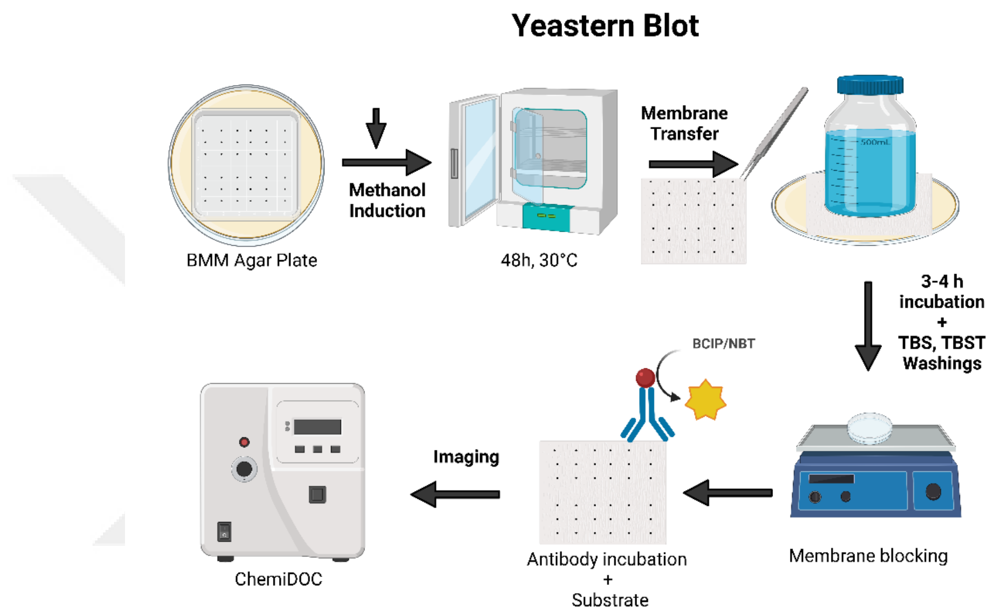


Figure 12. Yeastern blot steps to characterization. ("Created with BioRender.com").

3.2.3.4 Semi-dry western blot

Tris-Glycine SDS-PAGE protocol was followed to running of samples and gel was incubated with Towbin transfer buffer for 15 minutes. Filter papers were soaked with Towbin buffer as a one bottom one top. PVDF membrane was activated with 100% of methanol for 30 seconds. Transfer components were placed into transfer cassette TransBlot. First of all, one filter paper was placed, then PVDF membrane, SDS gel and finally the last filter paper were placed on system. In each step, roller was used to eliminate bubbles. Running was started with 1 Amper, 25V for 20 minutes. After the transfer, membrane was washed with TBS-T for 5 minutes. This step was repeated for three times to eliminate gel particles. Membrane was blocked with TBS-T containing 5% non-fat skim milk for 1 hour. Primer incubation was done as a

overnight at +4°C with 1:2000 diluted Novex Anti-His C Term AP/Ab antibody into TBS containing 0,1% (v/v) Tween-20 and 1% (w/v) non-fat skim milk. The next day, membrane was washed with 1X TBS-T for 10 minutes, three times. 1 ml of 1-Step NBT/BCIP substrate solution was added on membrane gently at dark and incubated for 10 minutes. Visualization was done via ChemiDoc MP (131).

3.2.4 Purification

Recombinantly produced fungal peptides were purified via His-Tag purification method. Poly-Histidine label is generally founded at last and consisted from 6-Histidine. The motif, provide the purification of proteins by affinity. Resin is mostly formed from sepharose or agarose and becomes functional with nitriloacetic acid (Ni-NTA). Ni-NTA is bound to 6-His via affinity and addition of imidazole is a way to increase yield. After purification, proteins are visualized by SDS-PAGE.

In this study, 1 ml of resin was added into 15 ml of falcon tubes and centrifugated for 2 minutes at 700 x g, supernatant was carefully discarded. Two-resin bed volume of equilibration buffer was added and mixed until resin fully resuspended. Falcons were centrifugated again for 2 minutes at 700 x g. Samples were prepared as a 4X volume with equilibration buffer as a totally 1 ml and placed on end-over-end rotator for 30 minutes. Then, centrifuge step was done again with the same conditions, supernatants were collected to downstream analysis as a flow through. Resins were washed with two resin-bed volume of wash buffer for 3 times, 2 minutes at 700 x g. Supernatants were saved. Lastly, samples were eluted with one resin-bed volume of elution buffer for three times and two minutes at 700 x g. Each of the fractions were collected to SDS-PAGE analysis (132).

3.2.5 Functional tests

3.2.5.1 Antimicrobial tests

Antimicrobial activity tests are the test which applied to determine the effectiveness of any antibacterial agent certain bacterial species. Here the purpose is, a new candidate drug can act in a broad spectrum with minimum adverse effect and this can be achieved with the minimal dose. By means of various antimicrobial activity tests, whether these candidate drugs are effective against pathogenic microorganisms and their Minimal Inhibition Concentration (MIC) are determined. The minimal inhibition concentration is the lowest concentration which affect against microorganisms. The determination can be made in a short time and at low cost by various methods such as agar dilution, liquid microdilution and liquid microdilution. The method which is used in this thesis was the liquid microdilution method. This method basically based on growing the microorganism strain to be tested overnight in suitable liquid culture, then seeding the next day into 96-well plates with the number of cells which are equivalent to 0,5 McFarland standard. Then, different concentrations of antimicrobial agents are applied and OD_{600} value is determined for 24 hours. The tested strains are as follows;

- *Escherichia coli (E. coli)* ATCC 25922
- *Staphylococcus aureus (S. aureus)* ATCC 29213
- *Pseudomonas aeruginosa (P. aeruginosa)* ATCC 27853

LB liquid culture was used for gram negative *E. coli.* MH liquid culture was used for gram positive *S. aureus* and *P. aeruginosa*. Their growth temperatures were 37°C and biosafety levels were 2. Antimicrobial candidates were prepared via serial dilution. Concentrations of recombinant peptides are 240 µg/ml, 120 µg/ml, 60 µg/ml, 30 µg/ml, 15 µg/ml, 7,5 µg/ml, 3,75 µg/ml, 1,87 µg/ml, 0 (blank) respectively.

Strains were grown into 5 ml of suitable liquid media as overnight at 37°C, 180 rpm shaker incubator. The next day, OD_{600} values were determined and cell suspensions

were prepared as equal 0,5 McFarland standard which have $5x10^5$ cells/ well. 50 µl cell, 50 µl candidate peptide and 100 µl of liquid media were added into 96-well plates as a triplicated. Microorganism containing wells were negative control, liquid media containing wells were blank. Lastly; 0, 3, 18 and 24 hours $0D_{600}$ values were read via VarioSkan (133).

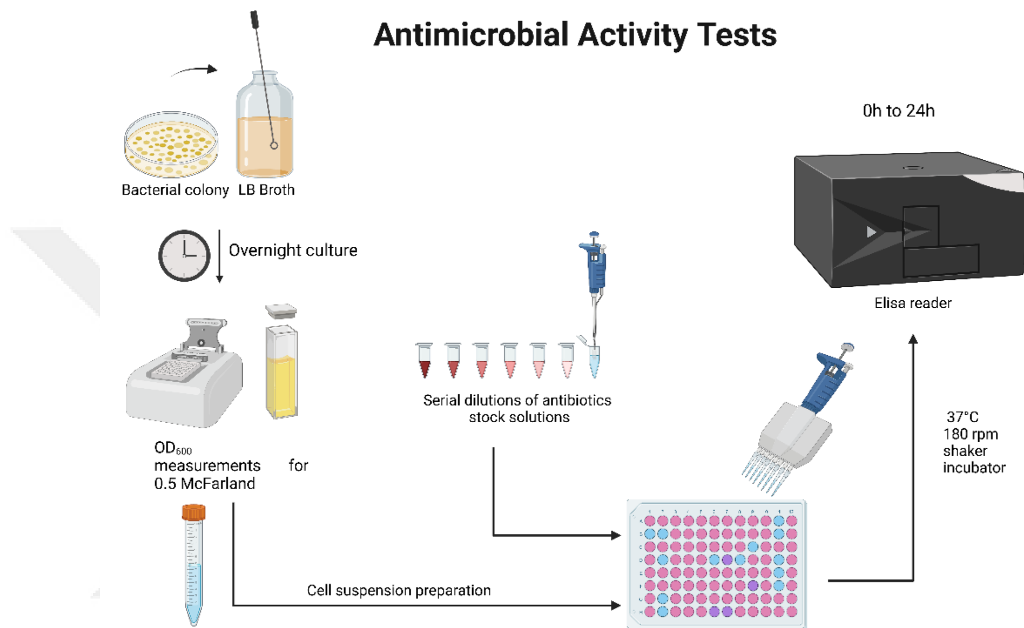


Figure 13. Antimicrobial activity test set-up and steps. ("Created with BioRender.com").

3.2.5.2 Antifungal tests

Antifungal activity test is a test which used to determine the effectiveness of the candidate antifungal agent against a particular fungal strain. From past to present, the frequency of fungal infections and their negative impact on quality of life have brough the use of antifungal drugs along with it rapidly. However, drugs that are used unnecessarily and strains develop resistance, except for doctor's advice, have reduced the effectiveness of these drugs over time and have led to the search for new antifungal drug treatments. At this point, antifungal susceptibility tests have great importance to determine the spectrum of action and to find the Minimal Inhibition Concentration. At this purpose, different concentrations of antifungal agents are applied to fungal strains.

Afterwards, appropriate doses are determined by measuring OD_{600} nm for 24 and 48 hours. *Candida albicans* ATCC 90028 strain was used to determine antifungal activity. Suitable liquid medium for this strain is YPD broth with an optimal growth temperature of approximately 30-35°C. Biosafety working level is 1.

Antimicrobial candidates were prepared via serial dilution. Concentrations of recombinant peptides are 240 µg/ml, 120 µg/ml, 60 µg/ml, 30 µg/ml, 15 µg/ml, 7,5 µg/ml, 3,75 µg/ml, 1,87 µg/ml, 0 (blank) respectively. Strains were grown into 5 ml of YPD liquid media as overnight at 35°C, 180 rpm shaker incubator. The next day, OD_{600} values were determined and cell suspensions were prepared as equal 0,5 McFarland standard which have $1x10^5$ cells/ well. 100 µl of *C. albicans* culture and 100 µl of liquid media with peptide were added into 96-well plates as a triplicated. Microorganism containing wells were negative control, YPD media containing wells were blank. Lastly; 0, 3, 18, 24 and 48 hours OD_{600} values were read via VarioSkan (134).

3.2.5.3 Cytotoxicity test

WST-8 test is a colorimetric method which used to determine cell viability and proliferation to observe cytotoxicity. This method is based on the principle of reducing WST-8 tetrazolium salts by cellular dehydrogenases to from orange colored formazan. The amount of formazan is related directly number of viable cells. After incubation, absorbance is measured at 460 nm.

HepG2 ATCC HB-8065 hepatocellular carcinoma cells were opened from DMSO stock and first of all were taken to T-25 flask. After the expected growth was achieved, the cells were divided into T-75 flasks and they were expected to reach appropriate number of cells to experiment. The cells were detached from flask by using 3 ml of 0,05% of Trypsin-EDTA which is a proteolytic enzyme used to adherent cells. Then, centrifugated at 100 g for 7 minutes. After centrifugation, cells previously optimized as 10.000 cells/well were distributed in 100 µl to each well and incubated for 24 hours at 37°C for 24 hours 5% CO_2. The next day, media was removed from the wells gently

via micropipette and appropriate doses of peptides were prepared into 100 µl of RPMI-1620 media. Then, peptides were applied and incubated again for 24 hours at 37°C 5% CO_2 incubator. Finally, 10 µl/well of WST-8 solution was added to each well and incubated at 37°C 5% CO_2 without contacting light. 0, 1, 2, 3, 4 hour measurements were taken at 460 nm by using VarioSkan (135).

4 RESULTS

4.1 Candidate Peptide Selection, Cloning And Transformation

4.1.1 Candidate selection and gene synthesis

Candidate peptides were selected from UniProt database according to their instability index, GRAVY index, aliphatic index, theoretical isoelectric point and net charge parameters and signal sequences were eliminated during selection (Table 19. Candidate peptide selection parameters.

Table 19. Candidate peptide selection parameters.

Name	Instability Index	GRAVY Index	Aliphatic Index	Theoritical PI	Net Charge
F2T142 with signal sequence	53,49	0,049	60,63	9,23	5,7
F2T142 without signal sequence	82,92	-0,927	21,35	9,64	5,8
F2T142Mut with signal sequence	45,11	0,129	61,61	9,18	4,7
F2T142Mut without signal sequence	69,03	-0,828	21,94	9,35	4,8
D6RKI7 with signal sequence	47,37	0,167	88,86	5,10	-14
D6RKI7 without signal sequence	48,94	0,118	88,47	5,04	-15
RodA with signal sequence	35,12	0,245	102,52	5,17	-2,1
RodA without signal sequence	38,33	0,048	99,65	4,87	-3,1

F2T142 was selected from *Trichophyton rubrum*, F2T142Mut was selected from *Trichophyton equinum*, D6RKI7 was selected from *Coprinopsis cinerea* and RodA was selected from *Aspergillus fumigatus* (Table 20).

Table 20. General information about selected proteins.

