ANTIMICROBIAL SPECTRUM DETERMINATION OF THE K5 TYPE YEAST KILLER PROTEIN ON BACTERIA CAUSING SKIN INFECTIONS AND ITS CELL KILLING ACTIVITY

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Approval of the Graduate School of Natural and Applied Sciences

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Tuğçe GÖNEN

ABSTRACT

ANTIMICROBIAL SPECTRUM DETERMINATION OF THE K5 TYPE YEAST KILLER PROTEIN ON BACTERIA CAUSING SKIN INFECTIONS AND ITS CELL KILLING ACTIVITY

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Some yeast strains secrete extracellular polypeptide toxins known to have potential growth inhibitory activity on sensitive yeast cells. These yeast strains are known as killer yeasts and their toxins are named as killer toxins or killer proteins. Yeast killer proteins are found inhibitory to Gram-positive bacteria in several studies which were based on microbial interactions of the producer strains tested with sensitive strains. K5 type yeast killer protein produced by *Pichia anomala* NCYC 434 was previously purified and characterized in our laboratory. The protein is glycosilated and has a pI value of 3,7 and molecular mass of 49 kDa, with exo β-1,3-glucanase activity. Antibacterial activity of the pure K5 type yeast killer protein was tested against 19 clinical isolates of gram-positive bacteria causing skin infections and 2 quality control strains and found to have inhibitory activity on the isolates of Methicillin-sensitive *Staphylococcus aureus* (MSSA) and *Enterococcus faecium*. Toxin MIC and MBC ranges were 32 - 256 µg/ml and 64 - >512 µg/ml

respectively. Cell killing analysis revealed that toxin has a bacteriostatic activity and the inhibitory effect starts between 8. and 12. hours. Regrowth of the bacteria is retarded with the increased dose of the toxin. K5 type yeast killer protein might be used as a topical antibacterial agent with its bacteriostatic activity for skin and wound infections caused by MSSA and *Enterococcus faecium* with appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study.

Key words: Antibacterial agent, K5 type yeast killer toxin, MIC, MBC, Bacteriostatic

ÖZ

K5 TİPİ ÖLDÜRÜCÜ MAYA PROTEİNİNİN DERİ ENFEKSİYONU MEYDANA GETİREN BAKTERİLER ÜZERİNDEKİ ANTİBAKTERİYEL SPEKTRUMUNUN TAYİNİ VE HÜCRE ÖLDÜRME AKTİVİTESİ

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Bazı maya suşları diğer duyarlı maya hücrelerinin potansiyel büyümelerini inhibe edici aktivitesi olduğu bilinen ekstrasellüler polipeptid toksinler salgılarlar. Bu tip mantarlara öldürücü mantarlar ve salgıladıkları toksinlere öldürücü proteinler veya öldürücü toksinler adı verilmiştir. Duyarlı hücreler ile üretici suşlar arasındaki mikrobiyal etkileşimlere dayalı çalışmalar sonucunda maya öldürücü proteinlerinin Gram-pozitif bakterilere karşı inhibe edici olduğu bulunmuştur. *Pichia anomala* NCYC 434 tarafından üretilen K5 tipi maya öldürücü proteini laboratuarımızda önceden saflaştırılmış ve karakterize edilmiştir. Protein glikolize olmuş ve 3,7 pI değerine, 49kDa moleküler ağırlığa ve exo- β-1,3-glucanaz aktivitesine sahiptir. K5 tipi öldürücü maya proteininin antibakteriyel aktivitesi deri hastalığı meydana getiren 19 gram-positif klinik bakteri izolatı ve 2 kontrol suşu üzerinde test edilmiş ve Metisiline duyarlı *Staphylococcus aureus* (MSSA) ve *Enterococcus faecium* isolatları üzerinde inhibasyon aktivitesi bulunmuştur. Toksin MİK ve MBK'ları,

sırasıyla, 32 - 256 µg/ml and 64 - >512 µg/ml'dir. Hücre öldürme analizleri toksinin bakteriostatik aktivitesinin olduğunu ve inhibasyon etkisinin 8. ve 12. saatler arasında başladığını göstermiştir. Bakterilerin yeniden büyümeleri toksin dozunun artışıyla gecikmiştir. K5 tipi maya öldürücü proteini bu çalışmadaki antibakteriyel spectrum tayini üzerine yapılacak uygun formülasyon çalışmaları sonrasında MSSA ve *Enterococcus faecium* un sebep olduğu deri ve yara enfeksiyonlarında bakteriostatik etkisi ile topikal antibakteriyel ajan olarak kullanılabilir.

Anahtar kelimeler: Antibakteriyel ajan, K5 tipi maya öldürücü proteini, MİK, MBK, Bacteriostatik

To My Family

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ATCC	American Type Culture Collection
AU	Arbitrary unit
bp	Base pair
CFU	Colony forming units
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraaceticacid
HPLC	High Performance Liquid Chromatography
CLSI	Clinical and Laboratory Standards Institute
K^+	Killer character
K_m	Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$).
kb	Kilo base
kDa	Kilo dalton
L dsRNA	Large size double stranded RNA
Linear dsDNA	Linear double stranded deoxyribonucleic acid
MBC	Minimum bactericidal concentration
MIC	Minimum inhibition concentration
MIC_{50}	The MIC at which 50% of the isolates were inhibited
MIC_{90}	The MIC at which 90% of the isolates were inhibited
MIC-0	The lowest concentration of an antimicrobial agent producing a clear well or 100 % growth inhibition
MIC-2	The lowest concentration producing prominent growth reduction or a 50% reduction in growth
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MWCO	Molecular Weight Cut-Off

M dsRNA	Medium size double stranded RNA
NCCLS	National Comitee for Clinical Laboratory Standarts
NCYC	National Collection of Yeast Cultures
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSSS	Staphylococcal scalded skin syndrome
TEMED	N, N, N' N'- Tetramethylethylenediamine
%T	Ratio of the sum of the weights of the acrylamide monomer and the cross linker in the solution , expressed as %w/v
%C	The ratio of cross-linker to acrylamide monomer
V_{max}	The maximal velocity at saturation
VRE	Vancomycin Resistant *Enterococcus faecium*
YEPD	Yeast extract-peptone-dextrose

CHAPTER I

INTRODUCTION

The microbial flora of human skin presents a distinct ecosystem which consists of a characteristic microflora. Although keratin, lipids and fatty acids make the skin a good habitat for microorganisms; dry conditions, salinity and inhibitory activities of some fatty acids make the skin best tolerated by gram-positive bacteria and some yeasts. Most of the organisms colonizing the skin are harmless and they constitute the normal flora of the skin [1, 2].

Pathogenic bacteria are normally unable to colonize the skin thus when applied to the surface of the skin the number of the bacteria decrease rapidly [3]. The natural antibacterial substances in the secretions, low pH of the skin, moisture content and normal mucocutaneus microflora limit the colonization of the skin by pathogenic strains. The colonization of the skin by pathogenic bacteria takes place if the skin integrity is disrupted by burns or wounds [3]. Most of the bacterial skin disease is seen in immunocompramized patients unable to develop normal immune response thus the probability of the infection is increased [3,4].

Skin diseases affect 20-33% of the population at any one time, seriously interfering with activities in 10% [5]. The infections of hospital-acquired wounds that are acquired surgically or by the use of intravenous medical devices, are among the leading nosocomial causes of morbidity [6].

1.1. Bacterial Skin Infections

There is a high number of bacterial diseases involving skin. They can be grouped as primary pyodermas, secondary bacterial infections, cutaneous involvement in systemic bacterial infections (exclusive of venereal diseases and mycobacterial infections) and infections due to unusual organisms (Figure 1.1.) [3].

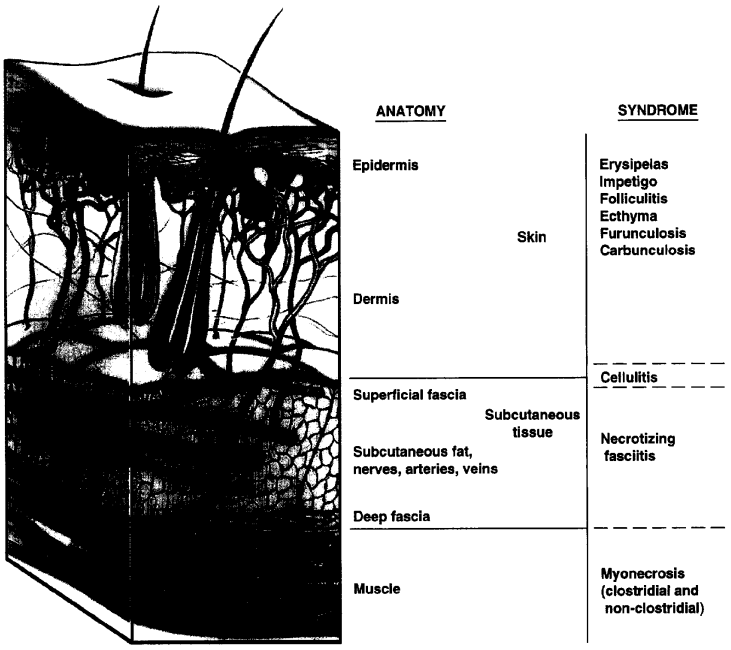

Figure 1.1. Anatomic and Clinical Classification of Skin and Soft Tissue Infections [7].

1.1.1. Gram Positive Bacterial Skin Diseases

Most common skin infections caused by gram-positive bacteria include impetigo, ecthyma, erysipelas, acute cellulitis, acute lymphangitis, necrotizing fasciitis, progressive bacterial synergistic gangrene, scarlet fever, bullous

impetigo, staphylococcal scalded-skin syndrome, folliculitis, fruncles and carbuncles, *Clostridium perfringens* infections, erythrasma, trichomycosis axillaris and pitted keratolysis [8, 9].

1.1.1.a. Impetigo

Impetigo is the superficial infection of the skin commonly caused by group A streptococci and sometimes by *Staphylococcus aureus*. The disease is mostly seen among economically poor children of below school age living in warm, humid climates. Lesions of the disease are initially vesicular with rapid pustulation. Later they rupture and the discharge forms soft, thick golden-yellow crust. Lesions are usually painless and mostly seen on face and extremities (Figure 1.2.) [3, 8, 9].

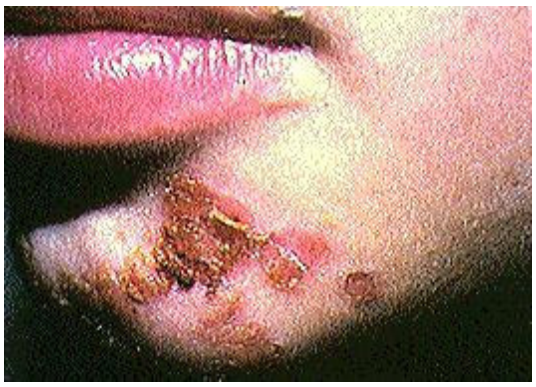

Figure 1.2. Lesions of the Disease Impetigo [10].

1.1.1.b. Ecthyma

Ecthyma differs from impetigo with penetration through the epidermis to produce shallow ulcers. Usually group A streptococci initiates the disease. The

initial lesion is a vesicle seen on lower extremities of children and elderly patients especially with poorly controlled diabetes. The vesicle enlarges and crusts over with grayish-yellow color. The lesions than extend into dermis, they are slow to heal and painful (Figure 1.3.) [3, 8, 11].

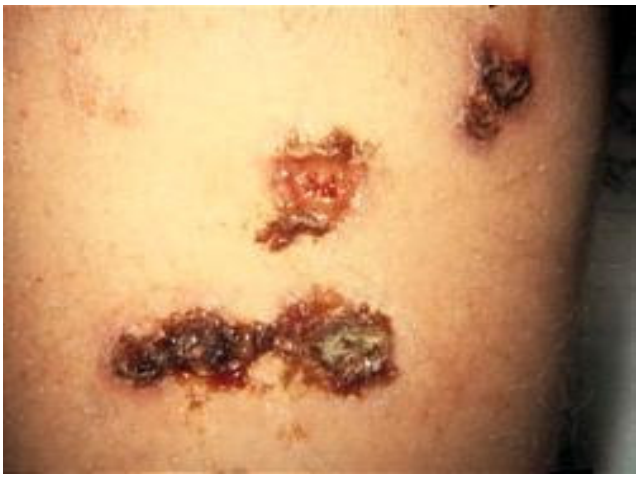

Figure 1.3. Lesions of the Disease Echytma [12].

1.1.1.c. Erysipelas

Erysipelas is a type of superficial cellulitis that involves lymphatic vessels commonly due to group A streptococci, but also streptococci of groups G, C, and B (in newborn) and, rarely, staphylococci [3, 8, 9].

It is mostly seen in young children and the elderly with diabetes mellitus, immunosupression, lymphatic obstruction. Also alcohol usage increases the risk of infection[3, 8, 9].

The lesions are hot, shiny, red, edematous and they spread periferally (Figure 1.4.). There is fever and leukocytosis as well [8].

Erysipelas involves more superficial layers of the skin especially legs and face. Cutaneous lymphatics and the area of inflammation is raised above the surrounding skin, and there is a distinct demarcation between involved and normal skin. The literature indicates that the face is the typical site of erysipelas, but up to 85 percent of cases occur on feet and legs [3, 8, 9].

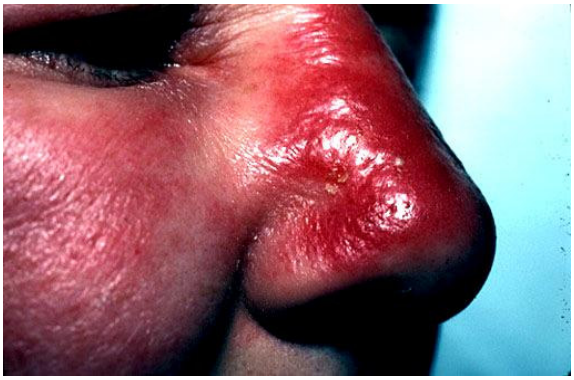

Figure 1.4. Lesions of the Disease Erysipelas [13].

1.1.1.d. Acute Cellulitis

The disease is caused by Group A streptococci, *Staphylococcus aureus* and group B streptococci in newborn. It is the painful, erythematous infection of the dermis and subcutaneous tissue that is characterized by hot, edematous appearence and superficial vesicles that can rupture easily [3].

Cellulitis commonly occurs near breaks on the skin, such as trauma, tinea infections, surgical wounds, or ulcerations (Figure 1.5.). When compared to erysipelas the disease involves deeper subcutaneus tissues and there is no clear distinctions between infected and uninfected skin in acute cellulitis [3, 8, 9].