Name	UniProt ID	Organism	Sequence
Uncharacterized F2T142	559305	*Trichophyton rubrum*	QCDASCRREGYTGGTCLKNAGFSYCACRGARPPGRR**R**
Uncharacterized F2T142Mut	559882	*Trichophyton equinum*	QCDASCRREGYTGGTCLKNAGFSYCACRGARPPGRR
Uncharacterized D6RKI7	240176	*Coprinopsis cinerea*	ASTVPGCYAECLEKAATAIGCAADDIECIKASSQFTTIVGEC VASGCTALAPGSAADADSITATFNLLSGLGLVDAADADFSA ADILEERDLTGLSRVLPVEKRQ**SCPTRRGLCFTSGLAACRA HCRGCHLGSNGRDCVRCPNG**AQCTGVIGQTCTCLNPCPPE GEIATFDVLYIPELWSAFMLSFQPYHQIAIIRKAELKPLIGNDP VAFNPNTVKHARSCPNAVPMSLDEPTNEVHEEPLVQGPARR FDEWPREVACELIVLDRLVPMVSLFGVEEAQEGVRFLFVKG LGATSPFPDLTVDEITSIIVQKLLFRNVNGFNGFRLFELKTGG SPDSHSSVEGSIYRRILQYLLSTLTMKLSTSLLAIVAVASTFIG NALSATTVPGCFAECIDKAAVAVNCAAGDIDCLQASSQFATI VSECVATSDCTALSPGSASDADSINKTFNILSGLGFIDEADAF SAADVPEERDLTGLGRVLPVEKRQNCPTRRGLCVTSGLTAC RNHCRSCHRGDLYMKPKG
RodA	33087	*Aspergillus fumigatus*	LPQHDVNAAGNGVGNKGNANVRFPVPDDITVKQATEKCGD QAQLSCCNKATYAGDVTDIDEGILAGTLKNLIGGGSGTEGL GLFNQCSKLDLQIPVIGIPIQALVNQKCKQNIACCQNSPSDAS GSLIGLGLPCIALGSIL

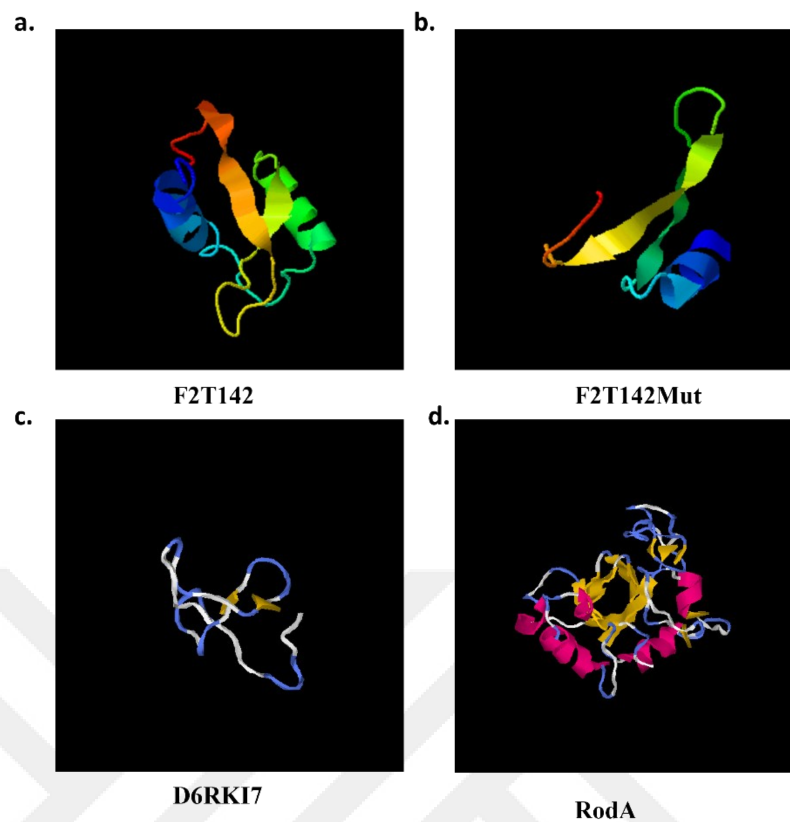


Figure 14. I-TASSER 3-D modals of the candidate peptides (a. F2T142, b. F2T142Mut, c. D6RKI7, d. RodA)

4.1.2 *E.coli* DH5α transformation via heat shock method

Synthetic genes which are provided by Gene Universal were came into *P. pastoris* pPICZαA plasmid vector. That genes were subcloned into *E. coli DH5a* competent cells via heat shock transformation method. Cells were spreaded via 0.2 mm glass beads on low-salt Zeocin containing (25 µg/ml) LB agar plates. Zeocin resistant colonies were shown at Figure 15. Colony selection was based on single colonies.

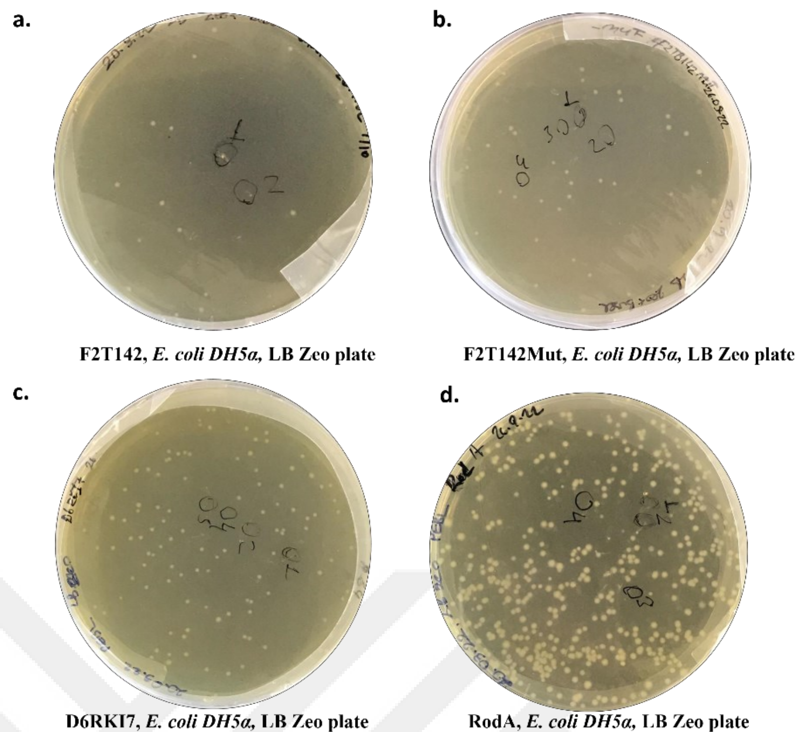


Figure 15. Low salt LB agar (Zeocin 25 µg/ml) *E. coli DH5α* transformation plates (a. F2T142, b. F2T142Mut, c. D6RKI7 d. RodA).

4.1.3 Colony PCR verification

Positive transformants were verified by colony PCR. 5'AOX and 3'AOX primers were used to amplification. Samples were run at 1,2% agarose gel within Tris-borate EDTA (TBE) buffer. Empty vector pPICZαA was used as a positive control. According to that, the empty vector pPICZαA with 588 base pairs, was run further back than the synthetic gene containing samples (588 bp + insertion) which confirms that positive transformants (Figure 16).

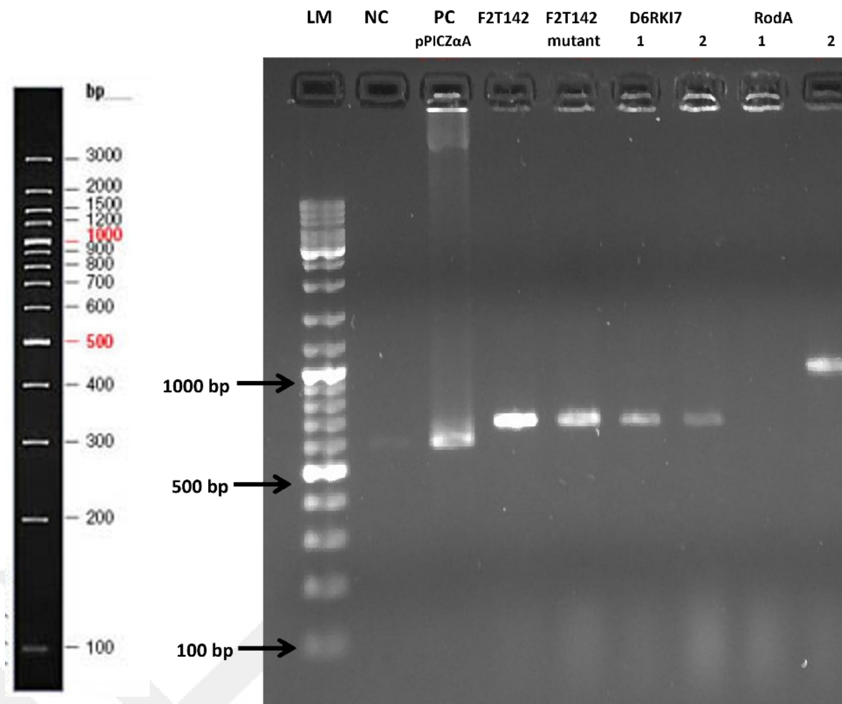


Figure 16. *E.coli* DH5α transformation verification via agarose gel electrophoresis. (LM: GeneRuler 1kb Ladder Mix, NC: Negative control, PC: Empty pPICZαA vector as a positive control, F2T142, F2T142Mut, D6RKI7 and RodA samples)

4.1.4 MiniPrep plasmid isolation

Transformed colonies were cultured overnight into LB broth at 37 °C to plasmid isolation. Qiagen MiniPrep plasmid isolation kit were used during isolation. Concentrations, A260/280 and A260/230 values were measured by NanoDrop1C. Results showed that the concentrations high and samples do not contain any contaminants (Table 21).

Table 21. MiniPrep plasmid isolation results.

Name	Concentration (ng/µl)	A260/280	A260/230
F2T142	232,3	1,97	2,1
F2T142	197,5	1,92	2,3
F2T142	213,9	1,91	2,1
F2T142Mut	176,8	1,90	2,17
F2T142Mut	180,1	1,91	2,2
F2T142Mut	197,6	1,85	1,9
D6RKI7	235,2	1,96	2
D6RKI7	192,2	1,90	2,2
D6RKI7	173,7	1,83	1,9
RodA	240,9	1,94	2,2
RodA	231,3	1,82	1,8
RodA	133,4	1,93	2,2

4.1.5 Plasmid linearization via *SacI* and agarose gel imaging

Isolated plasmids were linearized to *P. pastoris* KM71H transformation with *SacI* enzyme as an overnight at 37 °C heat block for 16 hours. Then, restricted samples were run via agarose gel electrophoresis at 1,2% agarose gel, 100 volt and 80 minutes. As a result, restricted plasmids ran ahead of the uncuts as expected (Figure 17).

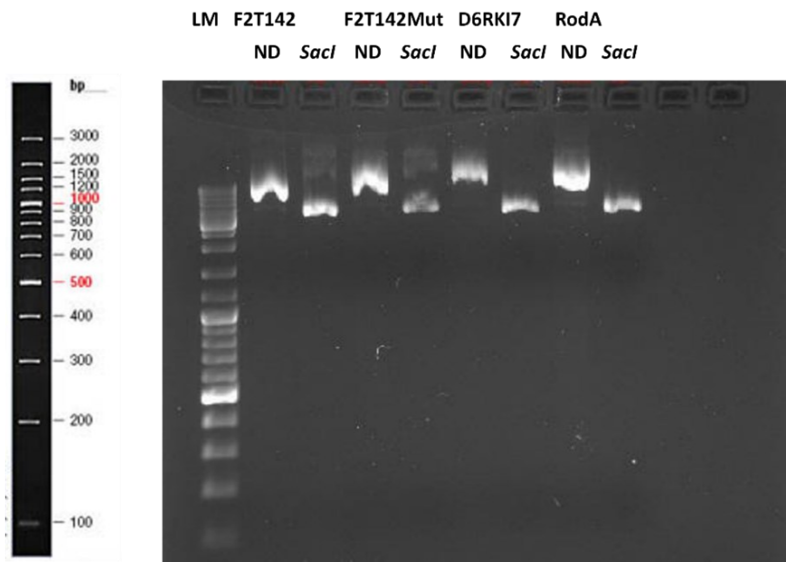


Figure 17. Agarose gel electrophoresis result after overnight *SacI* digestion (LM: GeneRuler 1kb Ladder Mix, ND: Non-digested samples, *SacI*: Digested samples).

Restricted plasmids were purified by Qiagen PCR purification kit and concentrations with A260/280, A260/230 were evaluated before *P. pastoris* transformation.

4.1.6 *Pichia pastoris* KM71H transformation via high efficiency lithium acetate transformation method

Purified samples were transformed into *P. pastoris* KM71H competent cells via high efficiency electroporation method. Transformation efficacy was increased via lithium acetate treatment. Samples were spreaded Zeocin containing (100 µg/ml) YPD agar plates. Zeocin resistant colonies were shown at Figure 18.