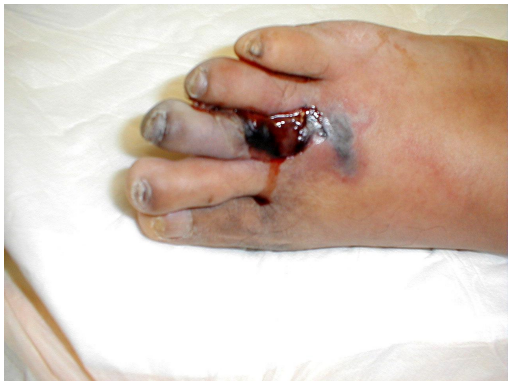

Figure 1.5. Acute Cellulitis in a Poorly Controlled Diabetic Patient [14].

1.1.1.e. Acute Lymphangitis

It is caused by group A streptococci and *Staphylococcus aureus.* The disease involves the subcutaneus lymphatic channels. It is characterized by linear red streaks that extend from the lesion towards the lymph node (Figure 1.6.) [3].

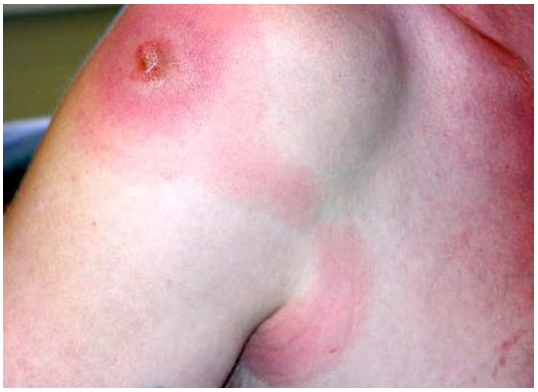

Figure 1.6. Lesions of Acute Lymphangitis [15].

1.1.1.f. Necrotizing Fasciitis (Streptococcal Gangrene)

Necrotizing fasciitis is a subcutaneous tissue and superficial fascia infection with a mortality rate of 30 to 60%. It is caused by group A beta-hemolytic streptococci and *Staphylococcus aureus* [16]. Predisposing factors for the development of necrotizing fasciitis include needle punctures, wounds, minor cuts, burns, splinters, surgical procedures and childbirth. At the beginning, it represents cellulitis but rapidly it turns to gangrene of subcutaneus tissue with the necrosis of overlying skin. The first symptom of the disease is the swelling of an arm or leg that is followed by the bullae filled with clear fluid. Than the area becomes dusky-blue and looks like a third-degree burn. Shock and organ failure can be seen (Figure 1.7.) [3, 8].

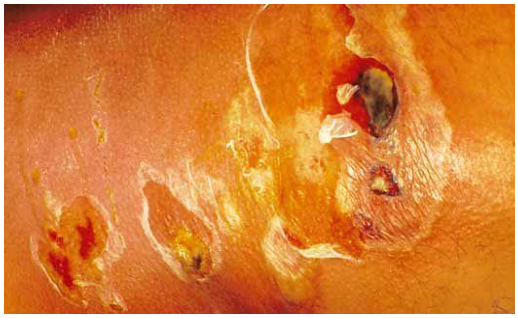

Figure 1.7. Necrotizing Fasciitis due to Group A Streptococcus in a Patient with Varicella [8].

1.1.1.g. Streptococcal Toxic Shock Syndrome

It is an acute multisystem sydrome that resembles toxic shock syndrome of *Staphylococcus aureus*. In 60 percent of patients, the portal of entry is skin or vaginal mucosa. Seeding of infection to deeper tissues from a transient bacteremia originating in the pharynx seems the most likely cause of the disease

in the others. In about 50 percent of patients necrotizing fasciitis or cellulitis is present [3].

First clinical symptoms include chills, fever, nausea, vomiting, diarrhea, hypotension, myalgias, tachycardia, multiorgan disfunction and mental changes. Than the disease continues with increasing pain at the site of infection, shock and organ failure [3, 8].

1.1.1.h. Scarlet Fever

The erythrogenic toxin of the group A streptococci causes an erythematous eruption. The bacteria located in pharyngeal infection causes the disease [8].

A strawberry tongue, red pharynx, sore throat, red rush of skin are the charactheristics of the disease that is most prevalent in children aged 4 to 7 years [3, 17].

1.1.1.i. Bullous Impetigo

The disease is caused by phage group II Staphylococci and it usually occurs in newborn and in older children. It is characterized by the rapid progression of vesicles to large thin-walled bulla (2 to 5 cm) containing dark-yellow fluid (Figure 1.8.). Than they rupture and form thin, varnish-like, brown crusts [3, 9, 18].

1.1.1.j. Staphylococcal Scalded-Skin Syndrome

Staphylococcal scalded skin syndrome (SSSS) is a generalized exfoliative dermatitis caused by exfoliative extoxin-producing strains of phage

group II *Staphylococcus aureus.* It is the most severe skin disease caused by bacteria [3, 20].

It is usually seen in newborns or in older children but it can be seen rarely in adults especially in those with chronic renal failure. The mortality in adults is usually higher when compared to the mortality in infants [3, 20].

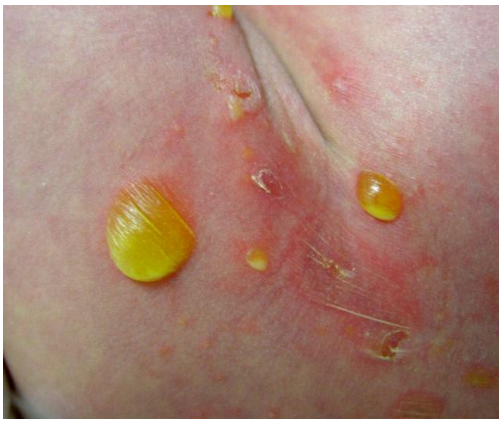

Figure1.8. Vesicles in Bullous Impetigo [19].

It starts with fever and skin tenderness and large bullous are formed that are filled with fluid. After some days bullous rupture and large sheets of skin seperates leaving an area bright red (Figure 1.9.) [3, 20].

1.1.1.k. Folliculitis

It is a form of impetigo with yellowish pustules seen at the openings of the hair follicules inflamed by physical injury or chemical irritation caused by *Staphylococcus aureus* (Figure 1.10.). The most common form is superficial folliculitis with painless pustules healing without scarring. Lesions can appear

on the head, neck, trunk, buttocks, and extremities. Patients with diabetes are more susceptible to the infection [3, 9, 11].

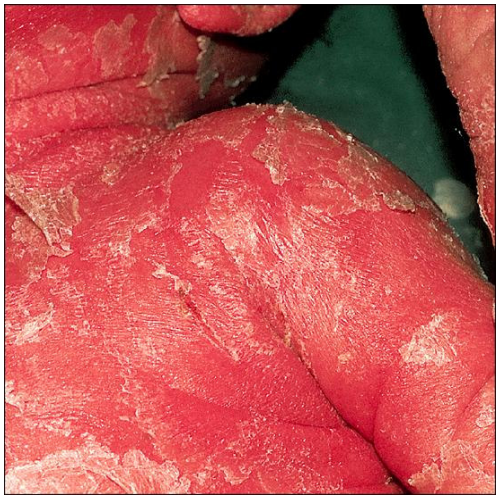

Figure 1.9. Staphylococcal Scalded-Skin Syndrome [21].

1.1.1.l. Furuncles and Carbuncles

Furuncles are the nodules growing near a hair follicule and carbuncles are the deeper ones.

Furuncles are (commonly known as an abscess or boil) painful, erythematous, firm or fluctuant masses of walled-off purulent material, arising from the hair follicle (Figure 1.11.). The lesions may occur anywhere on the body. The causative agent is usually *Staphylococcus aureus* [3, 9, 11].

Carbuncles are deep, broad, swollen, erythematous and painful masses that are usually seen near the hair follicles on the back of the neck in middle-aged and older men. They are the network of furuncles connected by sinus tracts. Fever and malaise are the other symptoms of the disease [3, 9, 11].

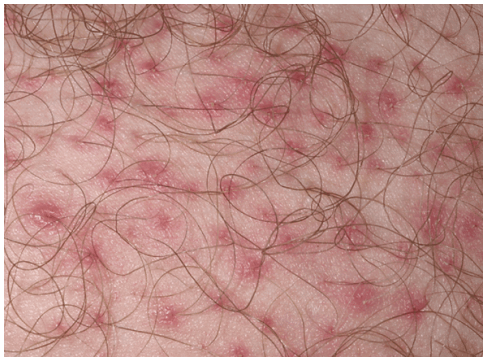

Figure 1.10. Lesions of Folliculitis [22].

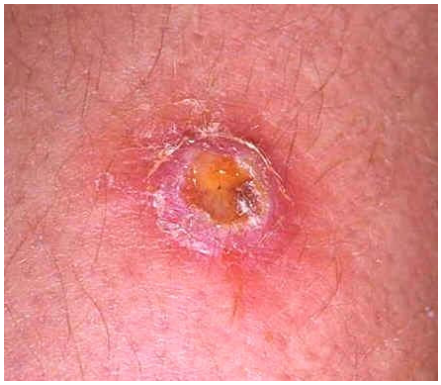

Figure 1.11. Furuncle (Boil) [23].

1.1.1.m. *Clostridium perfringens* Infections of Skin

Anaerobic cellulitis and gas gangrene are the infections involving the subcutaneus tissue and muscles that are caused by the bacteria *Clostridium perfringens.* Anerobic cellulitis starts in a dirty wound with pain and the bacteria causes gas formatian with brown, dark discharge. Infection causes high fever, hypotension, oliguria, tachycardia, severe soft tissue and muscle damage at the end [24].

1.1.1.n. Erythrasma

It is a superficial skin infection characterized by the formation of reddish-brown patches on axillary and genitocrural areas infected with the bacteria *Corynebacterium minutissimum* [3]

The symptoms include itching and discomfort. The production of porphyrins by the pathogen give the lesions a coral-pink color [3, 11].

1.1.1.o. Trichomycosis Axillaris and Pitted Keratolysis

Both are caused by *Corynebacterium spp.* Trichomycosis axillaris is characterized by disagreeable underarm odor. The underarm is coated with black, yellow-white or reddish deposits. Daily cleansing with soap and water generally cures the infection, and regular use of antiperspirants aids in prevention [11].

Pitted keratolysis is characterized by painful burning of the feet caused by wearing occlusive footwear in warm, damp environments. There are small, punctate, pitted lesions on calloused areas of the feet and a disagreeable foot odor [11].

There are some other less common skin disease which are also caused by gram-positive bacteria and they are not involved here.

1.1.2. Gram Negative Bacterial Skin Diseases

Some of the skin disease caused by gram-negative bacteria include, acute meningococcocemia, infections of *Pseudomonas aeroginosa, Haemophilus influenza* and *Salmonella spp.* [3].

1.1.2.a. Acute Meningococcocemia

The disease is caused by *Neisseria meningitidis*. Patients with this disease show consciousness and meningeal irritation and the cutaneus lesions which can be papular, macular or urticarial. Petechias, bullous and gangrenous hemorrhagic areas can develop [3].

1.1.2.b. Skin Infections of *Pseudomonas aeroginosa*

Pseudomonas aeroginosa infections occur in immunocompromized patients. Paronychial lesions of blue or green color that cause pain are the characteristics of the disease (Figure 1.12.). Also vesicles and bullae, ecthyma gangrenosum, gangrenous cellulitis and some nodular lesions are the other skin diseases caused by the same organism [3].

Figure 1.12. Green nail [25].

1.1.2.c. Skin Infections of *Haemophilus influenza*

The bacteria cause a cellulitis with blue-red or purple-red lesions on face, neck, and arms of especially young children [3].

1.1.2.d. Rose spots of *Salmonella* Infections

Salmonella causes enteric infections due to contaminated food or water. During these infections after 7 to 10 days pink papules may appear on nipple or extremities. These papules are named as rose spots and they will disappear after 3 to 4 days [3].

1.1.2.e. Skin Infections of Other Gram-Negative Bacteria

These infections can be caused because of the contamination of skin and subcutaneous tissue in the sites of injury or surgery. Gas-containing cellulitis is caused by *E. coli, Klebsiella* and *Aeromonas hydrophila* in the presence of *Bacteroides spp.* or anaerobic streptococci [3].

1.1.3. Skin Diseases Related to Intimate Contact with Animals

1.1.3.a. Anthrax

Anthrax, caused by *Bacillus anthracis*, is an often fatal bacterial infection that occurs when *Bacillus anthracis* endospores enter the body through abrasions in the skin or by inhalation or ingestion. Anthrax is a zoonosis to most mammals so that human infections result from exposure to animals and their products [3, 26].

The disease has two forms as cutaneous and systemic anthrax. Systemic infection resulting from inhalation of the organism has the symptoms of hypotension, shock, and sudden death with a mortality rate approaching 100 percent. Cutaneous anthrax, is usually curable. A small percentage of cutaneous infections become systemic, and these can be fatal. Disease starts with malaise and fever. Skin lesions are seen on exposure area. Characterized skin lesions of

anthrax are the vesicles that turn into hemorrhagic and necrotic ones. Disease continues with vomiting, nausea, fever, pain in abdominal area and hypotension. Mortality rate of the disease is 25 to 50 percent range [3, 26].

1.1.3.b. Erysipeloid

Erysipeloid is caused by *Erysipelothrix rhusiopathiae* and it is known as acute infection of traumatized skin mostly seen in butchers, fisherman and people handling raw fish and meat. Skin lesions are on hands and fingers and they are purple-red in color (Figure 1.13.). Ulceration can be seen [3].

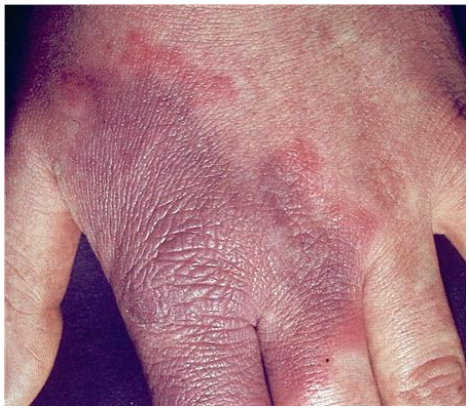

Figure 1.13. Erysipeloid [27].

1.1.4. Diseases Associated with Geographic Distrubituon

1.1.4.a. Bartonellosis

A disease caused by *Bartonella bacilliformis* with nodular cutaneus eruptions seen in South America [3].