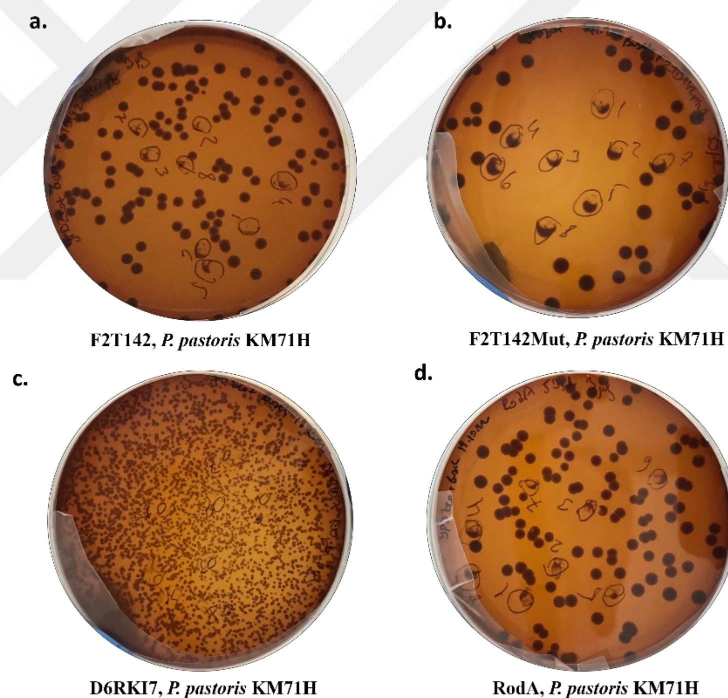


Figure 18. *P. pastoris* KM71H strain YPD Zeocin (100 µg/ml) transformation plates which were used to microwave colony PCR verification method (a. F2T142, b. F2T142Mut, c. D6RKI7 d. RodA).

4.1.7 Microwave colony PCR verification and replatings

Positive transformants were verified via microwave colony PCR. In this method, colony samples were taken and cooked into maximum level of microwave. Samples

were immediately taken to on ice and sterile ddH$_2$0 was added and that was used as a template DNA to standard colony PCR method. Samples were run at 1.2% agarose gel at 100V for 80 minutes. Empty vector pPICZαA was used as a positive control. Samples were transformed to successfully according to gel results. pPICZαA has 588 bp and because of the ran from ahead. On the other hand, samples were ran slow according to pPICZαA, that confirms the insertions. F2T142 had 728 bp, F2T142Mut had 725 bp, D6RKI7 had 719 bp and RodA had 1025 bp (Figure 19, Figure 20).

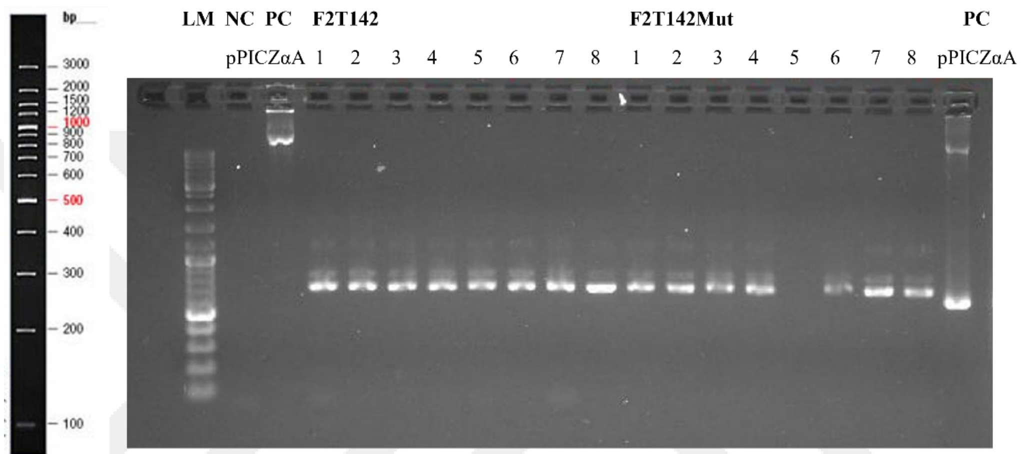


Figure 19. Microwave colony PCR results of the F2T142 and F2T142Mut samples (LM: GeneRuler 1kb ladder mix, PC: pPICZαA, NC: ddH$_2$O).

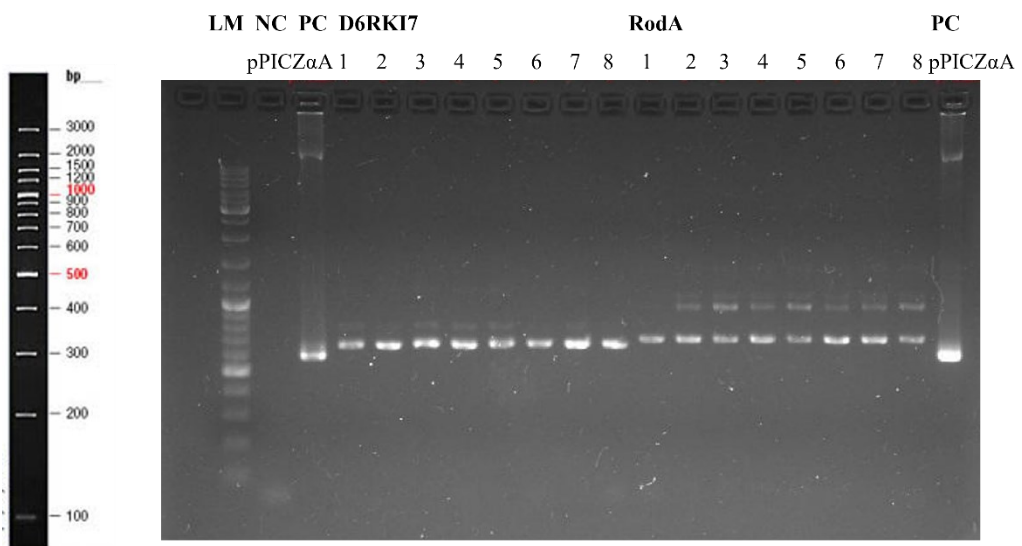


Figure 20. Microwave colony PCR results of the D6RKI7 and RodA samples (LM: GeneRuler 1kb ladder mix, PC: pPICZαA, NC: ddH$_2$O).

8 of positive colonies were selected and streaked to Zeocin containing (100 µg/ml) YPD agar plates for each sample and incubated for 3 days at 30°C. This step was repeated for three times to pure and single colony.

4.2 Biochemical Characterization

4.2.1 BCA assay

Bicinchoninic acid (BCA) assay was done to calculate total protein concentration of the samples which includes *Pichia pastoris* and our interested proteins.

4.2.1.1 96-deep well expression optimization BCA assay

96-deep well BCA assay was done to calculate total concentration of the recombinantly produced peptides. According to that, the colonies with the highest production efficiency was selected and baffled shake flask expression was started from that. BCA standards were used at different concentrations (Table 15). ddH$_2$0 was the blank of BCA standards, 2X BMM was the blank of samples. From 0 hour to 48 hours, total protein amount increased because of the methanol induction (Figure 21). F2T142.1, F2T142Mut.3, D6RKI7.8 and RodA.1 were selected as the most productive colonies to baffled-shake flask expression (Table 22, Table 23).

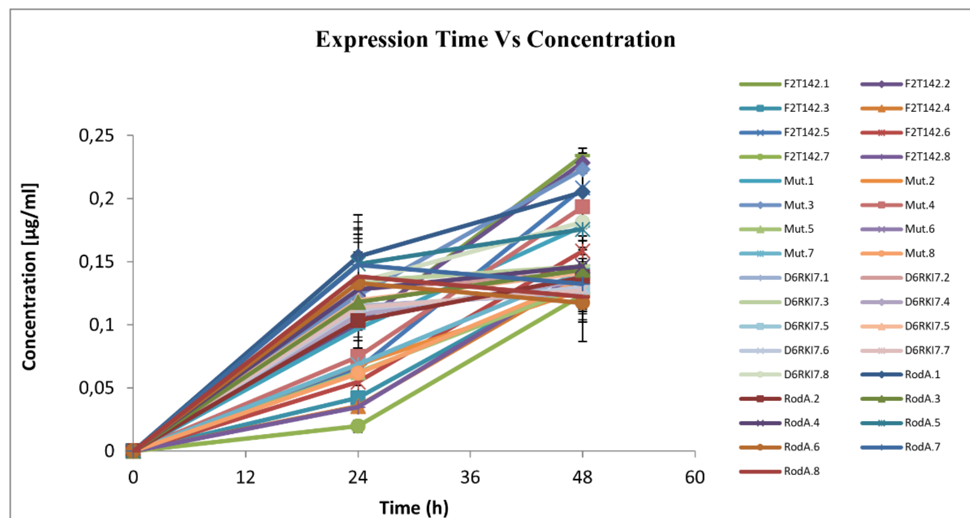


Figure 21. 96-deep well expression BCA assay results of the candidate peptides.

Table 22. 96-deep well expression total protein concentrations of the F2T142 and F2T142Mut candidate peptides.

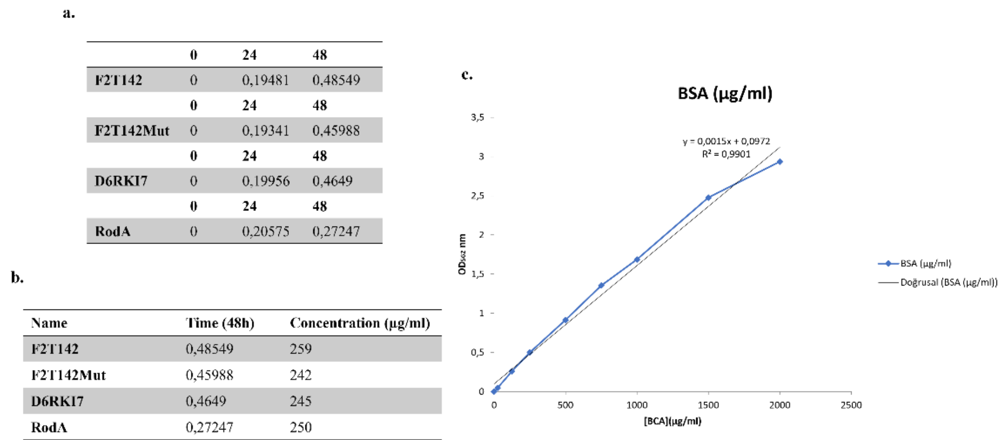
F2T142	Concentration (µg/ml)	F2T142Mut	Concentration (µg/ml)
F2T142.1	230	**F2T142Mut.1**	178
F2T142.2	228	**F2T142Mut.2**	127
F2T142.3	140	**F2T142Mut.3**	222
F2T142.4	138	**F2T142Mut.4**	193
F2T142.5	208	**F2T142Mut.5**	130
F2T142.6	158	**F2T142Mut.6**	161
F2T142.7	123	**F2T142Mut.7**	142
F2T142.8	142	**F2T142Mut.8**	135

Table 23. 96-deep well expression total protein concentrations of the D6RKI7 and RodA candidate peptides.

D6RKI7	Concentration (µg/ml)	RodA	Concentration (µg/ml)
D6RKI7.1	125	**RodA.1**	205
D6RKI7.2	141	**RodA.2**	137
D6RKI7.3	146	**RodA.3**	143
D6RKI7.4	135	**RodA.4**	146
D6RKI7.5	140	**RodA.5**	175
D6RKI7.6	129	**RodA.6**	117
D6RKI7.7	127	**RodA.7**	132
D6RKI7.8	181	**RodA.8**	122

4.2.1.2 Baffled shake flask expression BCA assay

BCA assay was done to calculate total protein concentrations of the recombinantly produced peptides. Here, from 0 hour to 48 hours, total protein concentration was regularly increased because of the methanol induction (Figure 23). At 48 hours; concentrations were measured. According to that, F2T142 had 259 µg/ml, F2T142Mut had 242 µg/ml, D6RKI7 had 245 µg/ml and RodA had 250 µg/ml total protein (Figure 22). However, R^2 value of the experiment was 0,9901.



a.

	0	24	48
F2T142	0	0,19481	0,48549
	0	24	48
F2T142Mut	0	0,19341	0,45988
	0	24	48
D6RKI7	0	0,19956	0,4649
	0	24	48
RodA	0	0,20575	0,27247

b.

Name	Time (48h)	Concentration (µg/ml)
F2T142	0,48549	259
F2T142Mut	0,45988	242
D6RKI7	0,4649	245
RodA	0,27247	250

Figure 22. Time dependent concentrations of the samples after shake baffled shake flask expression. (**a.** Average BCA standards readings at 562 nm, **b.** 48h readings at 562 nm and total protein concentrations, **c.** Equation of the BCA standards to calculate total protein.)

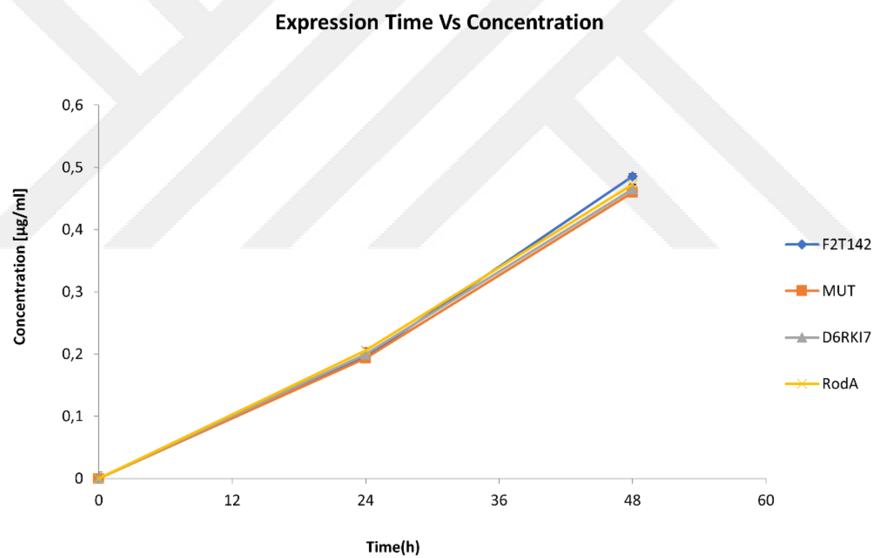


Figure 23. Shake flask BCA assay results of the peptides.

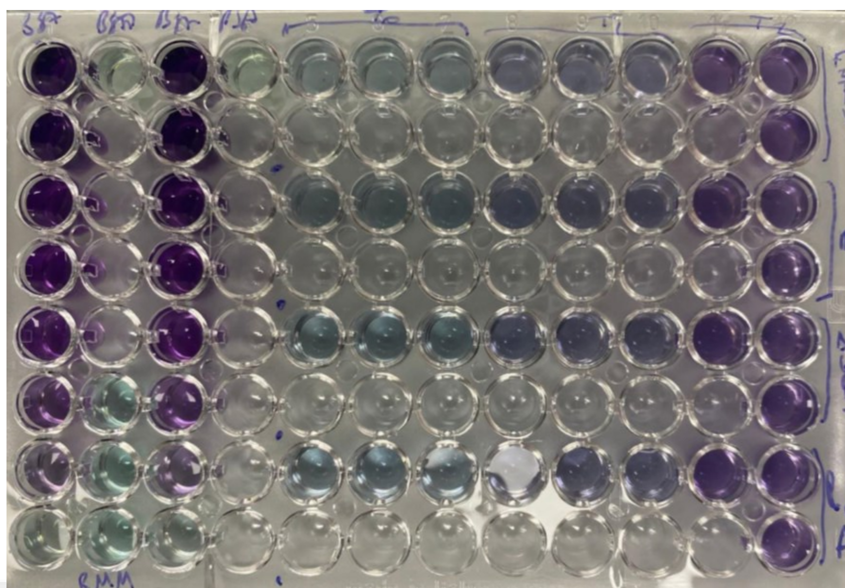


Figure 24. Shake flask BCA assay plate image of the peptides. Purple wells represents protein content.

4.2.2 Tris-glycine SDS-PAGE

Tris-Glycine SDS-PAGE method was done to characterize peptides. 0h, 24h and 48h samples were prepared with 2X urea sample buffer as a 1:1 ratio. Then boiled for 5 minutes at 95°C and loaded 1mm gel. Running was done for 150 minutes at 90 volt. 14,4-200 kDa range of Thermo/Pierce unstained protein ladder was used. To visualize bands, Thermo Pierce silver stain kit was used and images were taken from ChemiDoc MP. According to that, from 0 hour to 48 hours protein production increased with the methanol induction (Figure 25). Desired bands were visualized lower than 14.4 kDa (Figure 26).

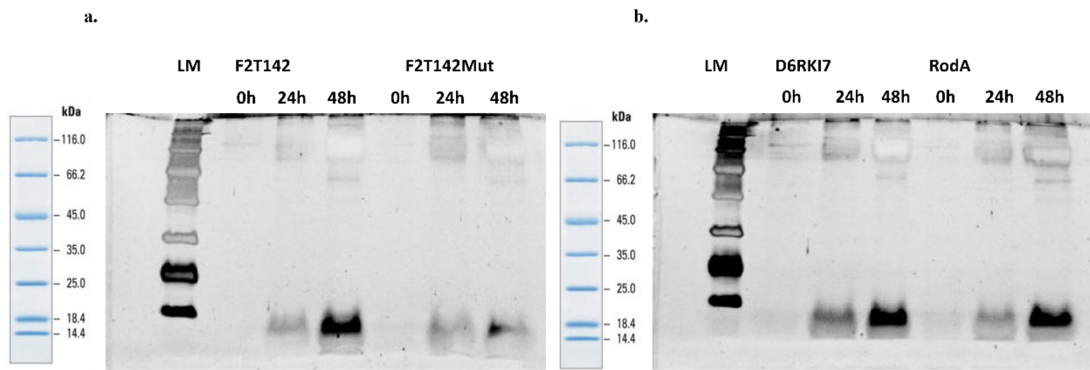


Figure 25. Baffled shake flask Tris-Glycine SDS-PAGE results of the samples. (**a.** LM: PageRuler unstained protein ladder mix, F2T142: 0h, 24h, 48h, F2T142Mut: 0h, 24h, 48h. **b.** LM: Thermo/Pierce unstained protein ladder mix, D6RKI7: 0h, 24h, 48h, RodA: 0h, 24h, 48h.)

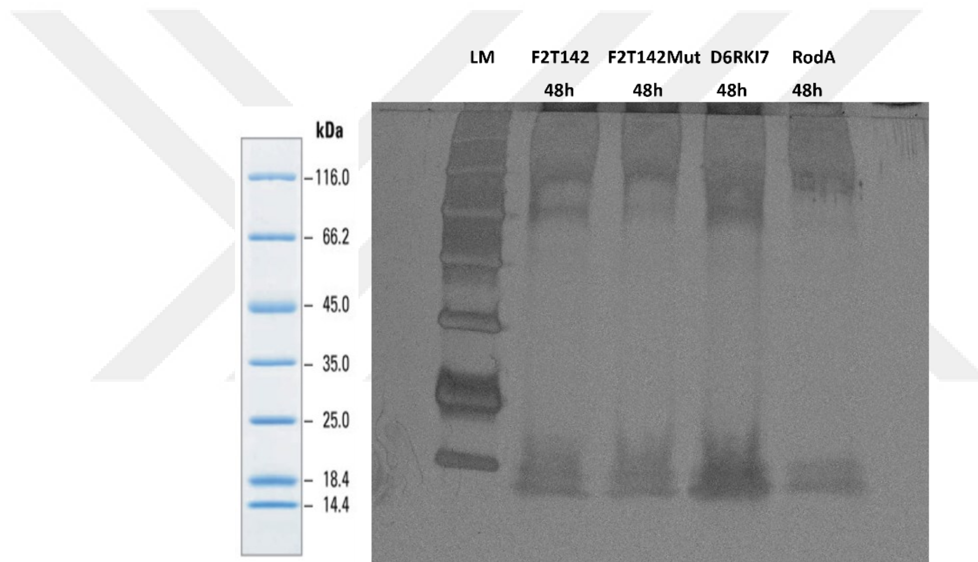


Figure 26. Baffled shake flask 48h Tris-Glycine SDS-PAGE results of the samples. (LM: Thermo/Pierce unstained ladder mix, F2T142 48h, F2T142Mut 48h, D6RKI7 48h and RodA 48h)**.**

4.2.3 Yeastern blot

Yeastern blot is a specific method to characterize the transformed colonies which has high production yield. Here, colonies were grown onto BMM agar plate for 24 and 48 hours with methanol induction. After that, colonies were transferred to 0,22 µm PVDF membrane with the help of weight for three hours. Finally, the other steps were followed as a standard western blot protocol. Results prove that the colonies were transferred to membrane from BMM agar plate at the end of the three hours incubation

(Figure 27). On the other hands colonies were expressed at different yields of proteins (Figure 28).

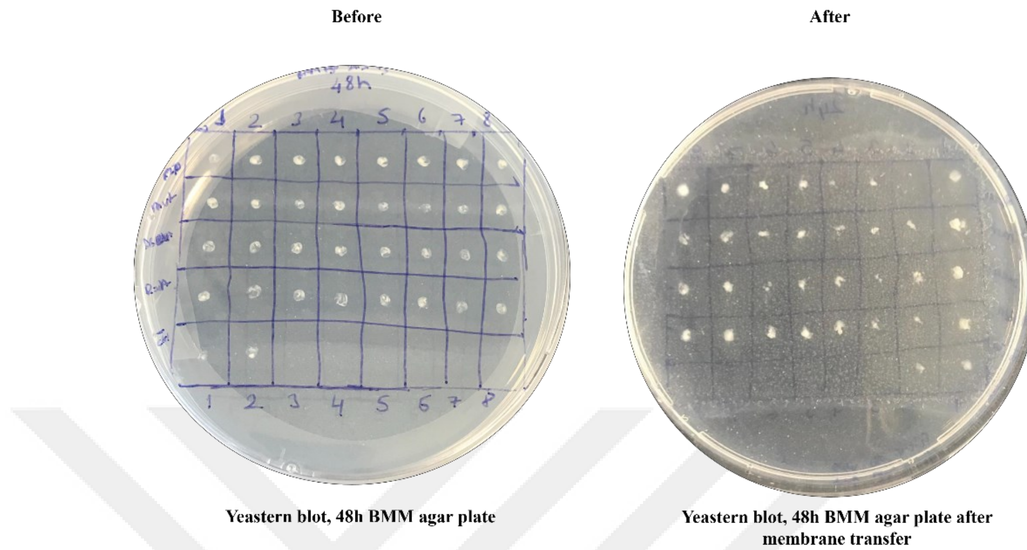


Figure 27. Yeastern blot BMM agar plates after membrane transfer.

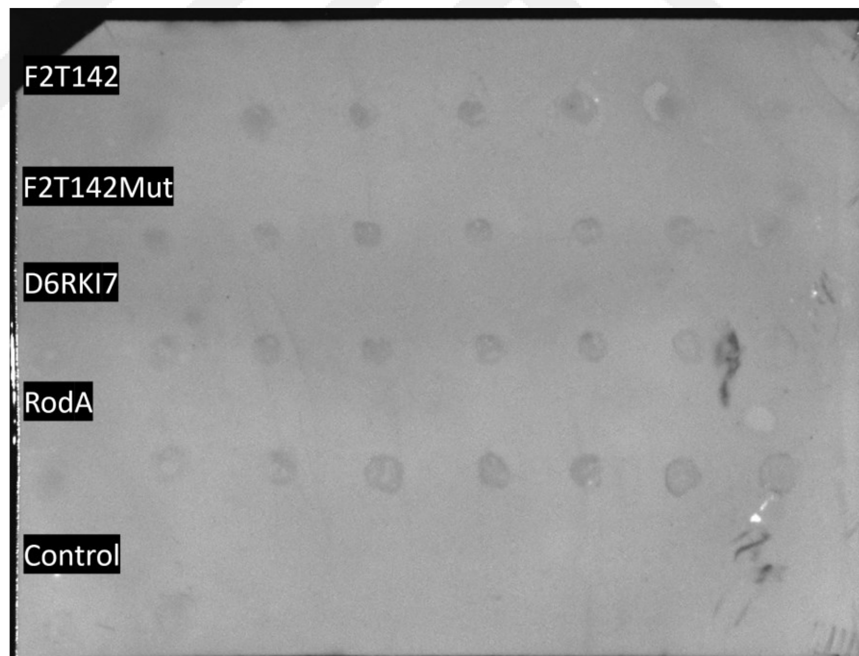


Figure 28. 48h Yeastern blot results of the candidates. 8 of each colony from samples were seen on membrane.

4.3 Functional Tests

4.3.1 Antimicrobial tests

Antimicrobial activity tests were done against *E. coli*, *S. aureus* and *P. aeruginosa* strains. Cells were grown overnight in LB and MHB broth which depends strains. The next day that were adjusted to 0,5 McFarland Standard. Then peptides were applied to observe antimicrobial activity for 0h, 3h, 24h at OD_{600} nm. Figure 29 showed that, peptides did not have antimicrobial activity against *E. coli*. Figure 30 evaluate that the F2T142 had antimicrobial effect on *S.aureus* strain at 240 µg/ml dose. Finally, antimicrobial activity did not observe against *P. aeruginosa* strain (Figure 31).

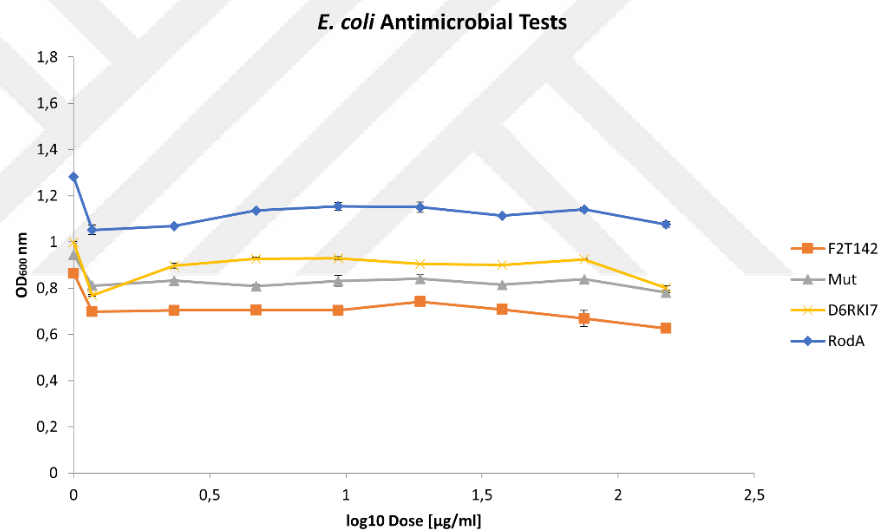


Figure 29. Antimicrobial activity results of the peptides against *E.coli* strain.