1.1.4.b. Plaque

Plaque is caused by *Yersinia pestis*. It is transmitted between human and rhodonts by the way of fleas. The disease is seen in western United States, Africa and Wietnam [3].

It starts with malaise, myalgia than continues with backache and high fever. Skin lesions include buboes [3].

1.1.5. Tularemia

It is caused by *Franciella tularensis* and characterized by chancre-like ulcers [3].

1.1.6. Diphteria

Diphteria is a disease caused by *Corynebacteriun diphtheriae*. Skin lesions are covered with the membraned zone of erythema. The membrane becomes dark brown [3].

1.1.7. Wound Infections Caused by *Vibrio* Species

Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus and Vibrio damsela cause wound infections [3].

1.1.8. Mycobacterial Infections

Mycobacterium spp. causes many different forms of tuberculosis which include skin lesions known as chancres. They turn into small ulcers with

abscesses that can heal up to 12 months without treatment. In some other forms of tuberculosis larger and severe ulcers can develop [3].

1.2. Treatment of Bacterial Skin Infections

The selection of the appropriate therapy is made initially on the basis of the appearence of the skin lesion, the characteristics of any systemic disease and a Gram-stained smear of material from a lesion if available to sample. The therapy is applied according to the results of the susceptibility testing of the isolated pathogen [3].

Primary cutaneous infections of mild to moderate severity can be treated with local measures, topical drugs, oral antibiotics or by a combination of these methods. Extensive infections of the skin, with or without systemic manifestations, are treated with parenteral antibiotics in adequate dosage [3].

A number of factors are considered in the adminstration of drugs. Oral treatment can be limited by gastrointestinal disturbances or adsorbtion. Hypotension and severe thrombopenia can prohibit the intramuscular route. If intravenous or intramuscular route is used, sterility will be very important [3].

Topical antibacterial agents have also frequently used to prevent and to suppress the bacterial growth in burns and other open lesions. The emergence of bacterial resistance and sensitization are the limitations of this treatment [3].

Streptococcus pyogenes continues to be exquisitely susceptible to β-lactam antibiotics and numerous studies have demonstrated the clinical efficacy of penicillin in treating most group A streptococcal infections. Penicillin is the first choice for the antibiotic therapy of most Streptococcal skin and soft tissue infections including erysipelas, impetigo, cellulitis and others. In more aggressive infections, such as necrotizing fasciitis, empyema and myositis, penicillin treatment is still associated with high mortality and extensive

morbidity. Patients with these disorders should be treated with broad-spectrum antimicrobial regimens which are effective against the pathogen such as expanded-spectrum penicillins and cephalosporins, clindamycin and aminoglycosides in combinations that are suggested by the clinical manifestation and epidemiologic features of the case [8].

A course of oral erythromycin, plus vigorous daily cleansing with soap and water generally cures erythrasma caused by corynebacteria and for trichomycosis axillaris and pitted keratolysis caused by the same microorganism, frequent cleansing and application of topical erythromycin are generally effective. Use of antiperspirants also aids in cure and prevents recurrence [11].

Frequent use of soap and water and the use of topical antibiotic agents, such as mupirocin or bacitracin (Neosporin, Polysporin), generally clear the lesions of folliculitis. Occasionally, the addition of a systemic antistaphylococcal agent is required. Topical therapy with erythromycin, clindamycin, mupirocin or benzoyl peroxide can be administered [9].

Treatment of fruncles and carbuncles often requires drainage of the lesion. Antibiotic therapy should be considered if the furuncle is not yet fluctuant, if there is evidence of surrounding cellulitis or lymphadenitis, or if the lesion is on the face [11]. In severe cases, parenteral antibiotics such as cloxacillin or a first generation cephalosporin are required [9].

The majority of bacterial skin infections are caused by *Staphylococcus* and *Streptococcus* species. Antibiotics should be used empirically with consideration for resistance patterns. Current antibiotic recommendations include penicillinase-resistant penicillins, first-generation cephalosporins, azithromycin, clarithromycin, amoxicillin clavulanic acid, or a second-generation fluoroquinolone in the skeletally mature patient. Gram-negative coverage with a second-, third-, or fourth-generation cephalosporin is usually

indicated in children under three years and in patients with diabetes or who are immunocompromised [9].

1.3. Antibacterial Drug Resistance and the Need for New Alternatives for Treatment of Skin Disease and Wound Infections

The emergence of antibiotic-resistant bacteria worldwide and the lack of new antibiotics to combat such pathogens continue to be matters of concern for the medical community [28]. Hospitals are now faced with the problem of controlling infections caused by multidrug-resistant organisms, including methicillin-resistant staphylococci, penicilin and erythromycin-resistant pneumococci and vancomycin-resistant enterococci [29].

For wound infections, the current standard of care involves using systemic antibiotics or topical antimicrobial agents, such as silver sulfadiazine, mafenide acetate and gentamicin sulfate which are initially introduced in the 1960s. These products have various limitations including a limited ability to penetrate partial-and full-thickness burns, limited efficacy against both gram-positive and gram-negative bacteria, potential toxicity to host cells and the emergence of antibiotic-resistant bacteria. Recent reports show that mortality remains significantly higher in patients who receive inadequate antimicrobial therapy and this supports the need for novel strategies to prevent and treat wound infections [28, 30].

In skin and soft tissue infections, the incidence of infections caused by multidrug-resistant gram-positive organisms, which are the major pathogens in these infections as *Staphylococcus aureus*, has been increasing over the last 20 years [31].

Methicillin-resistant *Staphylococcus aureus* (MRSA) first reported in 1961 following the introduction of the semisynthetic lactamase-resistant penicillins, has increased in prevalence and the proportion of isolates resistant to

methicillin in USA raised from 2.4% in 1975 to 29% in 1991 [29]. Methicillin-resistant *Staphylococcus aureus* which encompasses resistance to all currently available β-lactams, macrolides, lincosamides and aminoglycosids is a common phenotype among multiresistant nosocomial isolates [32, 33]. A recent survey of more than 7000 isolates of *S. aureus* from ten western European countries indicated that 12.8% were methicillin-resistant [29]. Today MRSA is recognized as a major nosocomial pathogen, accounting for 28% of surgical wound infections and 21% of skin infections in the USA [29]. Vancomycin has been widely used to treat MRSA infections, and unsurprisingly, this has led to the identification of vancomycin-intermediate *S. aureus* in 1997 and fully vancomycin-resistant *S. aureus* in 2002. *S. aureus* continues to be a significant health problem in the hospital and other settings, such as nursing homes. Nasal carriage of *S. aureus* is an important risk factor in the hospital setting, particularly in patients requiring surgery, implanted devices or hemodialysis [32]. Community-acquired MRSA is also becoming a problem, causing infective endocarditis in intravenous drug users and invasive infections in immunocompromised patients [29].

Enterococcus has been declared the pathogen of the 1990s. Enterococci is the second leading cause of nosocomial infections and an increase in the emergence of multidrug-resistant *Enterococcus faecium* has been described [34]. Since the first report of vancomycin-resistant enterococci (VRE) in 1988, VRE have spread worldwide with unanticipated rapidity [28]. Especially *Enterococcus faecium* is intrinsically resistant to β-lactams and aminoglycosides and has acquired high-level resistance to many other antibacterials [35]. Vancomycin has been used to treat such resistant gram-positive bacterial infections for some time. However, the wide spread of vancomycin-resistant enterococci (VRE) reduces the clinical efficacy of vancomycin, and the lack of effective drugs against such multiresistant pathogens is also a serious medical problem [35].

Unfortunately, effective therapy against VRE is presently lacking [34].

Consequently, the priority for the next decades should be focused in the development of alternative drugs and/or the recovery of natural molecules that would allow the consistent and proper control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance [36].

The new generation of native peptide and proteins, also known as antimicrobial peptides and proteins, isolated from a full range of organisms and species from bacteria to man are suggested as a new alternative for the treatment of pathogen-caused diseases. They have been termed as "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria and filamentous fungi in addition to protozoan and metazoan parasites [36].

Yeast killer proteins which are produced and secreted into the environment by certain yeast strains with a killer phenotype (K^+) are highly suggested as antimicrobial agents in the medical field and may be used as a new and natural alternative to chemical antibiotics after further studies [37]. Yeast killer proteins can not be used as systemic antibiotics because they are antigenic but the ones that are stable at wide pH and temperature ranges might be used as topical antimicrobials in the treatment of superficial infections [30, 32].

1.4. Yeasts and Yeast Killer System

Yeasts are unicellular, simple single-cell true fungi that are oval or spherical in shape whose individual cell size can vary widely from 2-3µm to 20-50µm in length and 1-10µm in width. Yeasts are facultative anaerobes and they reproduce asexually by budding or fission. Budding process starts when mother cell attains a critical cell size that will coincide with the onset of DNA synthesis. Localized weakining of the cell wall with the tension exerted by turgor pressure allows extrusion of cytoplasm in an area bounded by a new cell wall. Cell wall

polisaccharides are mainly synthesized by glucan and chitin synthetases. One of the nucleus migrates into the bud and after the formation of the cell wall between two cells the bud breaks away. In multilateral budding which is typical in ascomycetous yeasts, including *Saccharomyces cerevisiae*, buds may arise at any point on the mother cell surface but never again at the same site (Figure 1.14.) [38, 39].

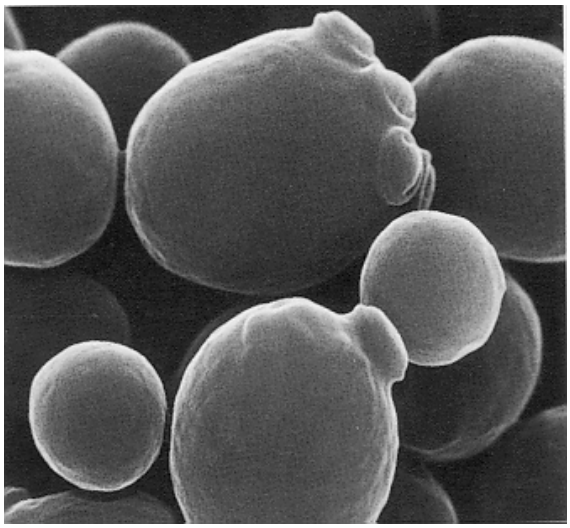

Figure 1.14. Scanning Electron Micrograph of the Budding Yeast *Saccharomyces cerevisiae* [40].

Some yeasts, such as *Saccharomyces cerevisiae,* can also reproduce sexually by a process called mating. The life cycle of *Saccharomyces cerevisiae* involves haploid forms of two distinct mating types (a and α type) that can fuse to form a diploid a/α cell, called zygote. These diploits will reproduce vegetatively by budding under rich growth conditions, but under conditions of nitrogen and fermentable carbon limitation diploit cells are induced to initiate meiosis and sporulation to form four haploid sexual spores (a and α) called ascospores and allow for genetic recombination (Figure 1.15.) [39, 41].

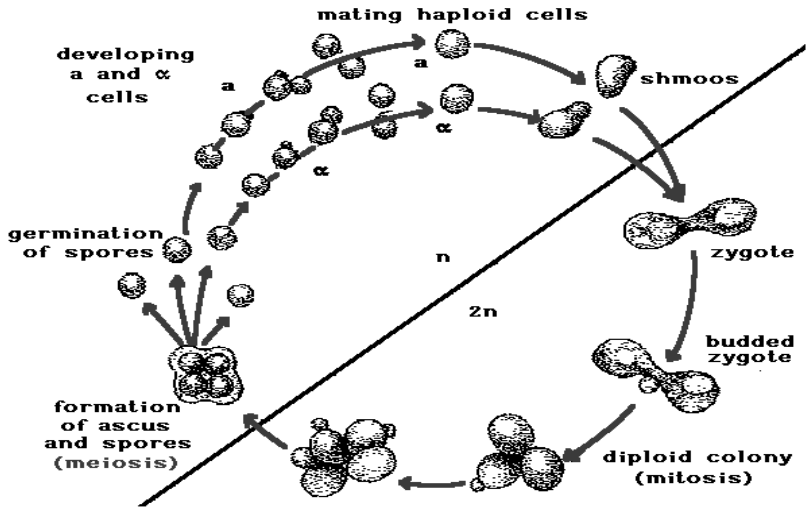

Figure 1.15. Yeast Life Cycle [42].

Yeasts are considered to be the best organisms for the study of basic eukaryotic genetics. Baker's yeast (*Saccharomyces cerevisiae*, 5,600 genes) was the first eukaryote genome to be sequenced fully, and fission yeast (*Schizosaccharomyces pombe*, 4,940 genes) was the second fungal genome to be completed. The information gleaned from fungal genomics and proteomics is providing valuable insight into human genetics and heritable disorders. The yeasts, most notably the yeast *S. cerevisiae*, play increasingly significant roles as model eukaryotic cells in furthering our fundemental knowledge of biological and biomedical science [39, 43].

Yeasts are the most important and the most extensively used microorganisms in industry. They are cultured for the cells themselves, for cell components, and for the end products that they produce during alcholic fermentation. Yeast cells are used in the manufacture of bread, and as sourses of food, food suplements, animal feed, vitamins as vitamin B and D. Yeast cells are also used in enzyme production including invertase and galactosidase,

glycerol production and the production of other growth factors. Large-scale fermentation by yeast is responsible for the production of alcohol for industrial purposes but yeast is better known for its role in the manufacture of alcholic beverages; beer, wine and liquors (Table 1.1.) [4].

Table 1.1. Industrial Uses of Yeast [4].

Production of Yeast Cells
Baker's yeast, for bread making
Dried food yeast, for food supplements, for animal feeds
Fermentation Products From Yeast
Ethanol for endustrial alchohol and as a gasoline extender
Glycerol
Yeast Products
Yeast extract for culture media,
Enzymes for food industry; invertase , galactosidase ,
B vitamins, vitamin D ,
Biochemicals for research; ATP , NAD , RNA
Beverage Alchohol
Beer, wine, whiskey, brandy, vodka, rum

The yeast *S. cerevisiae* was derived from wild yeast used in ancient times for the manufacture of wine and beer. The yeasts currently used are the descendants of the early *S. cerevisiae* [4]. The bakers use yeast as a leavening agent in the rising of the dough prior to baking and for the flavour of the bread [4].

Yeasts are attractive hosts with their rapid growth and ease of genetic manipulation for the production of heterologous proteins Their eukaryotic subcellular organization enables them to carry out many of the post–translational folding, processing and modification events required to produce bioactive mammalian proteins [44]. The gene for human interferon, human alpha-1-antitrypsin and somatostatin has been cloned and expressed in yeast [44-46].