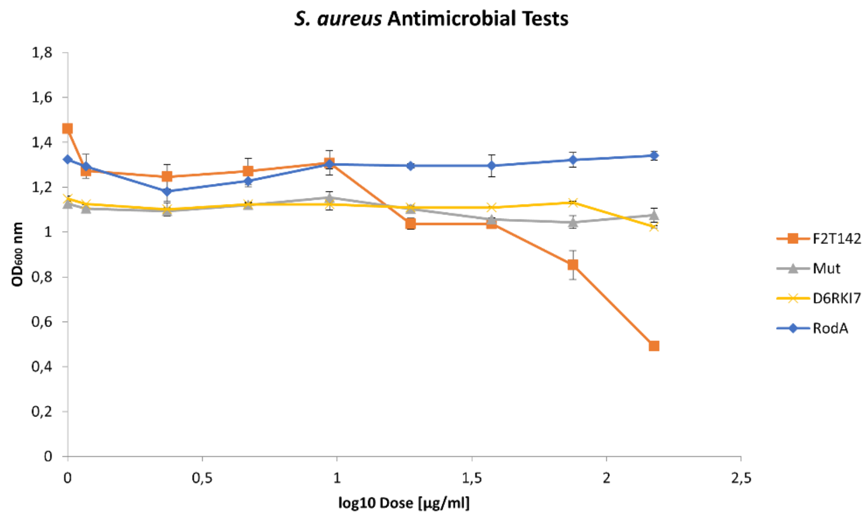


Figure 30. Antimicrobial activity results of the peptides against *S.aureus* strain.

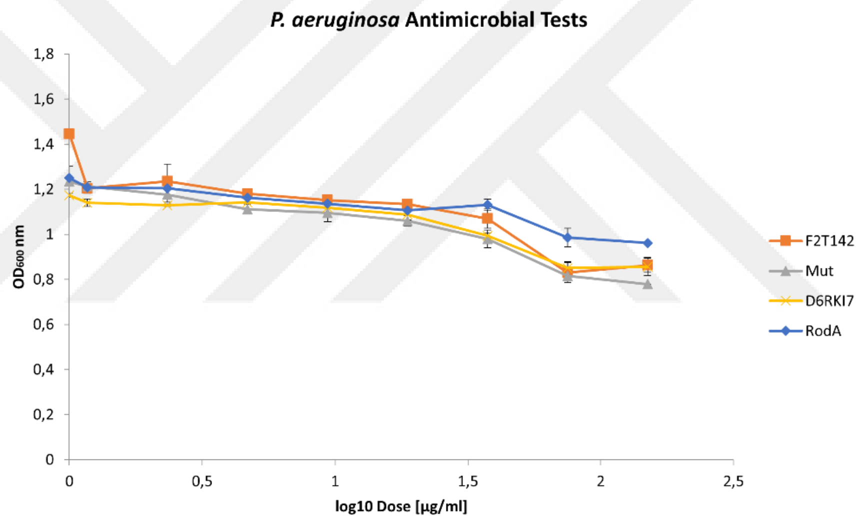


Figure 31. Antimicrobial activity results of the peptides against *P. aeruginosa* strain.

4.3.2 Antifungal tests

Antifungal tests were done agains *C. albicans*. According to results, F2T142 graph slightly decreased at lower dose. The other peptides did not have antifungal activity but reduce the cell growing (Figure 32).

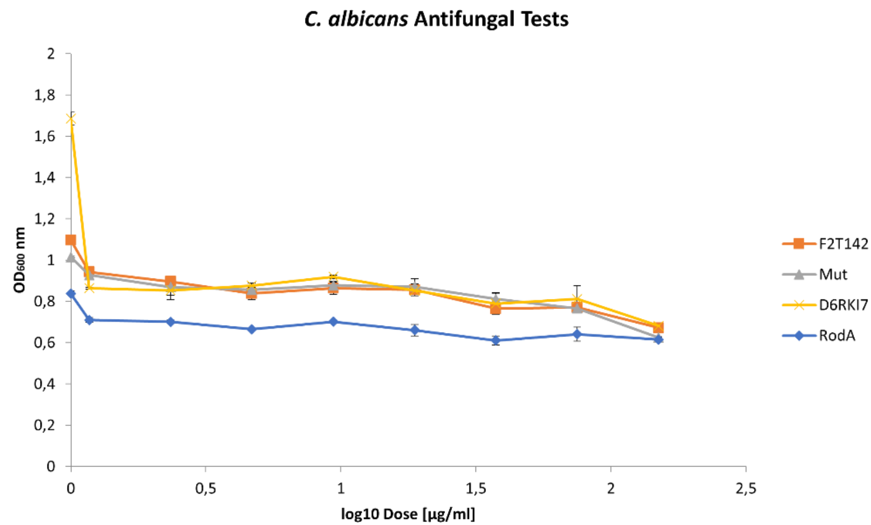


Figure 32. Antifungal test results of the peptides against *C. albicans* strain.

4.3.3 Cytotoxicity tests

WST- 8 test were done to determination of cytotoxicity profiles of the candidate peptides. During experiment, Hep-G2 hepatocellular cancer carcinoma cells were used. Cells were seeded as a 10,000 cell/well within RPMI1620 media and incubated at 37°C, 5% CO_2 for overnight. The next day, peptides were applied as a dose dependent manner and incubated again for 24 hours. The last day of the experiment, 10 µl/well of WST- 8 solution was added and measurement was done at 460 nm for four hours. As a result, all the peptides had cytotoxic effect with the dose dependent manner (Figure 33).

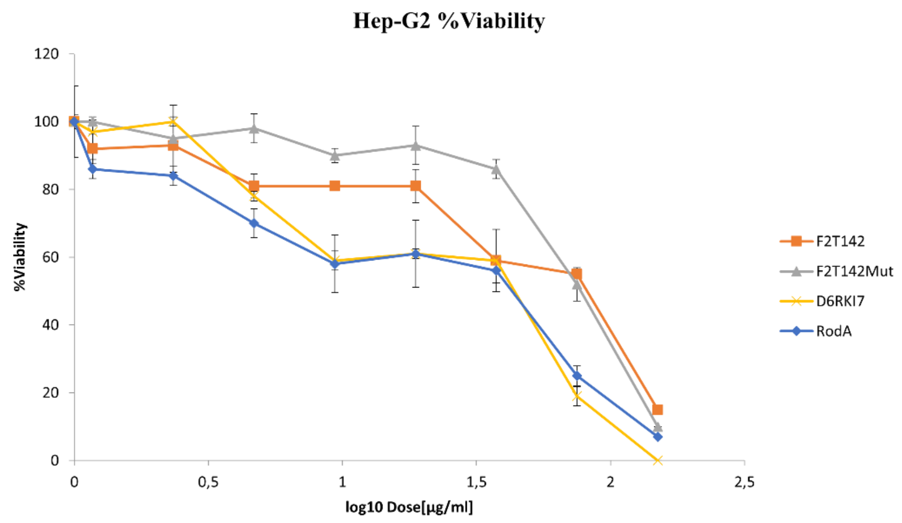


Figure 33. %Viability results of Hep-G2 WST- 8 cytotoxicity tests.

DISCUSSION

All antibiotics, antiviral and antifungal drugs are used in the treatments of infections categorized as antimicrobial drugs. Rapidly increasing antimicrobial resistance in the 21st century has become a global crisis that threatens human life. The development of antimicrobial resistance of microorganisms along with the evolutionary process over time renders existing antimicrobial drugs ineffective. This resistance complicates the treatment of important infections such as sepsis and pneumonia. Patients with organ transplantation, immune system disease and surgical operation are in the risk group. In most cases, the prognosis is very poor which resulting with death. However, it extends the length stay in the hospital and harms the individual and global economy. In order to cope with this problem, the search for a rapid, cost-effective and effective antimicrobial drugs are needed.

Antimicrobial peptides are small, cationic, amphiphilic and low molecular weight peptides that are a natural part of immune system of human, insect, vertebrate, fungi and bacteria. Fungal defensins are also a sub-branch of defensins which have similar properties with the CSαβ specific motif and disulfide bonds. Also these peptides have many biological activities, they have an important place in terms of antimicrobial activity. With the first identification of plectasin as a fungal defensin, studies gained momentum and many candidates emerged. Hydrophobins are small, surface-active peptides found in biofilm with both amphiphilic and amphipathic tails. They are called as hydrophobin because of the their hydrophobic amino acids which are produced by ascomycetes and basidiomycetes. These peptides also have the potential to have antimicrobial activity just like fungal defensins.

Pichia pastoris is a methylotrophic yeast that use methanol as a carbon source. That has strong potential for expression system and protein production. However, there are many advantages of to usage of *Pichia pastoris* in expression. The first advantage is that the capability of making complex proteins with post-translational modification, folding and disulfide bonds. N-Glycosylation very close to higher eukaryotes and proven system without virus and pyrogen. Soluble protein expression and purification

are easy, proteins are secreted into the media. However, fast, simple and production yield is high.

Based on all this information, the aim of this thesis is the recombinant expression, purification and characterization of a diverse set of previously unidentified fungal antimicrobial proteins in *Pichia pastoris* and analysis of their antibacterial-antifungal properties along with cytotoxicity profiles. All the candidate fungal antimicrobial proteins were expressed and characterized for the first time except RodA hydrophobin. Antibacterial activity was tested on *Staphylooccus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 strains. Additionally, antifungal activity tests were done against *Candida albicans* ATCC 90028 strains.

Candidate peptides are uncharacterized defensin F2T142 was selected from *Trichophyton rubrum*, uncharacterized defensin F2T142Mut was selected from *Trichophyton equinum*, uncharacterized defensin D6RKI7 was selected from *Coprinopsis cinerea* and RodA hydrophobin was from *Aspergillus fumigatus*. All these peptides were obtained by searching the NCBI Blast and UniProt protein database with some physicochemical parameters such as instability index, aliphatic index, GRAVY index and net charge. Expasy ProtParam and ProtScale tools were used to getting of the results. According to that, instability index represents peptide stability and must be lower than 40 or close to 40 (137). Candidate peptides are the closest to 40 in database (Table 19). Aliphatic index is related with peptide thermostability and should be high. GRAVY index showed that better association of water and peptide. The net charge is important for the antimicrobial effect of peptides. Because peptides with antimicrobial activity were generally found to be positively charged in literature (138–140). Also, candidate peptides had both positive and negative charges. Net charges as following; 5,8 to F2T142, 4,8 to F2T142Mut, -15 to D6RKI7 and -3,1 to RodA. In literature, positively charged defensins related with the high antimicrobial activity. Antimicrobial test results of the F2T142 defensin which has highest positive charge, was support that with the antimicrobial activity against gram positive bacteria.

Synthetic genes were provided by GeneUniversal which came into *P. pastoris* pPICZαA vector and they were subcloned into *E. coli DH5α* competent cells via heat shock transformation method. LB agar plates were contained 25 µg/ml Zeocin to culture only Zeocin resistant colonies onto plate. According to that, Zeocin resistant single colonies were obtained after transformation (Figure 15). Positive transformants were verified by colony PCR method with the 5'AOX and 3'AOX primers. Agarose gel electrophoresis result evaluate that the successful transformation. When 588 bp empty vector pPICZαA compared with the insertions, the empty vector ran faster than the others. Candidates base lengths were 728 bp for F2T142, 725 bp for F2T142Mut, 719 bp for D6RKI7 and 1025 bp for RodA respectively.

After the confirmation of positive transformants, colonies were cultured into LB broth at 37°C for overnight to MiniPrep plasmid isolation. Concentrations, A260/A280 and A260/A230 values were measured by NanoDrop1C. A260/A280 value represents purity of DNA and between 1,8 to 2,0 generally accepted as pure sample. Also, A260/A230 value represents carbohydrates and phenolic contaminants. Because all of them have absorbance near 230 nm. This value should be more than 2,0 (141). As a result, candidates were isolated with the high concentrations. A260/A280 values were between 1,8 to 2 as expected and 260/A230 values were more that 2,0 or very close to that (Table 21). Then isolated plasmids were linearized with *SacI* enzyme as overnight at 37°C to insert P. pastoris KM71H competent cells. Digested samples were run via 1.2% agarose gel electrophoresis. According to gel image, restriction was successful when samples were compared with non-digested controls (Figure 17).

Samples which were purified by "Qiagen PCR Purification kit" were transformed into *P. pastoris* KM71H competent cells via high efficiency electroporation method. Transformation efficiency was increased via lithium acetate treatment. After the transformation, cells were cultured onto 100 µg/ml Zeocin containing YPD agar plates at 30°C. Zeocin resistant genes colonies were observed as is seen Figure 18. That colonies were verified via microwave colony PCR. In this method, cells were cooked into maximum level of microwave oven. Because, yeast cells are durable compared to bacterial cells and it is more difficult to disrupt cell wall. With this cooking step, that

was aimed to damage the cell wall and its release DNA. These DNA was used as a template for colony PCR. Samples were run at 1,2% agarose gel and according to gel result, selected colonies were successfully transformed according to their band locations on gel when they compared to pPICZαA empty vector with the 588 bp and insertions (Figure 19,Figure 20).

Bicinchoninic acid (BCA) assay was done to calculate total protein concentration of the samples which includes *Pichia pastoris* and our interested proteins. Colonies were replated after the verification for three times to obtain single pure colony. Then they were expressed in 96-deep well plate to select which have highest production efficiency to produce baffled shake flask in next step. Expression time were taken for five days. First two days was important to cell growing and increasing of cell number. At the third day which was called 0 hour, methanol induction was started to express interested proteins. From third day to last day which were called as a 24 hours and 48 hours, expression increased because of methanol induction. Alcohol oxidase promoter made this during recombinant expression. During expression, 50 µl of samples were taken to BCA assay total protein concentration analysis. 96-deep well plate expression results prove that total protein concentrations increased from 0 hour to 48 hours (Figure 21). Total protein concentration was 230 µg/ml as a highest value for F2T142.1 sample. F2T142Mut.3 sample had 222 µg/ml total concentration. D6RKI7.8 had 181 µg/ml total protein concentration as a highest. Lastly, RodA.1 had 205 µg/ml total protein (Table 23). According to all these results, that colonies were suitable and selected to produce the most productive to the baffled shake flask expression. Samples which were selected according to production efficiency in 96-deep well plates were produced recombinantly in baffled shake flasks. The baffled shake flasks were preferred because it contributes to better growth of cells by providing better oxygenation. Here, production started on day three which was called 0 hour with the methanol induction just as in the 96-deep well plate expression. From 0 hour to 48 hours, total protein concentrations increased as expected in all samples (Figure 23). Concentrations were 259 µg/ml for F2T142, 242 µg/ml for F2T142Mut, 245 µg/ml for D6RKI7 and 250 µg/ml for RodA. The produced peptides were collected extracellularly from the medium by centrifugation, thanks to α-secretion signal in the

plasmid. In this way, the peptides were easily collected without an extra cell bursting step.

Tris-glycine SDS-PAGE method was used to characterize candidate peptides. Tris-tricine SDS-PAGE also tried another technique for imaging small peptides but it was not as successful as tris-glycine method. For this reason, tris-glycine gels were preferred. Coomassie blue, zinc-chloride and silver staining methods were tried to dye gel and the most effective one was silver staining which is known to be more sensitive than the others. When the gel results were examined, that was supported the increase in production from 0 hour to 48 hours just as in the BCA assay results. Bands displayed at the top of the gel indicated the proteins of *P. pastoris* itself. When compared to the 10 kDa to 200 kDa range of protein ladder, the bands below 14.4 kDa are the band images of the candidate peptides. Protein bands of the candidates were determined as 5,7 kDa for F2T142, 5,6 kDa for F2T142Mut, 5,3 kDa for D6RKI7 and 15,6 kDa for RodA (Figure 26). According to results, the protein bands of the defensin samples were displayed in the expected range in SDS-PAGE.

On the other hand, RodA hydrophobin was determined as approximately lower than 14,4 kDa instead of 15,6 kDa which is different from what was expected. At this point, when the *Pichia pastoris* colony PCR results were examined, band lengths of the RodA was determined between 700-800 bp instead of expected 1025 bp. Additionally, when the *E. coli* transformation colony PCR results were examined, RodA gave the expected band size on agarose gel which indicate a potential problem during transformation steps. On the other hands, in SDS-PAGE, used sample buffer was containing urea. With the combinantion of small peptides and urea may change molecular weights of the RodA. That cause the retarded running pattern of the sample and dimerization on SDS-PAGE gel. Further verification was planned as a mass spectrometer.

Yeastern blot is a specific method which is like western blot. In this technique, candidate colonies were grown on BMM agar plate for 24 and 48 hours and then transferred to 0,22 µM PVDF membrane with the force of bottle for 3 hours. After

that, protocol was followed as a standard western blot technique. As a result, colonies were transferred successfully to the PVDF membrane (Figure 27). Antibody binds to samples and bright areas were occurred around colonies at different sizes. According to 24 hours membrane, 48 hours membrane showed that more signal to samples (Figure 28).

With the discovery of plectasin, the potential of fungal antimicrobial proteins to be antimicrobial agent has emerged (7). In our study, candidate peptides were tested against *E. coli*, *S. aureus* and *P. aeruginosa* strains. OD_{600} values were taken after 24 hours of incubation with peptides. According to that, F2T142 peptide showed the antimicrobial activity against *S. aureus* at 240 µg/ml dose (Figure 30). For *E. coli* (Figure 29) and *P. aeruginosa* strains there was not any activity (Figure 31).

As a results of the literature review, many fungal antimicrobial proteins with antimicrobial effects were seen. For example, defensin Py4 showed antimicrobial activity against *Bacillus megaterium* at low concentration, but it was not effective on *S. aureus* as others (76). Zhang et al., evaluated the high activity with lower dose on *S. aureus* and *Streptococcus dysgalactiae* as 2 µg/ml dose (142). Fungal defensin micasin had the activity against *Bacillus megaterium* at low concentrations as 0,054 µM but did not show strong effect against *S. aureus* (8). The first identified defensin plectasin showed high activity against *Streptococcus pneumonie*, *Staphylococcus epidermis* and *Streptococcus suis*. On the other hands, that was not strong activity against *S. aureus* and other strains (6). Tachyplesin I peptide which produced *P. pastoris* expression system evaluated the activity against *E. coli*, *S. aureus*, *P. aeruginosa* (143). As a result, considering the antimicrobial test results F2T142 has been a promising candidate in terms of antimicrobial effect when compared to other peptides.

Antifungal tests of candidates were done against *C. albicans* ATCC 90028 strain. According to results, D6RKI7 had fungicidal activity at 1,87 µg/ml dose which was determined as lower dose. On the other hands, F2T142Mut, D6RKI7 and RodA showed the fungustatic activity by inhibiting their growth (Figure 32). When focused

on literature, DLP Coprisin was less effective against fungal strains than Mellitine control. Reactive oxygen species (ROS) was induced the cells because of the mitochondrial dysfunction to provoke apoptosis but this does not apply to the *C. albicans* strain (144). Ayroza et al. demonstrate that the antifungal peptide showed the activity against *C. albicans* MDM8 and IOC strains. However, Amphotericin B, which is commonly used as a conventional antifungal drug had the activity on *C. albicans* MDM8 strain but not effective against IOC strain (145).

Cytotoxicity profiles of the candidate peptides were tested against hepatocellular liver cancer cells (HepG2) via Water-Soluble Tetrazolium-8 (WST-8) test. The main purpose of using cancer cells, since cancer cells are immortal it is important to better identify and understand toxicity level. WST-8 test is a colorimetric way to determine cell viability. Also, it is not cell permeable and does not cause toxicity. For this reason, further experiments can be carried out. In this test, water- soluble formazan is produced by cellular reduction and no extra step is required to dissolve formazan.

Studies in literature, support that defensins with high antimicrobial activity also show high toxicity. The hemolytic activity results also support this, because the peptides which kill the epithelial mammalian cell or cancer cell also hemolyze human blood. According to that, the first identified defensin plectasin was showed antimicrobial activity against bacterial strains but unfortunately had high toxicity. Zhu et al. demonstrate that the Meucin-49 was highly toxic to cells (8). In another study, blapersin decreased the cell viability after 50 μM dose treatment (77). Qi et al., evaluated the high toxicity to Meucin-18 peptide at high dose treatment (76). On the other hand, Py4 showed lower toxicity. Defensin ZmD32 and NaD1 showed the activity against PC3 and U937 tumor cell lines. However, NaD1 was more active than ZmD32 (146). Our results supported that, F2T142 had low cytotoxic effect from the other candidates (Figure 33). Surprisingly the same peptide had antimicrobial activity, indicating that it may have the potential to be an important candidate. Peptide D6RKI7 showed the towering cytotoxic effect by killing all cells.

5 CONCLUSION

Antimicrobial resistance is a rapidly increasing global problem that threatens human life. For this reason, the treatment of many infections become harder or even impossible. Untreated cases result in death, treatment processes are quite and costly. This situation has increased the tendency towards new candidate biopharmaceuticals that are more efficient, wide spectrum, cost-effective and will emerge in a short time. Fungal antimicrobial peptides also peptides with an important potential in this direction. These peptides secreted by the defense mechanisms of organisms which have small and cationic properties. Fungal defensins show antimicrobial properties against many microorganisms as antimicrobial peptide family member in literature. With the discovery of plectasin as the first fungal defensin, these studies acceletared and new candidates emerged. Hydrophobins are also small hydrophobic peptides secreted by *ascomycetes* and *basidiomycetes* which have significant potential.

In this thesis, defensin F2T142, F2T142Mut, D6RKI7 and hydrophobin RodA were selected from protein databases according to various physicochemical parameters such as instability index, charge and hydropathicity. Gene sysnthesis of selected candidates were provided synthetically and subcloning was performed in *E. coli DH5α* competent cells. That were transformed into *P. pastoris* KM71H competent cells after colony PCR verification, plasmid isolation, linearization and clean-up. After the selection of high efficiency clone selection in 96-deep well plate expression, candidates were produced into baffled shake flask expression and characterized. Finally, functional tests as antimicrobial, antifungal and cytotoxicity were performed.

As a result, after the candidates were successfully cloned and verified they were produced. Characterizations were done with BCA assay, SDS-PAGE and yeastern blot. They were expressed up to 259 µg/ml total concentrations. According to that, F2T142 peptide showed the antimicrobial activity against *S. aureus* at 240 µg/ml dose For *E. coli* and *P. aeruginosa* strains there was not any activity. Antifungal tests of candidates were done against *C. albicans* ATCC 90028 strain. According to results, D6RKI7 had fungicidal activity at 1,87 µg/ml dose which was determined as lower

dose. On the other hands, F2T142Mut, D6RKI7 and RodA showed the fungustatic activity by inhibiting their growth. Our results supported that, F2T142 had low cytotoxic effect from the other candidates. Surprisingly the same peptide has antimicrobial activity, indicating that it may have the potential to be an important candidate against microorganisms. However, the high toxicity of the peptides against HepG2 hepatocellular cancer cell is promising in terms of determining and improving the potential anticancer properties of defensins. As an example, peptide D6RKI7 showed the towering cytotoxic effect by killing all cells with the dose dependent manner.

Considering these results, high yield recombinant production will be tried in the next stages of the study. High doses are also planned for antimicrobial and antifungal tests and the number of doses will be increased. Anticancer aspects of candidate peptides will also be explored on different cancer types.

6 REFERENCES

1. Tagliabue A, Rappuoli R. Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top. Front Immunol. 30 Mayıs 2018;9:1068.
2. Yang N, Teng D, Mao R, Hao Y, Wang X, Wang Z, vd. A recombinant fungal defensin-like peptide-P2 combats multidrug-resistant Staphylococcus aureus and biofilms. Appl Microbiol Biotechnol. Temmuz 2019;103(13):5193-213.
3. Fungal infections in humans: the silent crisis - PMC [İnternet]. [a.yer 04 Nisan 2023]. Erişim adresi: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7278517/
4. Koehler P, Cornely OA, Böttiger BW, Dusse F, Eichenauer DA, Fuchs F, vd. COVID-19 associated pulmonary aspergillosis. Mycoses. Haziran 2020;63(6):528-34.
5. Munk JK, Ritz C, Fliedner FP, Frimodt-Møller N, Hansen PR. Novel Method To Identify the Optimal Antimicrobial Peptide in a Combination Matrix, Using Anoplin as an Example. Antimicrob Agents Chemother. Şubat 2014;58(2):1063-70.
6. Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sönksen CP, Ludvigsen S, vd. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature. 13 Ekim 2005;437(7061):975-80.
7. Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, vd. Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. Science. 28 Mayıs 2010;328(5982):1168-72.
8. Zhu S, Gao B, Harvey PJ, Craik DJ. Dermatophytic defensin with antiinfective potential. Proc Natl Acad Sci. 29 Mayıs 2012;109(22):8495-500.
9. Li X, Wang F, Xu Y, Liu G, Dong C. Cysteine-Rich Hydrophobin Gene Family: Genome Wide Analysis, Phylogeny and Transcript Profiling in Cordyceps militaris. Int J Mol Sci. 11 Ocak 2021;22(2):643.
10. Kordts M, Kampe M, Kerth A, Hinderberger D. Structure Formation in Class I and Class II Hydrophobins at the Air–Water Interface under Multiple Compression/Expansion Cycles. ChemistryOpen. 21 Kasım 2018;7(12):1005-13.
11. Karbalaei M, Rezaee SA, Farsiani H. Pichia pastoris: A highly successful expression system for optimal synthesis of heterologous proteins. J Cell Physiol. Eylül 2020;235(9):5867-81.
12. Gould K. Antibiotics: from prehistory to the present day. J Antimicrob Chemother. 01 Mart 2016;71(3):572-5.
13. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. Curr Opin Microbiol. 01 Ekim 2019;51:72-80.
14. Ventola CL. The Antibiotic Resistance Crisis. Pharm Ther. Nisan 2015;40(4):277-83.
15. Andrei S, Droc G, Stefan G. FDA approved antibacterial drugs: 2018-2019. Discoveries. 7(4):e102.
16. Browne K, Chakraborty S, Chen R, Willcox MD, Black DS, Walsh WR, vd. A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. Int J Mol Sci. 24 Eylül 2020;21(19):7047.