Yeasts are also used in environmental technologies such as bioremediation [47].

Killer phenomenon [K^+] is widely distributed among yeasts and was first discovered by Bevan and Makower in 1963, in laboratory strains of *S. cerevisiae* which were isolated as brewery contaminant [48]. Certain yeast strains termed as killer yeasts, produce and secrete into the medium proteins or glycoproteins that are inhibitory to sensitive microbial cells. These compounds are designated as killer factors, killer toxins or killer proteins [49, 50]. These extruded killer proteins inhibit the growth of the same species or of different species of a single genus, and sometimes even representatives of other genera that are sensitive to them [51-53]. Also killer toxins of certain yeast strains have potential growth inhibitory affect on gram-positive pathogenic bacteria and plant pathogenic fungi [54, 55].

Killer phenomenon is widespread among many yeast genera such as *Candida, Hansenula, Pichia, Hanseniaspora, Kluyveromyces, Rhodotorula, Torulopsis, Trichosperon, Ustilago, Williopsis, Zygowilliopsis, Cryptococcus* and *Debaryomyces* [56-59].

Production of yeast killer toxin confers advantage to the yeast strains in competition with sensitive strains for nutrients available in their environment [60].

Killer yeast strains are immune to their own toxins and this phenomenon is known as self-immunity [56].

Yeast killer proteins are proteinaceous substances as they are protease-sensitive. Generally they are inactivated at elevated temperatures. Most of them are stable and act only at acidic pH values [56, 61].

Killer yeast strains were classified into 10 different types (K1-K10) by Young and Yagiu [56]. Later killer yeasts were classified into 11 distinct groups (K1-K11) based on the killing and immunity spectrum among them with the addition of a new killer strain (K11) to the previous classification (Table 1.2.) [62].

Table 1.2. Killer Yeasts and Their Protein Toxins [56].

Killer Protein Producing Strain	Classification
Saccharomyces cerevisiae	K1
Saccharomyces cerevisiae	K2
Saccharomyces capensis	K3
Candida glabrata	K4
Pichia anomala	K5
Kluyveromyces fragilis	K6
Candida valida	K7
Hansenula anomala	K8
Hansenula mrakii	K9
Kluyveromyces drosophilarum	K10
Candida glabrata	K11

1.4.1. Genetic Basis of Killer System

Yeasts are considered to be the best organisms for the study of basic eukaryotic genetics. The yeasts, most notably the yeast *S. cerevisiae*, play increasingly significant roles as model eukaryotic cells in furthering our fundemental knowledge of biological and biomedical science. *Saccharomyces cerevisiae*, was the first eukaryote genome fully sequenced [39, 43]. *Sacchromyces cerevisiae* contains a haploid set of 16 well-characterized chromosomes, ranging in size from 200 to 2,200 kb. The total sequence of chromosomal DNA, constituting 12,052 kb was released in April 1996 [63]. The yeast genome is highly compact, with genes representing 72% of the total sequence and in addition, chromosomes contain mobile DNA elements, retrotransposons, which vary in number and position in different strains of *S. cerevisiae*. Other nucleic acid entities considered as part of the yeast genome are shown in Figure 1.16. The 2-µm circle plasmids, present in most strains of *S. cerevisiae*, apparently function solely for their own replication [63-66].

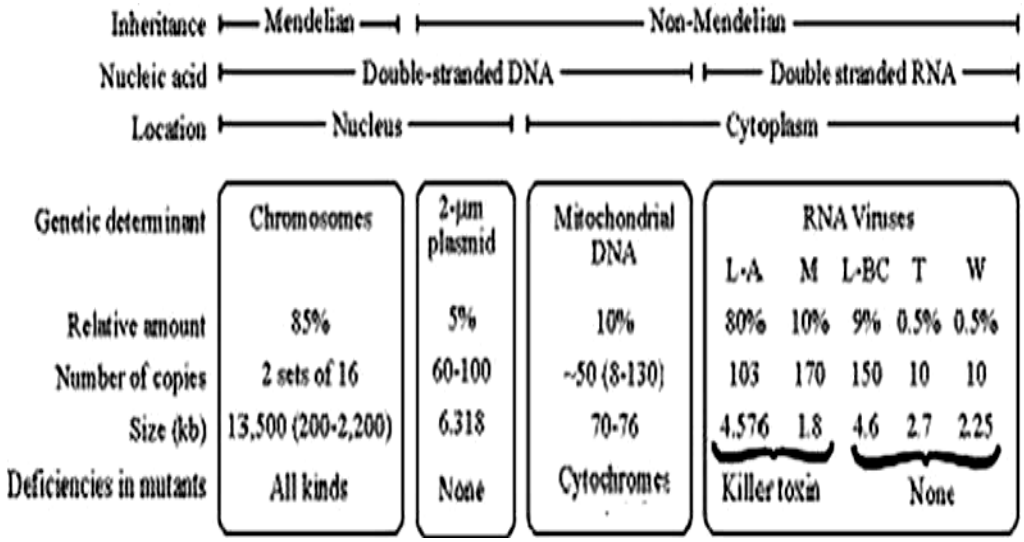

Figure 1.16. The Genome of a Diploid Cell of *S. cerevisiae* [64].

The genetic basis for killer phenotype expression can be quite variable; the genomes of the killer proteins have been mapped either on double-stranded RNA viruses, linear double-stranded DNA plasmids or on a chromosome [59]. Table 1.3 shows genetic basis for killer phenotype expression in yeast.

Table 1.3. Genetic Basis for Killer Phenotype Expression in Yeast [59, 62, 67, 68]

Yeast	*Genetic Basis*	*Toxin Gene*
Saccharomyces cerevisiae	dsRNA virus	M1- , M2- , M28
Hansenula uvarum	dsRNA virus	M-dsRNA
Zygosaccharomyces bailii	dsRNA virus	M-dsRNA
Ustilago maydis	dsRNA virus	M-dsRNA
Kluyveromyces lactis	linear dsDNA plasmid	pGKl1
Kluyveromyces fragilis	Chromosomal	Not identified
Pichia acaice	linear dsDNA plasmid	pPac1
Pichia inositovora	linear dsDNA plasmid	pPin1
Pichia kluyveri	Chromosomal	Not identified
Pichia farinosa	Chromosomal	SMK 1
Pichia anomala	Chromosomal	Not identified
Williopsis mrakii	Chromosomal	HMK
Williopsis saturnus	Chromosomal	Not identified
Schwanniomyces occidentalis	Chromosomal	Not identified
Debaryomyces hansenii	Chromosomal	Not identified
Candida glabrata	Chromosomal	Not identified

1.4.1.a. dsRNA Virus Based Yeast Killer Systems

The killer phenomenon is brought about by virus like particles occuring in yeast cytoplasm in cooperation with nuclear genes. The similarity of these particles with viruses consists in the fact that their genomes are usually dsRNA and encapsilated. The particles are non-infective but can be transfered by sexual hybridization, protoplast fusion or cytoduction. Their properties are similar to those of plasmids, and, therefore, the killer dsRNA determinants are often designated as virus-like plasmids [50].

Strains of *S. cerevisiae* carry as many as five non-homologous species of dsRNA called L_A, L_{BC}, T, W and M. All of them show non-Mendelian inheritance. L_A, L_{BC} and M are found in intracellular virus-like particles (VLPs) [66]. Production of the killer protein and the immunity to it are encoded by ribonucleic acid designated as MdsRNA. There are different MdsRNAs with different size M_1dsRNA (1.9 kB), M_2dsRNA (1.7 kB), M_3dsRNA (1.5 kB) correspond to individual types of strains; i.e., K1, K2, K3 respectively. These MdsRNAs are present in the cell in high copy number about 10-100 [50]. A larger dsRNA (4.7 kB) designated as LdsRNA is present in high copy number in cells. There are different types of LdsRNAs which are responsible from the replication and encapsidation of MdsRNAs. $L_A$dsRNAs code for capsid proteins while L_B, L_C and L_{BC}dsRNAs are associated with the synthesis of the protein of their own capsids. In a series of *S. cerevisiae* strains other dsRNA types are also present which are designated as T, W and XL but their function and their relation with killer phenomenon is not known but several studies showed that they can be M dsRNAs with several deletions [50, 69].

1.4.1.b. Linear dsDNA Plasmid Based Killer Systems

In some killer yeasts, killer character is encoded by linear dsDNA plasmids that have been identified for various yeast genera such as *Debaryomyces, Wingea, Kluyveromyces* and *Saccharomyces* [70-72].

The production of the killer toxin in *Kluyveromyces lactis* killer system depend on the presence of two linear, double-stranded DNA plasmids which have been termed as pGKL1 and pGKL2 respectively (Figure 1.17.). In this system, pGKL1 encodes for the precursors of the subunits of the killer toxin and involved in immunity phenotype and pGKL2 appers to be necessary for the replication and maintainance of the plasmids (Figure 1.18.) [70-72].

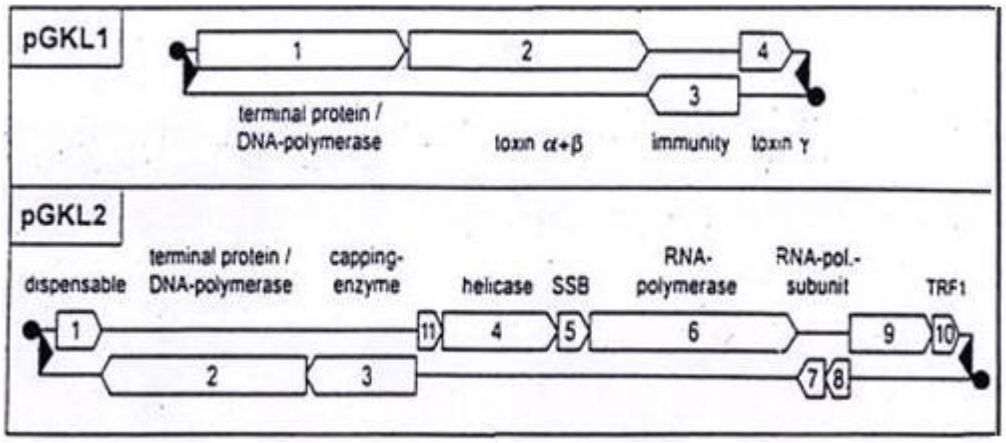

Figure 1.17. Schematic Representation of *K. lactis* Linear Plasmids [73].

The presence of three linear dsDNA plasmids, of approximately 18, 13, and 10 kbp, has been reported in a killer toxin producing strain of *Pichia inositovora*. Only two of them (p*Pin*1-1 and p*Pin*1-3) seem to be associated with the killer phenotype, while the loss of p*Pin*1-2 has no effect on toxin production or susceptibility [72].

Immunity function does not appear to be associated with the plasmids but is probably chromosomally encoded. The killer toxin apparently is an acidic heat-labile glycoprotein whose characterization and range of actions have not yet been determined [72].

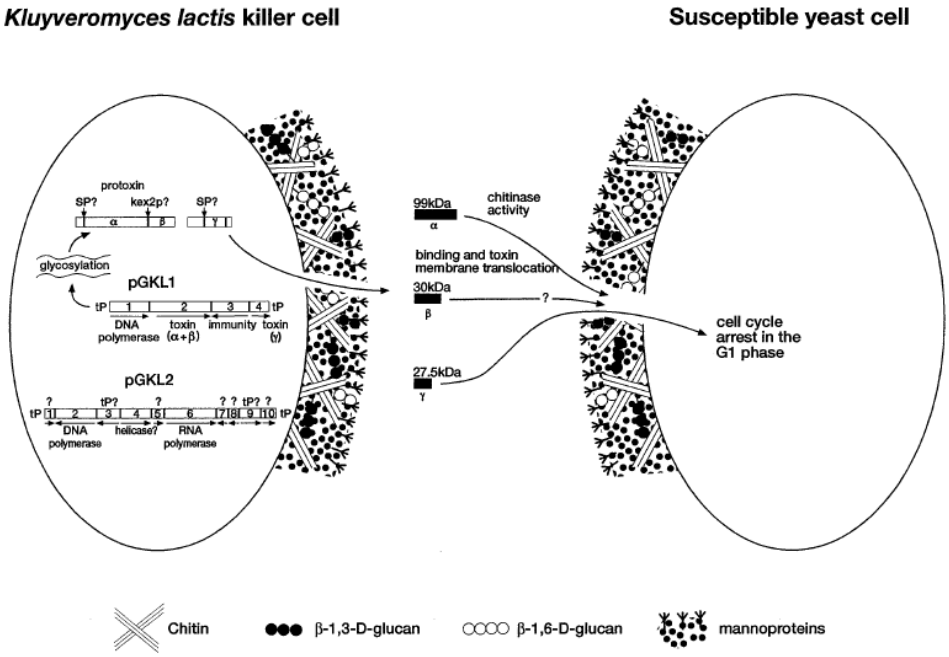

Figure 1.18. Sketch of the Genetics, Toxigenesis, Immunity and Activity of the *K. lactis* Killer System [72].

Pichia acaciae killer strains have been shown to possess two linear plasmids, p*Pac*1-1 (13.6 kbp) and p*Pac*1-2 (6.8 kbp) that are quite similar in both function and structural organization to those found in *K. lactis.* The larger one, p*Pac*1-2 is thought to be involved in toxin production [72].

1.4.1.c. Chromosomally Encoded Yeast Killer Systems

In some killer yeasts such as strains of *Williopsis, Pichia, Candida, Debaryomyces* and *Torulopsis* the killer character is not encoded by dsRNA viruses or dsDNA plasmids so it is suggested that the genes responsible for killer character are located on a chromosome. In one of the killer strains of *Saccharomyces cerevisiae*, the killer character was found to be encoded by two

different genes which are mapped on chromosome V and chromosome IX [74, 75].

In 1990 *Goto et al.* discovered two new killer activities. The killer genes were found to be encoded on chromosomal DNA of a strain of *S. cerevisiae*. KHR (killer of heat resistant) gene, is encoded on chromosome IX and KHS (killer of heat susceptible) gene is encoded on chromosome V and they differ in their thermostability and optimum pH [75]. Since the mature toxins have molecular masses lower than those of their precursors, some protein processing is thought to occur during maturation. KHR encodes a preprotoxin of 33 kDa, which has a possible hydrophobic signal sequence in the N-terminal site region, four competent sites for glycosylation, and five cleavage sites that might be cut by kex2 protease. The mature KHR toxin does not have clearly hydrophobic regions, and so its mode of action is probably different from that of K1. KHS encodes for a 79-kDa precursor with a hydrophobic N-terminal sequence that is probably spliced to produce the mature toxin. The mature KHS toxin shows three clusters of hydrophobic amino acid sequences that might have an ionophore function similar to that of K1 or K2 toxins. These killer genes might also contain regions responsible for immunity of the yeast to its own killer toxin [72, 75].

The HMK gene, encoding a killer toxin (HMK) of *Hansenula mrakii*, and the HSK gene, encoding a killer toxin of *H. saturnus* were cloned. Killer protein encoded by these chromosomal genes has higher thermostability and wider pH-stability than other killer toxins [76].

1.4.1.d. Structure, Processing and Secretion of Killer Toxins

Studies of the killer system particularly in *S. cerevisiae* resulted in substantial progress in different fields of biology providing important insights into basic and more general aspects of eukaryotic cell biology, virus-host cell interactions and yeast biology. Since yeast killer toxins resemble naturally secreted proteins or glycoproteins, detailed analysis of their structure and

synthesis give information about the mechanisms of post-translational prepro-toxin processing in the eukaryotic secretion pathway [59].

Toxin secretion pathways are fully identified for K1 and K28 which are both secreted by *S. cerevisiae*. The best-studied and best-known killer toxin, K1 (19 kDa), is secreted as a molecule consisting of two distinct disulfide bonded unglycosylated subunits, termed α (9.5 kD) and β (9.0 kD) derived from a 42-kDa glycosylated precursor molecule (protoxin). The α and β domains flank a segment called γ, which is not part of the mature toxin and assumed to be the immunity determinant. Protoxin is glycosylated at several sites within the γ segment [50, 77].

The primary translation product of M1dsRNA is a 35-kDa polypeptide (preprotoxin, M1p) consisting of a 44-amino-acid N-terminal leader sequence called d, which includes a 26-residue signal peptide, followed by the 103-residue α domain and the 83-residue β domain of toxin subunits, which are separated by a central γ peptide carrying all three potential N-glycosylation sites [50, 66, 69].

Preprotoxin enters the endoplasmic reticulum with the help of a highly hydrophobic signal peptide in the N-terminal region. In the endoplasmic reticulum cleavage of the leading hydrophobic sequence by a signal peptidase and glycolysation of γ subunit takes place to produce protoxin. In golgi compartment the Kex2p, which is the product of KEX2 gene, cleaves the proregion and removes the intramolecular γ sequence. A carboxypeptidase Kex1p, which is the product of KEX1 gene, removes the dipeptide region on the C terminal of the α subunit. Then mature toxin is transferred to a secretory vesicle and secreted outside the cell via the established yeast secretory pathway as a dimeric molecule in which the subunits are covalently linked by disulfide bonds that probably link the three cysteine residues in α and β. Processing of the killer toxin also requires the products of SEC genes, responsible for general secretion. Mutations in these genes result in accumulation of the glycosylated

protoxin in the endoplasmic reticulum or secretory vesicles [50, 59, 66, 69, 77, 78].

At the same time, the killer cell is effectively protected against its own toxin. Several lines of evidence suggest that the protoxin itself may be the immunity protein. However, the detailed mechanism of immunity is still largely unknown; the protoxin could, for example, bind to toxin receptors on the cell membrane much more tightly than toxin itself and would thus prevent the active toxin from binding and acting [50, 37].

Secretion pathway of K28 is shown in Figure 1.19. and processing of K1 type yeast killer toxin is shown in Figure 1.20.

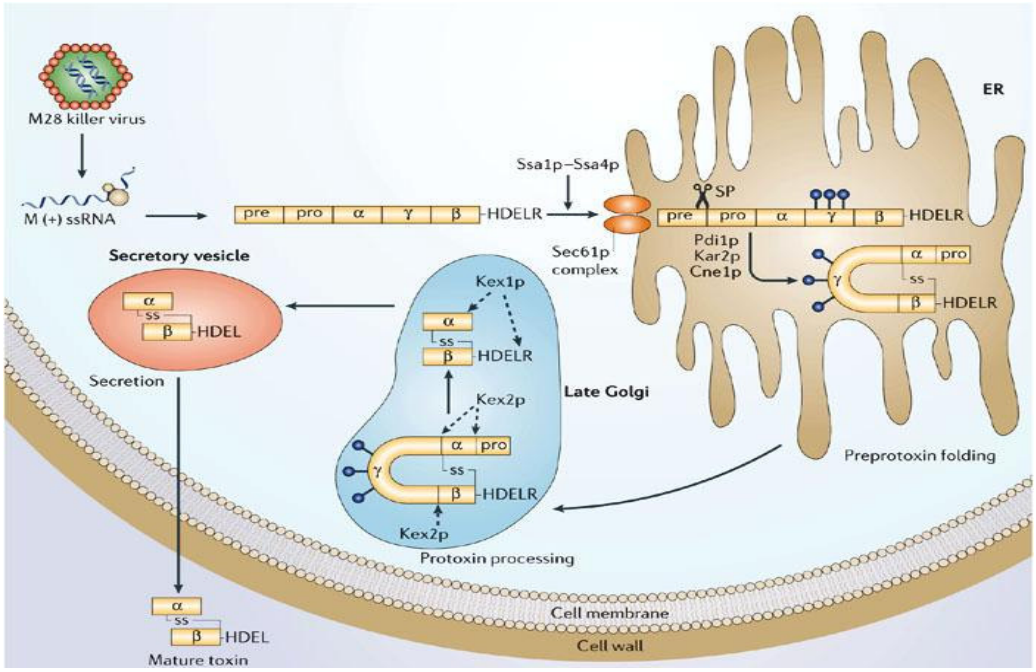

Figure 1.19. Secretory Pathway of Killer Toxin K28 in *S. cerevisiae* [79].

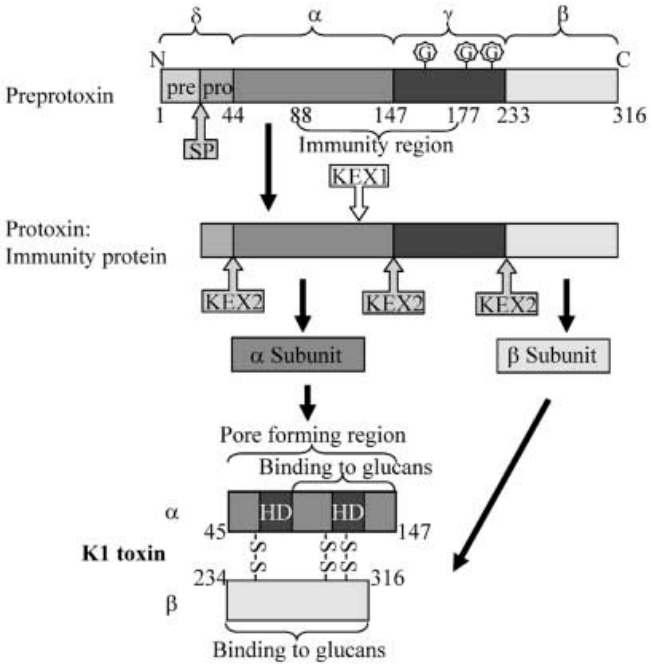

Figure 1.20. Structure and Processing of the K1 Type Yeast Killer Toxin [80].

1.4.2. Mode of Action of the Yeast Killer Proteins

Although the killer toxins possess different modes of action, all viral toxins kill the sensitive yeast cells in a receptor-mediated two-step process. The first step involves a fast and energy-independent binding to a toxin receptor within the cell wall of a target cell. It is suggested that toxin binding to the primary cell wall receptor concentrates the toxin at the level of the cell wall or mediates close contact between the toxin and the target cell membrane. The second step is energy-dependent and involves the toxin translocation to the cytoplasmic membrane and interaction with a secondary membrane receptor [59].

Mannoproteins, chitin, (1-3)-β-D-linked glucans and (1-6)-β-D-linked glucans of the cell wall have been suggested as primary cell wall reseptors of killer toxins [53, 62, 81-83]. Mannoproteins are the receptors for K28 killer toxin of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii,* (1-6)-β-D-

linked glucans of the cell wall are the receptors for K1 and K2 toxins of *S. cerevisiae* and for the toxins from genera *Candida, Torulapsis, Pichia, Kluyceromyces* and *Debaryomyces*. (1-3)-β-D-linked glucans are the receptors for the *Hansenula mrakii* and *Pichia anomala* killer toxin and chitin is the primary receptor for the toxin of *Kluyveromyces lactis* and *Pichia acaciae* [53, 81, 83, 84].

The first step of the binding of the K1 type killer toxin of *S. cerevisiae* to the (1-6)-β-D-linked glucan residues on the yeast cell wall as primary receptors is strongly pH dependent. The second step is a high-affinity, low-velocity, energy dependent interaction of the toxin with a probable plasma membrane receptor that leads to the actual lethal effect. After binding to the yeast cell wall, K1 toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cation transmembrane channels, which causes ion leakage and subsequent cell death [72].

K2 type killer toxin of *S. cerevisiae* has virtually identical toxin activity to that of K1, but K28 killer toxin of *S. cerevisiae* seems to act on the cell cycle in a different way. The K28 toxin binds primarily to the α-1,3-linked mannose residues of a 185-kDa cell wall mannoprotein leading to nonseparation of mother and daughter cells. DNA synthesis is rapidly inhibited, cell viability is lost more slowly and cells eventually arrest, apparently in the S phase of the cell cycle with a medium-sized bud, a single nucleus in the mother cell and a pre-replicated (n) DNA content. Morphogenesis in moribund cells is uniformly blocked in early S-phase with an immature bud. Toxin action causes either independent blockage of both DNA synthesis and the budding cycle, or inhibits some unknown steps required for both [62, 72, 84].

Genetics, toxigenesis, immunity, and activity of the *S. cerevisiae* killer system is shown in Figure 1.21.

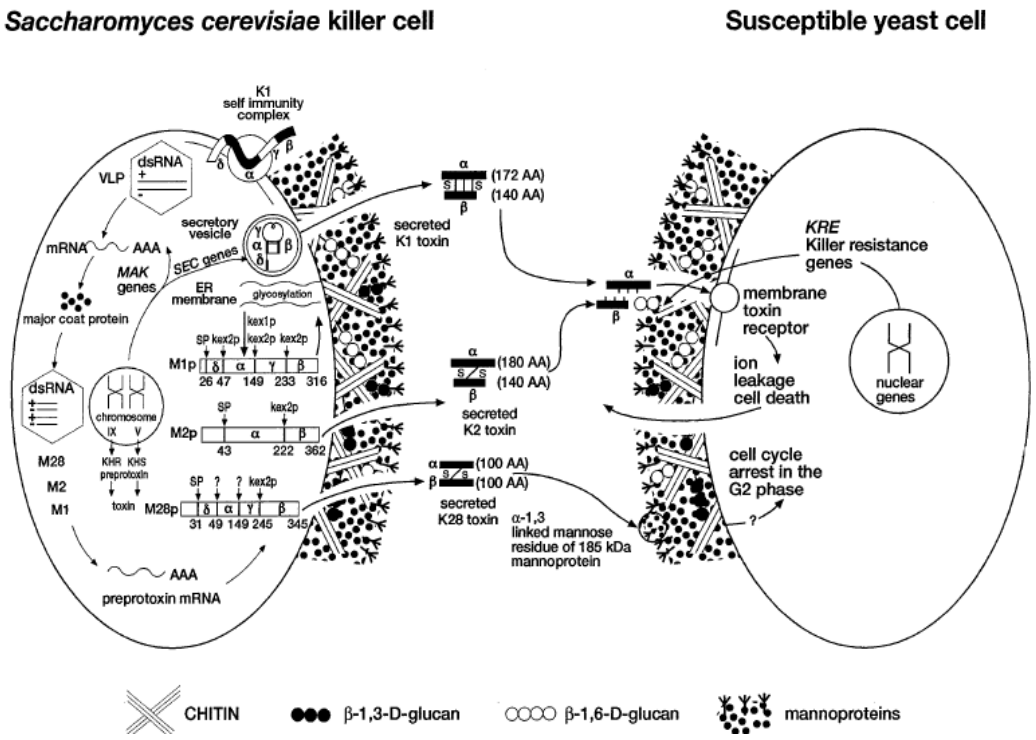

Figure 1.21. Sketch of the Genetics, Toxigenesis, Immunity and Activity of the *S. cerevisiae* Killer System [72].

K5 type yeast killer protein produced by *Pichia anomala* uses (1-3)-β-D-linked glucans as a target and inhibits the growth of sensitive microbial cells by hydrolyzing this major cell wall component which causes cell burst [81].

The killer toxin of *Kluyveromyces lactis* leads to the permanent arrest of susceptible cells in the unbudded (G1) phase of the cell cycle, in such a manner that they can never resume mitotic division. The toxin causes a rapid and progressive loss of viability that is sufficient to explain the blockage of cell division. The mode of action of the toxin is still poorly understood. However, two effects, arrest in G1 and loss of viability, are apparently mediated by different mechanisms [70, 72].

P. acaciae toxin seems to be composed of three subunits with an associated chitinase activity. Chitin binding is essential to the activity of the toxin, which causes G1 cell cycle arrest [72].

The *P. kluyveri* killer toxin, a 19-kDa acidic glycoprotein, induces the formation of ion-permeable channels, as does *S. cerevisiae* K1, which causes leakage of potassium ions and ATP, decrease of the cellular pH, and inhibition of amino acid uptake [72].

HM-1 killer toxin of *Williopsis mrakii* kills susceptible strains by a unique mechanism, presumably involving interference with the synthesis of β-1,3-glucan, thus rendering the wall osmotically fragile or defective and ultimately resulting in lytic cell death. HM-1 inhibits the in vitro activity of β-1,3-D-glucan synthase, suggesting that the toxin can perturb the synthesis of the yeast cell wall by inhibiting the glucan synthesis at the budding sites or the conjugating tubes, which results in cell lysis (Figure 1.22.) [72].