17. Sun J, Xia Y, Li D, Du Q, Liang D. Relationship between peptide structure and antimicrobial activity as studied by de novo designed peptides. Biochim Biophys Acta. Aralık 2014;1838(12):2985-93.
18. Antimicrobial Peptides Services - Profacgen [İnternet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.profacgen.com/antimicrobial-peptides-services.htm
19. Huan Y, Kong Q, Mou H, Yi H. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. Front Microbiol [Internet]. 16 Ekim 2020 [a.yer 29 Nisan 2023];11. Erişim adresi: https://www.frontiersin.org/articles/10.3389/fmicb.2020.582779/full
20. Mattiuzzo M, Bandiera A, Gennaro R, Benincasa M, Pacor S, Antcheva N, vd. Role of the Escherichia coli SbmA in the antimicrobial activity of proline-rich peptides. Mol Microbiol. Ekim 2007;66(1):151-63.
21. Walrant A, Bauzá A, Girardet C, Alves ID, Lecomte S, Illien F, vd. Ionpair-π interactions favor cell penetration of arginine/tryptophan-rich cell-penetrating peptides. Biochim Biophys Acta Biomembr. 01 Şubat 2020;1862(2):183098.
22. Lee TH, Hall KN, Aguilar MI. Antimicrobial Peptide Structure and Mechanism of Action: A Focus on the Role of Membrane Structure. Curr Top Med Chem. 2016;16(1):25-39.
23. Lei J, Sun L, Huang S, Zhu C, Li P, He J, vd. The antimicrobial peptides and their potential clinical applications. Am J Transl Res. 2019;11(7):3919-31.
24. Mahlapuu M, Håkansson J, Ringstad L, Björn C. Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. Front Cell Infect Microbiol. 27 Aralık 2016;6:194.
25. Chen Y, Guarnieri MT, Vasil AI, Vasil ML, Mant CT, Hodges RS. Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. Antimicrob Agents Chemother. Nisan 2007;51(4):1398-406.
26. Stark M, Liu LP, Deber CM. Cationic Hydrophobic Peptides with Antimicrobial Activity. Antimicrob Agents Chemother. Kasım 2002;46(11):3585-90.
27. Sengupta D, Leontiadou H, Mark AE, Marrink SJ. Toroidal pores formed by antimicrobial peptides show significant disorder. Biochim Biophys Acta BBA - Biomembr. 01 Ekim 2008;1778(10):2308-17.
28. Matsuzaki K, Murase O, Fujii N, Miyajima K. Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. Biochemistry. 16 Mayıs 1995;34(19):6521-6.
29. Leontiadou H, Mark AE, Marrink SJ. Antimicrobial Peptides in Action. J Am Chem Soc. 01 Eylül 2006;128(37):12156-61.
30. Wimley WC. Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. ACS Chem Biol. 15 Ekim 2010;5(10):905-17.
31. Oren Z, Shai Y. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. Biopolymers. 1998;47(6):451-63.
32. Rozek A, Friedrich CL, Hancock RE. Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. Biochemistry. 26 Aralık 2000;39(51):15765-74.

33. Sitaram N, Nagaraj R. Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. Biochim Biophys Acta. 15 Aralık 1999;1462(1-2):29-54.
34. Mardirossian M, Grzela R, Giglione C, Meinnel T, Gennaro R, Mergaert P, vd. The host antimicrobial peptide Bac71-35 binds to bacterial ribosomal proteins and inhibits protein synthesis. Chem Biol. 18 Aralık 2014;21(12):1639-47.
35. Mardirossian M, Barrière Q, Timchenko T, Müller C, Pacor S, Mergaert P, vd. Fragments of the Nonlytic Proline-Rich Antimicrobial Peptide Bac5 Kill Escherichia coli Cells by Inhibiting Protein Synthesis. Antimicrob Agents Chemother. Ağustos 2018;62(8):e00534-18.
36. Graf M, Wilson DN. Intracellular Antimicrobial Peptides Targeting the Protein Synthesis Machinery. Adv Exp Med Biol. 2019;1117:73-89.
37. Erdem Büyükkiraz M, Kesmen Z. Antimicrobial peptides (AMPs): A promising class of antimicrobial compounds. J Appl Microbiol. Mart 2022;132(3):1573-96.
38. Subbalakshmi C, Sitaram N. Mechanism of antimicrobial action of indolicidin. FEMS Microbiol Lett. 01 Mart 1998;160(1):91-6.
39. Le CF, Fang CM, Sekaran SD. Intracellular Targeting Mechanisms by Antimicrobial Peptides. Antimicrob Agents Chemother. Nisan 2017;61(4):e02340-16.
40. Li L, Sun J, Xia S, Tian X, Cheserek MJ, Le G. Mechanism of antifungal activity of antimicrobial peptide APP, a cell-penetrating peptide derivative, against Candida albicans: intracellular DNA binding and cell cycle arrest. Appl Microbiol Biotechnol. Nisan 2016;100(7):3245-53.
41. Cruz GF, de Araujo I, Torres MDT, de la Fuente-Nunez C, Oliveira VX, Ambrosio FN, vd. Photochemically-Generated Silver Chloride Nanoparticles Stabilized by a Peptide Inhibitor of Cell Division and Its Antimicrobial Properties. J Inorg Organomet Polym Mater. 01 Temmuz 2020;30(7):2464-74.
42. Silva PM, Gonçalves S, Santos NC. Defensins: antifungal lessons from eukaryotes. Front Microbiol. 20 Mart 2014;5:97.
43. Hiemstra PS. DEFENSINS. Içinde: Laurent GJ, Shapiro SD, editörler. Encyclopedia of Respiratory Medicine [Internet]. Oxford: Academic Press; 2006 [a.yer 30 Nisan 2023]. s. 7-10. Erişim adresi: https://www.sciencedirect.com/science/article/pii/B0123708796001101
44. Bolatchiev A. Antibacterial activity of human defensins against Staphylococcus aureus and Escherichia coli. PeerJ. 25 Kasım 2020;8:e10455.
45. Varkey J, Nagaraj R. Antibacterial Activity of Human Neutrophil Defensin HNP-1 Analogs without Cysteines. Antimicrob Agents Chemother. Kasım 2005;49(11):4561-6.
46. Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB, Ganz T. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J Clin Invest. 15 Nisan 1998;101(8):1633-42.
47. Bank RPD. RCSB PDB - 1MR4: Solution Structure of NaD1 from Nicotiana alata [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/1MR4
48. Bank RPD. RCSB PDB - 2KOZ: Solution structure of nasonin-1 [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/2KOZ

49. Bank RPD. RCSB PDB - 1FJN: SOLUTION STRUCTURE AND ACTIVITY OF THE FOUR DISULFIDE BOND MEDITERRANEAN MUSSEL DEFENSIN MGD-1 [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/1FJN
50. Bank RPD. RCSB PDB - 2LXZ: Solution Structure of the Antimicrobial Peptide Human Defensin 5 [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/2LXZ
51. Bank RPD. RCSB PDB - 1IJV: HUMAN BETA-DEFENSIN-1 [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/1IJV
52. Bank RPD. RCSB PDB - 2RNG: Solution structure of big defensin [Internet]. [a.yer 02 Mayıs 2023]. Erişim adresi: https://www.rcsb.org/structure/2RNG
53. White SH, Wimley WC, Selsted ME. Structure, function, and membrane integration of defensins. Curr Opin Struct Biol. Ağustos 1995;5(4):521-7.
54. Satchell DP, Sheynis T, Shirafuji Y, Kolusheva S, Ouellette AJ, Jelinek R. Interactions of Mouse Paneth Cell α-Defensins and α-Defensin Precursors with Membranes: PROSEGMENT INHIBITION OF PEPTIDE ASSOCIATION WITH BIOMIMETIC MEMBRANES *. J Biol Chem. 18 Nisan 2003;278(16):13838-46.
55. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol. Ağustos 2000;1(2):113-8.
56. Diamond G, Zasloff M, Eck H, Brasseur M, Maloy WL, Bevins CL. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proc Natl Acad Sci U S A. 01 Mayıs 1991;88(9):3952-6.
57. Bensch KW, Raida M, Mägert HJ, Schulz-Knappe P, Forssmann WG. hBD-1: a novel β-defensin from human plasma. FEBS Lett. 1995;368(2):331-5.
58. White SH, Wimley WC, Selsted ME. Structure, function, and membrane integration of defensins. Curr Opin Struct Biol. 01 Ağustos 1995;5(4):521-7.
59. Harder J, Bartels J, Christophers E, Schröder JM. A peptide antibiotic from human skin. Nature. Haziran 1997;387(6636):861-861.
60. Li D, Zhang L, Yin H, Xu H, Trask JS, Smith DG, vd. Evolution of primate α and θ defensins revealed by analysis of genomes. Mol Biol Rep. 01 Haziran 2014;41(6):3859-66.
61. Daly NL, Chen YK, Rosengren KJ, Marx UC, Phillips ML, Waring AJ, vd. Retrocyclin-2: Structural Analysis of a Potent Anti-HIV θ-Defensin,. Biochemistry. 01 Eylül 2007;46(35):9920-8.
62. Garcia AE, Ösapay G, Tran PA, Yuan J, Selsted ME. Isolation, Synthesis, and Antimicrobial Activities of Naturally Occurring θ-Defensin Isoforms from Baboon Leukocytes. Infect Immun. Aralık 2008;76(12):5883-91.
63. Tran D, Tran PA, Tang YQ, Yuan J, Cole T, Selsted ME. Homodimeric θ-Defensins from Rhesus macaqueLeukocytes: ISOLATION, SYNTHESIS, ANTIMICROBIAL ACTIVITIES, AND BACTERIAL BINDING PROPERTIES OF THE CYCLIC PEPTIDES *. J Biol Chem. 01 Şubat 2002;277(5):3079-84.

64. Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, vd. The global threat of antimicrobial resistance: science for intervention. New Microbes New Infect. 16 Nisan 2015;6:22-9.
65. World Health Organization: WHO global strategy for... - Google Akademik [İnternet]. [a.yer 09 Nisan 2023]. Erişim adresi: https://scholar.google.com/scholar_lookup?title=WHO+global+strategy+for+containment+of+antimicrobial+resistance&publication_year=2001&
66. Rolain JM, Abat C, Jimeno MT, Fournier PE, Raoult D. Do we need new antibiotics? Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. Mayıs 2016;22(5):408-15.
67. O'Neill J. Review on antimicrobial resistance: tackling drug-resistant infections globally: final report and recommendations. Rev Antimicrob Resist Tackling Drug-Resist Infect Glob Final Rep Recomm [Internet]. 2016 [a.yer 30 Nisan 2023]; Erişim adresi: https://www.cabdirect.org/globalhealth/abstract/20163354200
68. Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol. 1993;11:105-28.
69. Hultmark D, Steiner H, Rasmuson T, Boman HG. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. Eur J Biochem. Mayıs 1980;106(1):7-16.
70. Zasloff M. Antimicrobial Peptides of Multicellular Organisms: My Perspective. Adv Exp Med Biol. 2019;1117:3-6.
71. Tomita T, Hitomi S, Nagase T, Matsui H, Matsuse T, Kimura S, vd. Effect of Ions on Antibacterial Activity of Human Beta Defensin 2. Microbiol Immunol. 2000;44(9):749-54.
72. Mathew B, Nagaraj R. Antimicrobial activity of human α-defensin 5 and its linear analogs: N-terminal fatty acylation results in enhanced antimicrobial activity of the linear analogs. Peptides. 01 Eylül 2015;71:128-40.
73. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, vd. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science. 15 Ekim 1999;286(5439):525-8.
74. Sathoff AE, Lewenza S, Samac DA. Plant defensin antibacterial mode of action against Pseudomonas species. BMC Microbiol. 19 Haziran 2020;20(1):173.
75. Velivelli SLS, Islam KT, Hobson E, Shah DM. Modes of Action of a Bi-domain Plant Defensin MtDef5 Against a Bacterial Pathogen Xanthomonas campestris. Front Microbiol [İnternet]. 2018 [a.yer 01 Mayıs 2023];9. Erişim adresi: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00934
76. Qi S, Gao B, Zhu S. A Fungal Defensin Inhibiting Bacterial Cell-Wall Biosynthesis with Non-Hemolysis and Serum Stability. J Fungi. Şubat 2022;8(2):174.
77. Wu J, Qu C, Zhang S, Chen W, Wang S. The second N-terminal aromatic residue of the fungal defensin, blapersin, of Blastomyces percursus is essential for its antibacterial activity. Peptides. Kasım 2020;133:170400.

78. Fizil Á, Sonderegger C, Czajlik A, Fekete A, Komáromi I, Hajdu D, vd. Calcium binding of the antifungal protein PAF: Structure, dynamics and function aspects by NMR and MD simulations. PLoS ONE. 15 Ekim 2018;13(10):e0204825.
79. Garrigues S, Gandía M, Castillo L, Coca M, Marx F, Marcos JF, vd. Three Antifungal Proteins From Penicillium expansum: Different Patterns of Production and Antifungal Activity. Front Microbiol. 05 Ekim 2018;9:2370.
80. M S, Ah K, Md T, Re B, Jp M. Structural analysis of hydrophobins. Micron Oxf Engl 1993 [Internet]. Ekim 2008 [a.yer 01 Mayıs 2023];39(7). Erişim adresi: https://pubmed.ncbi.nlm.nih.gov/17875392/
81. Oeemig JS, Lynggaard C, Knudsen DH, Hansen FT, Nørgaard KD, Schneider T, vd. Eurocin, a new fungal defensin: structure, lipid binding, and its mode of action. J Biol Chem. 07 Aralık 2012;287(50):42361-72.
82. Contreras G, Braun MS, Schäfer H, Wink M. Recombinant AfusinC, an anionic fungal CSαβ defensin from Aspergillus fumigatus, exhibits antimicrobial activity against gram-positive bacteria. PloS One. 2018;13(10):e0205509.
83. Hv W, M Z, Jl R, Rw H, A DW, A VG, vd. Functional synergism of the magainins PGLa and magainin-2 in Escherichia coli, tumor cells and liposomes. Eur J Biochem [Internet]. 03 Ocak 1995 [a.yer 03 Mayıs 2023];228(2). Erişim adresi: https://pubmed.ncbi.nlm.nih.gov/7705337/
84. Darveau RP, Cunningham MD, Seachord CL, Cassiano-Clough L, Cosand WL, Blake J, vd. Beta-lactam antibiotics potentiate magainin 2 antimicrobial activity in vitro and in vivo. Antimicrob Agents Chemother. Haziran 1991;35(6):1153.
85. Wessels JG, de Vries OM, Asgeirsdóttir SA, Springer J. The thn mutation of Schizophyllum commune, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene. J Gen Microbiol. Ekim 1991;137(10):2439-45.
86. Bayry J, Aimanianda V, Guijarro JI, Sunde M, Latgé JP. Hydrophobins--unique fungal proteins. PLoS Pathog. 2012;8(5):e1002700.
87. Kwan AH, Macindoe I, Vukasin PV, Morris VK, Kass I, Gupte R, vd. The Cys3-Cys4 loop of the hydrophobin EAS is not required for rodlet formation and surface activity. J Mol Biol. 10 Ekim 2008;382(3):708-20.
88. Sorrentino I, Gargano M, Ricciardelli A, Parrilli E, Buonocore C, de Pascale D, vd. Development of anti-bacterial surfaces using a hydrophobin chimeric protein. Int J Biol Macromol. 01 Aralık 2020;164:2293-300.
89. Sorrentino I, Gargano M, Ricciardelli A, Parrilli E, Buonocore C, de Pascale D, vd. Development of anti-bacterial surfaces using a hydrophobin chimeric protein. Int J Biol Macromol. 01 Aralık 2020;164:2293-300.
90. Wang L, Lu C, Fan M, Liao B. Coriolopsis trogii hydrophobin genes favor a clustering distribution and are widely involved in mycelial growth and primordia formation. Gene. 15 Kasım 2021;802:145863.