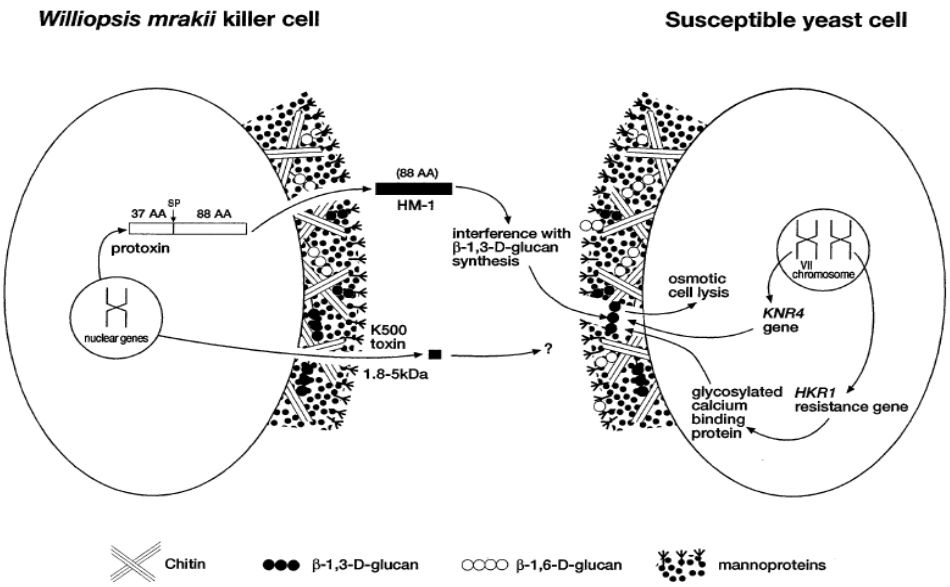

Figure 1.22. Sketch of the Genetics, Toxigenesis, and Activity of the *W. mrakii* Killer System [72].

1.4.3. Ecology of Yeast Killer Systems

The nature of the yeast killer phenomenon implies a potential role for competition, considering that yeast killer toxins may prevent antagonistic microorganisms from gaining access to resources that would provide a selective advantage during the early phases of microbial growth. Yeast killer toxins are produced optimally by growing cells and are exquisitely active against cells in the same stage when nutrients are available and pH is low [72].

The ecological role of the yeast killer phenomenon (toxin production and susceptibility) has been extensively studied in yeast communities, particularly in decaying stems and fruits and in slime growth on trees. In particular, fruits appear to be very important habitats for the killer phenomenon within yeast communities, since one-quarter of the yeast strains isolated from them are killers. This habitat is characterized by a low pH and high sugar concentration [72].

Generally, natural communities of yeasts are made up of only one predominant killer species, even though susceptible strains are more widespread than the killer counterpart. Few species, if any, appear to be immune to all yeast killer toxins. Ecologically defined natural yeast killer systems showed a definite seasonal fluctuation in their occurrence that markedly increased during the cooler winter period, possibly because of the seasonal nature of fruit production at certain latitudes [72].

Studies of some environmental factors, such as the pH of the habitat in which the killer yeast lives, have shown that killer and susceptible yeasts are not independently distributed and that some killer toxins function in nature to limit the proliferation of other yeasts in the same community [72].

The possibility exists that yeast interference competition and mutualism are both important determinants of the composition of yeast communities and are related to variations among the habitats in which the yeasts coexist.

Experimental studies of yeast interference competition in cacti have demonstrated that the composition of the yeast community differed in the presence of a killer or a nonkiller strain. The killer yeast was present in a higher density than the nonkiller strain because of selection against susceptible yeasts. The density of the population of susceptible yeasts grown in the presence of the killer strain was much lower than that of the same susceptible yeast strain grown in association with the nonkiller strain [72].

The probability that a killer toxin produced by a yeast may kill a certain susceptible yeast would also depend on ecological characteristics such as the region, the host plant, and the habitat from which both the killer and susceptible yeasts were collected [72].

1.4.4. Toxin Immunity

The question of how immunity occurs in killer yeast strains has still not been answered.

Production of *K. lactis* toxin is also associated with the expression of an immunity to the secreted product and killer strains are insensitive to the presence of toxin in the extracellular medium. This phenomenon was shown to depend on pGKL1 but the biochemical basis of immunity is stil unknown [70].

Although the precise molecular basis for toxin immunity is stil unknown, it has been thought that it might be conferred by the toxin precursor itself. There are studies showing that the unprocessed toxin precursor is responsible from immunity in K1 and K28 strains [59].

K1 killer strains of *Saccharomyces cerevisiae* secrete a polypeptide toxin to which they are themselves immune. The K1 preprotoxin includes α and β components. The intervening segment is called γ. It is shown that α toxin was the lethal component and that its secretion in the mature form caused severe

growth inhibition while secretion of α fused to just an N-terminal fragment of γ was sufficient to confer immunity. Expression of immunity requires that component and the N-terminal of γ. An additional C-terminal extension, either eight residues of γ or three of four unrelated peptides, is also required [59, 85].

A completely different mechanism must be postulated for K28 killers, since it has been shown that K28 killers take up their own toxin after it has been secreted. The toxin re-enters the secretory pathway of a killer cell and reaches the cytosol, just as in a sensitive cell. However the killer cell is not killed but rather protected from the lethal effect of the toxin. K28 immunity is likely to affect an unknown step either within the yeast cell cytosol or within the nucleus [59].

1.4.5. Applications of Yeast Killer Phenomenon

Killer yeasts and their toxins have many potential applications in environmental, medical and industrial biotechnology. They are also suitable to study the mechanisms of protein processing and secretion, and toxin interaction with sensitive cells [62].

1.4.5.a. Killer Yeasts in Food and Fermantation Industry

The killer phenomenon can be utilized for the protection of fermentation process against contaminating yeasts. Killer yeasts could be used as starter cultures to control the growth of contaminating yeasts during the early stages of wine fermentation. Genetically modified wine yeasts producing different killer toxins simultaneously have an increased antagonistic ability in mixed yeasts fermentations, exhibit a significantly broader killing activity and are thus capable of outcompeting potentially contaminating yeasts like *Candida, Hanseniaspora, Kloeckera* and *Pichia* in mixed culture [59].

In wine making, killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation to improve the process of wine making and wine quality. However, the main limit of the killer toxin of *S. cerevisiae* wine yeast (K2 type) resides in its narrow antiyeast spectrum which, being restricted to sensitive *Saccharomyces* strains, does not affect wild yeasts, such as *Hanseniaspora/Kloeckera, Pichia*, and *Saccharomycodes* [86].

The killer toxin secreted by *Kluyveromyces phaffii* (KpKt) is active against spoilage yeast under winemaking conditions and thus has potential applications in the biocontrol of undesired microorganisms in the wine industry. KpKt has an extensive anti-*Hansenispora/Kloeckera* activity under winemaking conditions and, therefore, is of particular interest for its potential application as an antimicrobial agent in wine industry. At present, the inhibition of wild spoilage yeast at the pre-fermentative stage is achieved by the addition of SO_2 to freshly pressed must. This antiseptic agent, which has been shown to have a toxic action on human, is also re-added at the end of fermentation for its antioxidant properties. The use of KpKt as a substitute for SO_2 during the pre-fermentative stage would limit SO_2 use to only the post-fermentative stage, thus reducing the total amount of this antimicrobial in the final product. Moreover, as KpKt is also active against yeasts belonging to the species *Saccharomycodes ludwigii*, *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*, the possible use of this toxin for the control of spoilage yeasts in sweet beverages may also be promising [87].

Grey mould is a well-known disease caused by *Botrytis cinerea*. *B. cinerea* is a ubiquitous fungus with a wide host range, causing yield losses in wine grapes, lettuce, onion, potato, strawberry, tomato and other species of commercial interest. *Pichia membranifaciens, Pichia anomala* and *Debaryomyces hansenii* have inhibitory effect against *Botrytis* strains. In small-scale trials, post-harvest application of *P. membranifaciens* to apple wounds inhibits *B. cinerea* and also the purified killer toxin from *P. membronifaciens* inhibits *B. cinerea* too. Results of previous studies indicate that certain yeasts,

or their toxins such as *P. membranifaciens* killer toxin, might have potential as novel agents to control *B. cinerea* [88, 89].

1.4.5.b. Biotyping of Microorganisms

Killer yeasts have found applications in the biotyping of the pathogenic yeasts such as *Candida albicans*, *Cryptococcus* and micellal fungus such as *Aspergillus niger* by using their sensitivity or resistance to a wide range of killer toxins [90-91].

1.4.5.c. Heterologous Protein Secretion

Traditionally, procaryotic systems such as *Escherichia coli* or *Bacillus subtilis* are used for expression and purification of heterologous proteins. However bacterial systems turn out to be ineffective hosts because of their limited capacity to perform post-translational modifications such as protein N-glycosilation, phosphorilation and acetylation when expression of eukaryotic proteins are desired. Therefore unicellular eukaryotes such as yeasts are used as hosts for the expression of heterologous proteins [59].

Many secretory proteins have been expressed as extracellular proteins by using homologous secretion signals derived from plasmid-derived killer toxin of *K. lactis.* The secretion and processing signal derived from *S. cerevisiae* ScV-M28 killer virus is functional in fission yeast and can be used to target foreign proteins for secretion into the extracellular environment [44, 59].

1.4.5.d. Killer Toxin Expression in Transgenic Plants

Transgenic plants are used to engineer pathogen resistance in crop plants which are capable of producing substances that are toxic to disease-causing

pathogens. For this aim, *Ustilago maydis* killer system is an attractive model for the introduction of fungal resistance into tobacco plants. Different strains of *Ustilago maydis*, a fungal pathogen of *Zea mays*, harbour different double-stranded RNA viruses encoding antifungal proteinaceous killer toxins, e.g. three subtypes P1, P4 and P6 of *U. maydis* producing KP1, KP4 and KP6 killer toxins respectively. *U. maydis* strains are resistant to the toxin produced within themselves but sensitive to the killer toxins of other strains. High level secretion of KP4 or KP6 killer toxin in transgenic tobacco plants rendered them resistant to fungal pathogens [92, 93].

1.4.5.e. Medical Use of Yeast Killer Proteins

Secreted yeast killer toxins show a broad spectrum of killing activity against a great number of human pathogens. Since yeast cell wall contains mannoproteins, chitin, (1-3)-β-D-linked glucans and (1-6)-β-D-linked glucans and these are the primary binding sites for yeast killer toxins antifungal research is currently focused on the possible use of yeast killer proteins as novel antifungals. Especially toxins of the genus *Hansenula*, *Pichia*, and *Kluyveromyces* have a wide range of killing spectrum on human and animal fungal pathogens [55, 59, 94].

The killer toxins from *Williopsis subsuffciens*, *Hanseniaspora uvarum, W. beijerinckii*, *W. mrakii* and *Hansenula anomala* are active against *Candida* [95, 96].

Some killer yeast strains have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Streptococcus pyogenes*, *Bacillus subtilis, Sarcina lutea* and *Staphylococcus aureus* [54].

Despite a century of often successful prevention and control efforts, infectious diseases remain an important global problem in public health, causing over 13 million deaths each year. The emergence and rapid dissemination of

infectious agents resistant to currently used antibiotics have emphasized the need for new alternatives for the treatment of diseases [97]. Yeast killer toxins are probably not suitable for oral and/or intravenous administration because they are antigenic, the ones that are stable at wide pH and temperature ranges might be used as a new alternative in the topical treatment of superficial infections [59, 62].

1.4.6. K5 Type Yeast Killer Protein

K5 type yeast killer protein is produced by *Pichia anomala* NCYC 434 according to the Young and Yagiu classification (Figure 1.23.).

Pichia anomala is a fungi that belongs to the phylum; *Ascomycetes*, class; *Hemiascomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Pichia* (*Hansenula*), species; *anomala* (Figure 1.24.) [98].

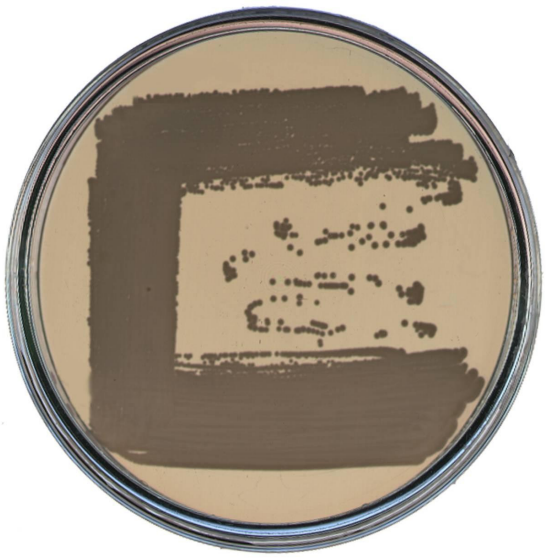

Figure 1.23. *Pichia anomala* NCYC 434 Cells on YEPD Agar Medium

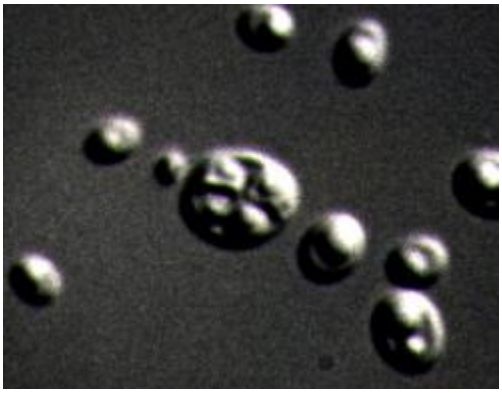

Figure 1.24. *Pichia anomala* Cells [98].

The ascomycetous yeast *Pichia anomala* is frequently associated with food and feed products, either as a production organism or as a spoilage yeast. It belongs to the non-*Saccharomyces* wine yeasts and contributes to the wine aroma by the production of volatile compounds. The ability to grow in preserved food and feed environments is due to its capacity to grow under low pH, high osmotic pressure and low oxygen tension. Although classified as a biosafety class-1 organism, cases of *P. anomala* infections have been reported in immunocompromised patients. The yeast can use a broad range of nitrogen and phosphor sources, which makes it a potential agent to decrease environmental pollution by organic residues from agriculture [99].

A new application of *P. anomala* is its use as a biocontrol agent, which is based on the potential to inhibit a variety of moulds in different environments [37].

On the other hand, *P. anomala* killer toxins have a potential as antimicrobial agents [99]. In several studies, *P. anomala* toxins have been reported to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts in several studies [94, 99-101]. Among *P. anomala*, the strain NCYC 434 especially has been extensively studied for various applications [55, 101].

K5 type yeast killer protein was previously purified and characterized in our laboratory. K5 type yeast killer protein is a glycosilated protein with a molecular mass of 49kDa and pI value of 3.7 [81].

Internal aminoacid sequences of the toxin share 100% homology with exo-β-1,3-glucanase of *P. anomala* Strain K which is a glycoprotein of 45.7 kDa with a pI of 4.7. (Figure 1.25.) [81].

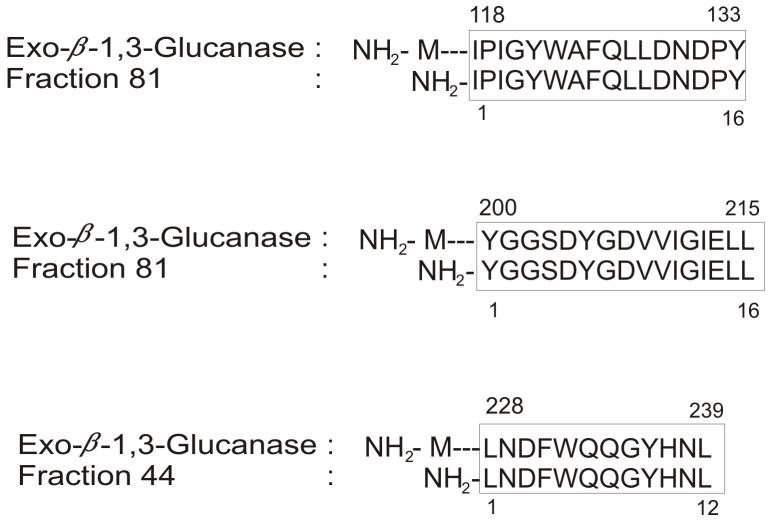

Figure 1.25. Internal Amino Acid Sequence Homology of the K5 Type Yeast Killer Toxin with the Exo-β-1,3-glucanase of *P.anomala* Strain K [81].

The pH and temperature optima of cultivation for the production of K5 type toxin is pH 4.5 and 20-22°C in YEPD medium [81].

The optimum pH value for K5 type yeast killer protein is 4.5 and about 70% of the activity remains even at pH 2.5. However toxin is readily inactivated at pH values above 6.5 (Figure 1.26.).

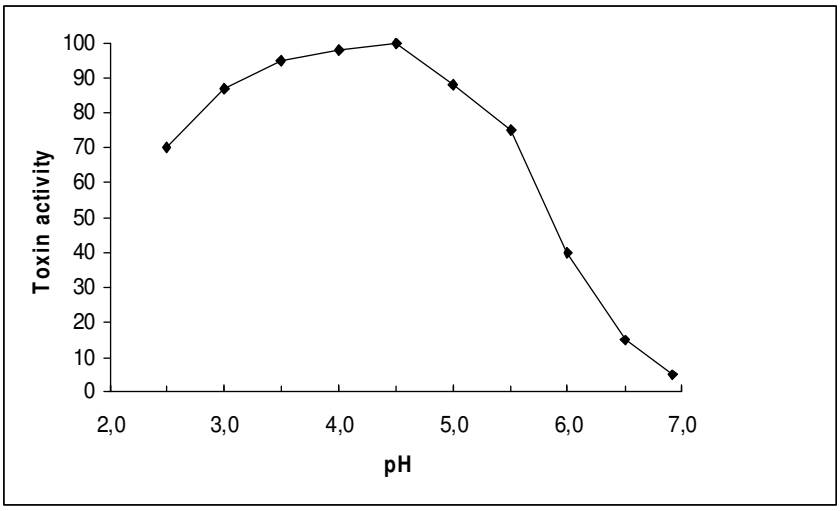

Figure 1.26. pH Stability of the K5 Type Yeast Killer Toxin [81].

The optimum temperature for the toxin activity is 25 °C. There is no loss of activity at temperatures up to 30°C and about 90% of the activity is retained at 37 °C whereas at 100 °C half of the toxin activity is lost (Figure 1.27.) [81].

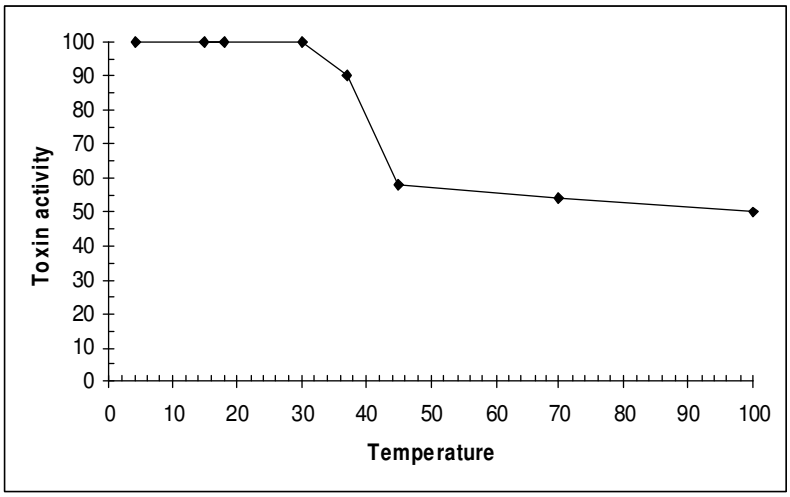

Figure1.27. Temperature Stability of the K5 Type Yeast Killer Toxin [81].

K5 type yeast killer toxin production by *P. anomala* NCYC 434 cells varies depending on the medium composition. The production of the toxin is stimulated in the the presence of β-glucans, and is dependent on the amount of β-glucan in the medium and its linkage (Figure 1.28.). Glucose is also required to maintain the highest level of K5 type toxin production (Figure 1.29.)[102].

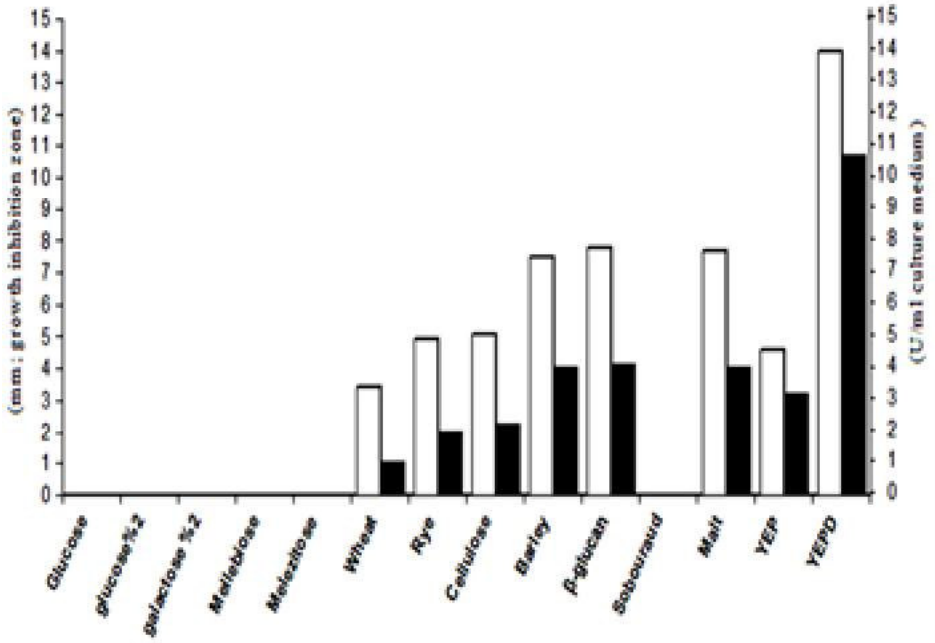

Figure 1.28. Effect of Different Carbon Sources on Production of K5 Type Yeast Killer Protein [102]

□ Determined by Agar Diffusion Assay, Killer Activity on *S. cerevisiae* cells (mm; growth inhibition zone)

■ Determined by Exo-β-1,3-Glucanase Assay, Exo-β-1,3-Glucanase Activity (U/ml culture medium)

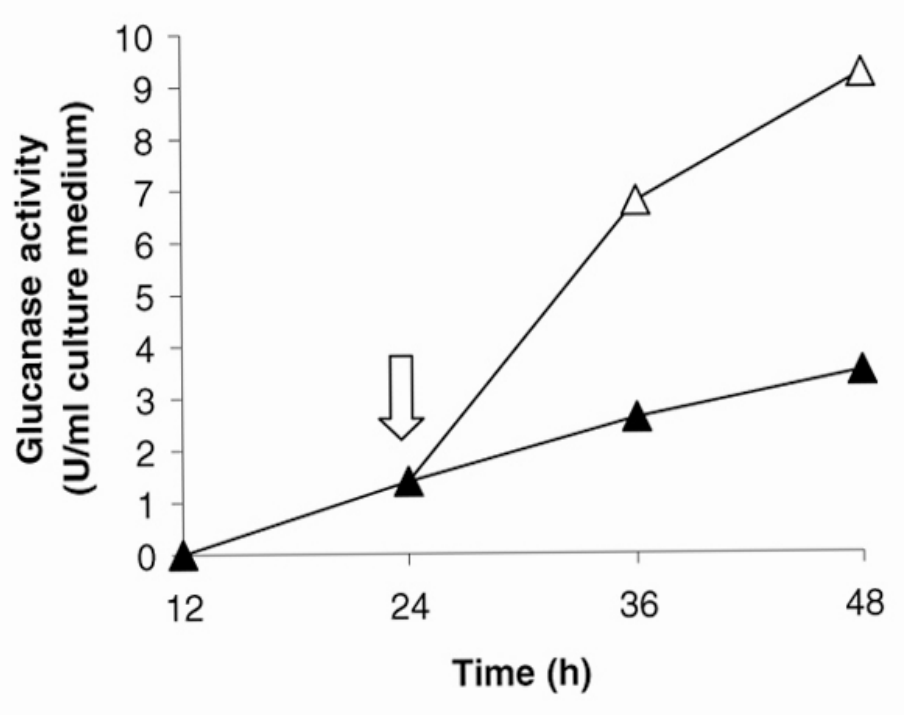

Figure1.29. Effect of Glucose on β-glucanase Induction [102]

Two parallel *P.anomala* NCYC 434 (K5) cell cultures in YEP broth were performed. At the time indicated by the arrow, 2% glucose was added to one of the cultures (Δ), the other culture was used as control (▲), and both cultures were incubated for an additional 24 hours.

K5 type yeast killer protein is most effectively absorbed by laminarin and β-glucan indicating that the main target of the K5 type yeast killer protein is the cell wall β-1,3-glucans. Some of the toxin bound also to chitin and pustulan since β-1,3-glucanases have slight affinity towards these polysaccharides. Also it is shown that the toxin exerts its hydrolytic activity in an exo-like fashion. The toxin exerts its lethal affect by hydrolyzing β-1,3-glucan residues of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure [102].

Specific activity of the K5 type yeast killer toxin on laminarin is 120 U/mg and the Michaelis Menten constants K_m and V_{max} are 0.25 mg/ml and 370 µmol/min/mg respectively [102].

The study about the affects of metal ions on the enzymic activity of the toxin shows that activity is fully inhibited by Hg^{+2} indicating that enzyme contains sulfhydryl groups that are important for its activity. The enzymic activity is increased by some other metal ions such as Ba^{+2}, Ni^{+2}, Cr^{+2}, Zn^{+2}; most of all by Pb^{+2}. A slight increase in the enzymic activity is observed in the presence of DDT. Probably DDT protects sulphydryl groups from oxidation and stabilizes the enzyme. EDTA has no effect on enzyme activity suggesting that enzymic activity is not dependent on a bivalent ion as cofactor (Table 1.4.) [102].

Table 1.4. Affect of Various Compounds on the Exo- β-1,3- glucanase Activity towards Laminarin [102].

Affecter	Relative Activity (%)
None	100
Ca^{2+}	115
Mn^{2+}	126
Mg^{2+}	120
Zn^{2+}	117
Pb^{2+}	163
Cu^{2+}	122
Cd^{2+}	134
Ba^{2+}	123
Cr^{2+}	119
Fe^{2+}	132
Ni^{2+}	127
EDTA	100
DTT	115
Hg^{2+}	-

Certain killer yeast strains are known to have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Bacillus subtilis, Streptococcus pyogenes and Staphylococcus aureus* besides certain pathogenic yeast and fungal strains [54]. However, previous studies comprising the effect of killer proteins against human pathogenic bacteria are limited to the interactions of the killer protein producing strains with human pathogenic bacteria or performed with crude toxin preparations [54, 94]. Further studies with the purified killer proteins are required for the potential use of these agents as novel antibacterial agents.

There are several studies reporting the wide-range intergeneric killing spectrum of *Pichia anomala* NCYC 434. In several studies *P. anomala* toxins have been reported to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts [94, 99-101]. Among *P. anomala*, the strain NCYC 434 especially has been extensively studied for various applications [55, 101].

High stability of the K5 type toxin at pH values between 2.5-6.5 and temperatures up to 37 ºC is appropriate for the use of this protein in medical and industrial biotechnology.

Recently, we have purified and characterized the killer protein of *P. anomala* NCYC 434 and determined its mode of action in our laboratory [81, 102]. In vitro susceptibility studies of *P. anomala* NCYC 434 killer protein on human pathogenic fungi along with analyses of its kinetics of cell killing were done in our laboratory. Antifungal activity of the K5 type yeast killer protein was tested against 32 clinical isolates and 9 standart strains of dermathophytes and found to be affective on all of the tested strains. MIC (minimum inhibition concentraition) values were determined. The MIC-2 values (the lowest K5 type yeast killer protein concentration producing prominent growth reduction or a 50% reduction in growth) ranged from 0.25 to 2 µg ml whereas MIC-0 values (corresponds to the lowest K5 type yeast killer protein concentration producing a clear well or 100 % growth inhibition) ranged from 1

to 8 µg ml [103]. Antifungal activity of the K5 type yeast killer protein was tested against 21 *Candida* isolates including 9 quality control strains and found to be affective on all of the tested strains with the MIC range of 0.5-8 µg/ml. The *C. albicans* strains that were resistant to azole derivatives such as flucanazole or itracanazole, was susceptible to the toxin in the MIC range of 1-2 µg/ml. Studies showed that both the clinical isolates and the standard strains of *C. krusei* were susceptible to the K5 type yeast killer toxin in the MIC range of 2-8 µg/ml [104]. The results of the in vitro susceptibility studies of *P. anomala* NCYC 434 killer protein on human pathogenic fungi highlights the use of the K5 type yeast killer protein against human fungal infections with appropriate formulations [103,104].

In vitro susceptibility testing of pure *P. anomala* NCYC 434 killer protein against human pathogenic bacteria remained to be explored and now it became possible to determine the effect of pure K5 type yeast killer protein on human pathogenic bacteria.