91. Artini M, Cicatiello P, Ricciardelli A, Papa R, Selan L, Dardano P, vd. Hydrophobin coating prevents Staphylococcus epidermidis biofilm formation on different surfaces. Biofouling. Ağustos 2017;33(7):601-11.
92. Kuvarina AE, Rogozhin EA, Sykonnikov MA, Timofeeva AV, Serebryakova MV, Fedorova NV, vd. Isolation and Characterization of a Novel Hydrophobin, Sa-HFB1, with Antifungal Activity from an Alkaliphilic Fungus, Sodiomyces alkalinus. J Fungi. Temmuz 2022;8(7):659.
93. Bawa Z, Routledge SJ, Jamshad M, Clare M, Sarkar D, Dickerson I, vd. Functional recombinant protein is present in the pre-induction phases of Pichia pastoris cultures when grown in bioreactors, but not shake-flasks. Microb Cell Factories. 04 Eylül 2014;13(1):127.
94. Kulagina N, Besseau S, Godon C, Goldman GH, Papon N, Courdavault V. Yeasts as Biopharmaceutical Production Platforms. Front Fungal Biol [İnternet]. 2021 [a.yer 09 Mayıs 2023];2. Erişim adresi: https://www.frontiersin.org/articles/10.3389/ffunb.2021.733492
95. Baeshen M, Al-Hejin A, Bora R, Ahmed M, Ramadan H, Saini K, vd. Production of Biopharmaceuticals in E. coli: Current Scenario and Future Perspectives. J Microbiol Biotechnol. 01 Temmuz 2015;25:953-62.
96. Dumont J, Euwart D, Mei B, Estes S, Kshirsagar R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit Rev Biotechnol. 01 Kasım 2016;36(6):1110-22.
97. Walsh G. Biopharmaceutical benchmarks 2018. Nat Biotechnol. Aralık 2018;36(12):1136-45.
98. Tripathi NK, Shrivastava A. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. Front Bioeng Biotechnol [İnternet]. 2019 [a.yer 10 Mayıs 2023];7. Erişim adresi: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00420
99. Overview of Protein Expression Systems - TR [Internet]. [a.yer 10 Mayıs 2023]. Erişim adresi: https://www.thermofisher.com/tr/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-protein-expression-systems.html
100. Table 1. List of biopharmaceuticals produced in E. coli. [Internet]. ResearchGate. [a.yer 09 Mayıs 2023]. Erişim adresi: https://www.researchgate.net/figure/List-of-biopharmaceuticals-produced-in-E-coli_tbl2_281027153
101. Baghban R, Farajnia S, Rajabibazl M, Ghasemi Y, Mafi A, Hoseinpoor R, vd. Yeast Expression Systems: Overview and Recent Advances. Mol Biotechnol. 01 Mayıs 2019;61(5):365-84.
102. Tesfaw A, Assefa F. Current Trends in Bioethanol Production by *Saccharomyces cerevisiae*: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. Int Sch Res Not. 08 Aralık 2014;2014:e532852.
103. Llopis S, Hernández-Haro C, Monteoliva L, Querol A, Molina M, Fernández-Espinar MT. Pathogenic Potential of Saccharomyces Strains Isolated from Dietary Supplements. PLoS ONE. 30 Mayıs 2014;9(5):e98094.

104. Matheson K, Parsons L, Gammie A. Whole-Genome Sequence and Variant Analysis of W303, a Widely-Used Strain of Saccharomyces cerevisiae. G3 Bethesda Md. 05 Temmuz 2017;7(7):2219-26.

105. Schacherer J, Ruderfer DM, Gresham D, Dolinski K, Botstein D, Kruglyak L. Genome-Wide Analysis of Nucleotide-Level Variation in Commonly Used Saccharomyces cerevisiae Strains. PLoS ONE. 28 Mart 2007;2(3):e322.

106. van Dijken JP null, Bauer J, Brambilla L, Duboc P, Francois JM, Gancedo C, vd. An interlaboratory comparison of physiological and genetic properties of four Saccharomyces cerevisiae strains. Enzyme Microb Technol. 01 Haziran 2000;26(9-10):706-14.

107. Nomura M, Nakamori S, Takagi H. Characterization of novel acetyltransferases found in budding and fission yeasts that detoxify a proline analogue, azetidine-2-carboxylic acid. J Biochem (Tokyo). Ocak 2003;133(1):67-74.

108. Young CL, Raden DL, Robinson AS. Analysis of ER resident proteins in Saccharomyces cerevisiae: implementation of H/KDEL retrieval sequences. Traffic Cph Den. Nisan 2013;14(4):365-81.

109. Machens F, Balazadeh S, Mueller-Roeber B, Messerschmidt K. Synthetic Promoters and Transcription Factors for Heterologous Protein Expression in Saccharomyces cerevisiae. Front Bioeng Biotechnol. 2017;5:63.

110. Vickers CE, Bydder SF, Zhou Y, Nielsen LK. Dual gene expression cassette vectors with antibiotic selection markers for engineering in Saccharomyces cerevisiae. Microb Cell Factories. 25 Ekim 2013;12:96.

111. Looser V, Bruhlmann B, Bumbak F, Stenger C, Costa M, Camattari A, vd. Cultivation strategies to enhance productivity of Pichia pastoris: A review. Biotechnol Adv. 01 Kasım 2015;33(6 Pt 2):1177-93.

112. Irani ZA, Kerkhoven EJ, Shojaosadati SA, Nielsen J. Genome-scale metabolic model of Pichia pastoris with native and humanized glycosylation of recombinant proteins. Biotechnol Bioeng. Mayıs 2016;113(5):961-9.

113. Zhou X, Yu Y, Tao J, Yu L. Production of LYZL6, a novel human c-type lysozyme, in recombinant Pichia pastoris employing high cell density fed-batch fermentation. J Biosci Bioeng. Ekim 2014;118(4):420-5.

114. Daly R, Hearn MTW. Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. J Mol Recognit JMR. 2005;18(2):119-38.

115. Cos O, Ramón R, Montesinos JL, Valero F. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast Pichia pastoris under different promoters: A review. Microb Cell Factories. 06 Nisan 2006;5(1):17.

116. EasySelect^{TM} Pichia.

117. BLAST: Basic Local Alignment Search Tool [İnternet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://blast.ncbi.nlm.nih.gov/Blast.cgi

118. UniProt [İnternet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://www.uniprot.org/

119. Expasy - ProtParam tool [İnternet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://web.expasy.org/protparam/
120. Expasy - ProtScale [İnternet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://web.expasy.org/protscale/
121. I-TASSER server for protein structure and function prediction [İnternet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://zhanggroup.org/I-TASSER/
122. Sambrook J, Russell DW. The Inoue Method for Preparation and Transformation of Competent E. Coli: “Ultra-Competent” Cells. Cold Spring Harb Protoc. 06 Ocak 2006;2006(1):pdb.prot3944.
123. Colony PCR [Internet]. [a.yer 03 Mayıs 2023]. Erişim adresi: https://murraylab.fas.harvard.edu/colony-pcr
124. QIAgen. Qiagen QIAprep Spin Miniprep Kit (27104 or 27106) with a microcentrifuge. 15 Kasım 2015 [a.yer 17 Nisan 2023]; Erişim adresi: https://www.protocols.io/view/Qiagen-QIAprep-Spin-Miniprep-Kit-27104-or-27106-wi-d7f9jm
125. SacI (10 U/µL) [İnternet]. [a.yer 17 Nisan 2023]. Erişim adresi: https://www.thermofisher.com/order/catalog/product/ER1131
126. QIAquick PCR Purification Kit [Internet]. [a.yer 17 Nisan 2023]. Erişim adresi: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-pcr-purification-kit
127. Wu S, Letchworth GJ. High efficiency transformation by electroporation of Pichia pastoris pretreated with lithium acetate and dithiothreitol. BioTechniques. Ocak 2004;36(1):152-4.
128. Pierce™ BCA Protein Assay Kit [Internet]. [a.yer 17 Nisan 2023]. Erişim adresi: https://www.thermofisher.com/order/catalog/product/23225
129. Pierce™ Silver Stain Kit [İnternet]. [a.yer 09 Nisan 2023]. Erişim adresi: https://www.thermofisher.com/order/catalog/product/24612
130. Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T. Expression in the yeast Pichia pastoris. Methods Enzymol. 2009;463:169-89.
131. Western Blot Protocol | Bio-Rad [İnternet]. [a.yer 17 Nisan 2023]. Erişim adresi: https://www.bio-rad-antibodies.com/western-blot-protocol.html
132. HisPur™ Ni-NTA Resin [Internet]. [a.yer 31 Mart 2023]. Erişim adresi: https://www.thermofisher.com/order/catalog/product/88221
133. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75.
134. Roscetto E, Contursi P, Vollaro A, Fusco S, Notomista E, Catania MR. Antifungal and anti-biofilm activity of the first cryptic antimicrobial peptide from an archaeal protein against Candida spp. clinical isolates. Sci Rep. 04 Aralık 2018;8:17570.
135. Cell Counting Kit 8 (WST-8 / CCK8) (ab228554) | Abcam [İnternet]. [a.yer 17 Nisan 2023]. Erişim adresi: https://www.abcam.com/products/assay-kits/cell-counting-kit-8-wst-8--cck8-ab228554.html

136. Li J, Koh JJ, Liu S, Lakshminarayanan R, Verma CS, Beuerman RW. Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. Front Neurosci. 14 Şubat 2017;11:73.

137. Neelima S, Archana K, Athira PP, Anju MV, Anooja VV, Bright Singh IS, vd. Molecular characterization of a novel β-defensin isoform from the red-toothed trigger fish, Odonus niger (Ruppel, 1836). J Genet Eng Biotechnol. 12 Mayıs 2021;19:71.

138. Yang M, Zhang C, Zhang MZ, Zhang S. Beta-defensin derived cationic antimicrobial peptides with potent killing activity against gram negative and gram positive bacteria. BMC Microbiol. 05 Haziran 2018;18(1):54.

139. Yu W, Ning N, Xue Y, Huang Y, Guo F, Li T, vd. A Chimeric Cationic Peptide Composed of Human β-Defensin 3 and Human β-Defensin 4 Exhibits Improved Antibacterial Activity and Salt Resistance. Front Microbiol [İnternet]. 2021 [a.yer 20 Mayıs 2023];12. Erişim adresi: https://www.frontiersin.org/articles/10.3389/fmicb.2021.663151

140. Ganz T. Defensins: antimicrobial peptides of vertebrates. C R Biol. 01 Haziran 2004;327(6):539-49.

141. Manchester KL. Value of A260/A280 ratios for measurement of purity of nucleic acids. BioTechniques. Ağustos 1995;19(2):208-10.

142. Zhang Q, Yang N, Mao R, Hao Y, Ma X, Teng D, vd. A recombinant fungal defensin-like peptide-P2 combats Streptococcus dysgalactiae and biofilms. Appl Microbiol Biotechnol. Şubat 2021;105(4):1489-504.

143. Li H, Ali Z, Liu X, Jiang L, Tang Y, Dai J. Expression of recombinant tachyplesin I in Pichia pastoris. Protein Expr Purif. Mayıs 2019;157:50-6.

144. Lee J, Hwang JS, Hwang IS, Cho J, Lee E, Kim Y, vd. Coprisin-induced antifungal effects in Candida albicans correlate with apoptotic mechanisms. Free Radic Biol Med. 01 Haziran 2012;52(11-12):2302-11.

145. Ayroza G, Ferreira ILC, Sayegh RSR, Tashima AK, da Silva PI. Juruin: an antifungal peptide from the venom of the Amazonian Pink Toe spider, Avicularia juruensis, which contains the inhibitory cystine knot motif. Front Microbiol. 10 Eylül 2012;3:324.

146. Kerenga BK, McKenna JA, Harvey PJ, Quimbar P, Garcia-Ceron D, Lay FT, vd. Salt-Tolerant Antifungal and Antibacterial Activities of the Corn Defensin ZmD32. Front Microbiol [İnternet]. 2019 [a.yer 23 Mayıs 2023];10. Erişim adresi: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00795

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