Yeast killer toxins are probably not suitable for oral and/or intravenous administration because they are antigenic, the ones that are stable at wide pH and temperature ranges might be used as a new and natural alternative in the topical treatment of superficial infections caused by gram-positive pathogenic bacteria thus it is known that certain killer yeast strains are known to have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Bacillus subtilis, Streptococcus pyogenes and Staphylococcus aureus* [59, 62].

Due to the high stability of the K5 type yeast killer toxin at pH values between 2.5-6.5 and temperatures up to 37 °C, it might be possible to use this protein as a topical antibacterial agent in the treatment of pathogenic gram-positive bacteria associated skin and soft tissue infections and wound infections, after in vitro susceptibility tests and appropppriate formulation studies if further studies confirm the inhibitory effect determined by the interactions of the killer toxin producer cells and pathogenic microorganisms.

In this study our aim is: (i) to determine the antibacterial spectrum of the purified K5 type yeast killer protein on gram-positive bacteria causing skin infections including the clinical isolates of *Staphylococcus aureus* (including methicillin sensitive and resistant strains), *Enterococcus faecium* and *Enterococcus fecalis* and two quality control strains including *Staphylococcus aureus* (MSSA) ATCC 29213 and *Enterococcus fecalis* ATCC 29212 (ii) to examine the MIC (minimum inhibition concentration) values and MIC range of the purified K5 type yeast killer protein (iii) to determine the MBC (minimum bactericidal concentration) values of the purified K5 type yeast killer protein as an alternative means of detecting the bactericidal activity (iv) to evaluate the cell killing activity of the purified K5 type yeast killer protein on *Staphylococcus aureus* A1 and *Staphylococcus aureus* A3 clinical isolates in order to determine if the toxin had bactericidal or bacteriostatic activity and in order to distinguish whether bacterial killing was concentration and/or time dependent.

CHAPTER II

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Fungal Strains

Fungal strains used in this study were *Pichia anomala* (NCYC 434, K5) as K5 type yeast killer toxin producing strain and *Saccharomyces cerevisiae* (NCYC 1006) as killer toxin sensitive strain both purchased from National Collection of Yeast Cultures (NCYC), Norwich, U.K.

2.1.2. Bacterial Strains

A total of 19 human pathogenic bacteria isolated fron skin infections and 2 quality control strains which were used in antibacterial susceptibility tests are given in Table 2.1.

2.1.3. Culture Media

All media used in this study were prepared by distilled water. Killer toxin sensitive strain *Saccharomyces cerevisiae* (NCYC 1006) and K5 type

yeast killer toxin producing strain *Pichia anomala* (NCYC 434, K5) were grown on yeast extract-peptone-dextrose (YEPD) agar plates buffered at pH 5.5 and consisting of 1% Bacto-yeast extract, 1% Bacto-peptone and 2% dextrose along with 2% Bacto-agar at 25 °C for the maintenance and routine growth. For the production of the K5 type yeast killer protein, *P. anomala* cells were grown in YEPD broth medium buffered to pH 4.5 with citrate-phosphate buffer and supplemented with 5% (v/v) glycerol. Killer activity assay was determined in YEPD medium with 2% Bacto-agar buffered to pH 4.5 with citrate-phosphate buffer.

Trypticase soy broth with 0.3% Bacto-yeast extract supplemented with 16% (v/v) glycerol was used for the cryopreservation of the bacterial strains. For routine growth and maintenance of bacterial cells 7% horse blood agar (1,4% Bacto-trypton, 0,45% Bacto-pepton, 0,45% Bacto-yeast extract, 0,5% NaCl and 1,25% Bacto-agar) plates were used. Prior to antibacterial susceptibility testing and analyses of cell killing activity, all bacterial strains were freshly subcultured on 7% horse blood plates at 37 °C for 20 to 24 hours to ensure purity and viability.

The culture medium recommended by the reference document CLSI (Clinical and Laboratory Standards Institue) (formerly NCCLS) M7-A6 (Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically) was cation adjusted Mueller-Hinton broth. Binding of the peptide and proteins to complex carbonhydrates such as starch in culture media used in the antibacterial susceptibility tests can cause reduced protein or peptide activity. Also the interference of the cations with proteins or peptides can cause the same effect too [105, 106]. Nutrient Broth, a culture media for the cultivation of fastidious bacteria, was used as test media in order to eradicate the decrease in the activity of the K5 type yeast killer protein due to the binding of the protein to starch in the medium and the effects of cations in cation adjusted Mueller-Hinton broth recommended by CLSI M7-A6.

Table 2.1. Bacteria Used in Antibacterial Susceptibiliy Tests

STRAIN	STRAIN NUMBER\ SOURCE	ISOLATION
Staphylococus aureus (MSSA)	ATCC 29213	Wound
S. aureus (MSSA)*	A1	Breast abscesses
S. aureus (MSSA)*	A2	Human
S. aureus (MSSA)*	A3	Caesarean wound
S. aureus (MSSA)*	A4	Wound
S. aureus (MSSA)*	A5	Wound
S. aureus (MRSA)*	A6	Wound (hand)
S.aureus (MRSA)*	A7	Surgical wound
S.aureus (MRSA)*	A8	Wound
*S.aureus (MRSA)**	A9	Wound
*E. faecium**	A10	Surgical wound
*E. faecium**	A11	Surgical wound (breast)
*E. faecium**	A12	Wound (abdomen)
Enterococcus faecalis	ATCC 29212	Urine
*E. faecalis**	A13	Surgical wound
*E. faecalis**	A14	Wound
*E. faecalis**	A15	Surgical wound (chest)
*E. faecalis**	A16	Wound
*E. faecalis**	A17	Wound
*E. faecalis**	A18	Wound
*E. faecalis**	A19	Wound

*Clinical isolates were collected either from Gülhane Military Medical Academy, Bayındır Hospital or Düzen Laboratories and all clinical isolates were analysed with API test system (Bio Merieux, Hazelwood, Mo.).

ATCC: American Type Culture Collection, USA; A: Our laboratory collection number, MSSA: Methicillin-sensitive *Staphylococcus aureus.*

2.1.4. Chemicals

The chemicals and the suppliers are listed in the Appendix A.

2.1.5. Buffers

Buffers and solutions used in the experiments are given in Appendix B.

2.2. METHODS

2.2.1. Sterilizations

The glassware were sterilized on dry-cycle at 200 ^{o}C for two hours. The media for stock cultures and for routine growth of the yeasts and bacteria were sterilized at 121 ^{o}C for 15 minutes on liquid cycle. Buffers used for the chromatographic purification steps were filtered using 0.45µm cellulose acetate filter disks (Sartorius, AG, Germany) using filter device (Sartorius, AG, Germany) prior to sterilization on liquid cycle.

2.2.2. Maintenance of the Fungal Cultures

Freeze-dried cultures of *Pichia anomala* (NCYC 434, K5) and *Saccharomyces cerevisiae* (NCYC 1006) in glass ampoules were opened aseptically and 0.5 ml of YEPD broth medium at pH 5.5. was added to dissolve the dried culture using a sterile pasteur pipette. Dissolved yeast cells were plated onto petri dishes containing YEPD agar at pH 5.5 and incubated at 25 ºC until the colonies were formed [107].

All plate cultures were than stored at 4 °C. The plates were subcultured onto new plates after one month maximum.

2.2.3. Maintenance of the Bacterial Cultures

Pathogenic bacteria obtained as passaged cultures on agar plates were subcultured onto 7% blood agar plates and incubated at 37 ºC until the colonies are formed.

All of the bacterial strains were stored in trypticase soy broth with 3% yeast extract supplemented with 16% glycerol as cryoprotectant in cryo tubes at -80 °C. For antibacterial susceptibility testing all bacterial strains preserved at -80 °C were streaked onto 7% blood agar plates for reactivation. Plate cultures of bacteria were than stored at 4 °C. The plates were subcultured onto new plates after 15 days maximum.

Pathogenic bacteria were subcultured onto 7% blood agar plates to ensure purity and viability prior to antibacterial susceptibility tests and analyses of cell killing activity.

2.2.4. Production of the K5 Type Yeast Killer Toxin and Crude Toxin Preparation

Production of the K5 type yeast killer toxin and crude toxin preparation was done as described previously by İzgü and Altınbay [81]. *Pichia anomala* NCYC 434 (K5) cells were cultivated into 10 ml of YEPD broth medium at pH 5.5. and incubated for 24 hours at 25 ^{o}C. One ml of cell suspension was further inoculated into 100 ml of YEPD broth medium at pH 5.5 and incubated for 24 hours at 25 ^{o}C at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). Ten ml of cell suspension was further transferred to 1L of YEPD broth medium buffered to pH 4.5 with citrate-phosphate buffer and supplemented

with 5% glycerol and *Pichia anomala* NCYC 434 (K5) cells were grown to stationary phase at 20 ^{o}C for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). The cells were removed by centrifugation (KR 22i, Jouan, France) at 5000 rpm for 10 min. at 4 ^{o}C to obtain the cell free culture medium and filtered through 0.45µm and 0.2µm cellulose acetate membranes (Sartorius, AG, Germany) respectively for the sterilization of the medium [81].

The filtrate containing the K5 type yeast killer protein was further concentrated 400-fold by using a centrifugal 30kDa-cutoff ultrafilter (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 rpm, 4 ^{o}C (MR23i, Jouan, France). In order to obtain the YEPD free sample the concentrated crude protein was buffer-exchanged to 30mM N-methylpiperazine-HCl at pH 4.8 by using a centrifugal 5kDa-cutoff ultrafilter (Vivaspin VS2012, Sartorius ,AG, Germany) at 4200 rpm, 4^{o}C (MR23i, Jouan, France). This step was performed three times. The samples were than filtered by 0.2µm syringe filter (Sartorius, AG, Germany) prior to injection onto the HPLC and tested for its killing activity with an agar diffusion assay [81].

2.2.5. Assesment of Killer Toxin Activity

At various stages of the study killer toxin activity was checked with an agar diffusion assay according to Brown et al [108]. Suspensions of the killer toxin sensitive strain *Saccharomyces cerevisiae* (NCYC 1006) were prepared in sterile saline water and standardized to an optical density of 0.5 on the McFarland standard (Densimat, Bio Merieux, France). Twenty five ml of molten YEPD agar (pH 4.5) was seeded with 1 ml from this suspension containing approximately 10^5 cells/ml and poured into petri dishes.

Protein samples of 30 µl were spotted onto petri dishes and incubated at 22 ^{o}C. Killer activity was measured by the occureance of the clear zone of growth inhibition of the seeded killer toxin sensitive strain after 48 hours of

incubation. Killer toxin which gave a clear zone of 10 mm in diameter was defined as 1 arbitrary unit (AU).

2.2.6. K5 Type Yeast Killer Toxin Purification

Toxin purification steps were done on a a fully automated HPLC system (Biocad 700E Perseptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at 20 ^{o}C [81].

Buffer-exchanged crude toxin obtained from the previous step was subjected to ion exchange chromatography. 750µL of the buffer-exchanged protein sample was injected into an anion-exchange column (POROS HQ/M 4.6 mmD/100mmL, Perseptive Biosystems, USA) that is previously equilibrated with 30 mM N-methylpiperazine-HCl buffer at pH 4.8. The column was washed to 20 column volumes (CV) with the same buffer. Elution was done with a linear gradient of 0 to 500 mM NaCl in the same buffer at a flow rate of 10 ml/min. Killer protein containing fractions (1600µL) that corresponds to 120mM NaCl were pooled. These eluted fractions were concentrated and buffer-exchanged to 100mM Na_2HPO_4- citric acid buffer, pH 4.5 by using 5 kDa molecular cut-off ultrafilter (Vivaspin VS2012, Sartorius ,AG, Germany) at 4200 rpm, 4^{o}C (MR23i, Jouan, France) and assayed for killer activity [81].

The sample obtained from the previous anion exchange step was then subjected to gel permeation chromatography using a TSK G2000SW, 7.5mmD/300mmL (TosoHaas, Japan) column. The column was equilibrated with 100mM Na_2HPO_4- citric acid buffer at pH 4.5, containing 100mM Na_2SO_4 at a flow rate of 1 ml/min prior to injection of the sample. Ninety µl of sample was injected into the column and elution was done with the same buffer at a flow rate of 1 ml/min. The eluted fractions (1300µl) that corresponds to 8.5 ml were pooled.

Normally, pure K5 type yeast killer protein obtained from gel permeation chromatography was buffer exchanged to 100mM Na_2HPO_{4-} citric acid buffer at pH 4.5, containing 100mM Na_2SO_4 [81]. However except *E. faecalis* isolates no acceptable bacterial growth occured when bacterial inoculum and 100mM Na_2HPO_{4-} citric acid buffer at pH 4.5, containing 100mM Na_2SO_4 were mixed in equal amounts in order to control the bacterial growth for the antibacterial susceptibility tests. In order to determine the optimum molarity for acceptable bacterial growth, the growth of 2 clinical isolates (A1, A6) and 1 quality control strain of *S. aureus* (ATCC 29213) and two clinical isolates of *E. faecium* (A10, A11) were tested in an assay performed in nutrient broth. Equal amounts of bacterial inoculum in nutrient broth and Na_2HPO_{4-} citric acid buffer at pH 4.5 with the molarities of 10, 20, 30, 40 and 50mM, containing Na_2SO_4 with the molarities of 10, 20, 30, 40 and 50mM were mixed. It was shown that acceptable bacterial growth occurs only in 10mM Na_2HPO_{4-} citric acid buffer at pH 4.5, containing Na_2SO_4 with the molarity of 10mM.

The active fractions obtained from gel permeation chromatography were then concentrated (totally 500-fold concentrated protein was obtained) and buffer exchanged to 10mM Na_2HPO_{4-} citric acid buffer at pH 4.5, containing 10mM Na_2SO_4 by using 5 kDa molecular cut-off ultrafilters (Vivaspin VS2012, Sartorius, AG, Germany). This step was performed three times. Thirty µl of the purified protein obtained from gel permeation chromatography was spotted on to YEPD (pH 4.5) agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells for the assessment of the killer toxin activity [81].

2.2.7. Assessment of Protein Concentration

Protein concentration was determined as described previously by Bradford [109]. Bradford reagent used for the assesment of protein concentration was prepared as follows. A hundered mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95 % ethanol then mixed with 100 ml of

85% (w/v) phosphoric acid. This solution was completed to a total volume of 1 L with distilled water and filtered through Whatman #1 paper.

Different concentrations of bovine serum albumin (Fraction V) was used as a protein standard. Different concentrations of bovine serum albumin was prepared in 10 mM Na_2HPO_4- citric acid buffer ranging from 5 to 25 µg/ml in a total volume of 800µl. These standard solutions were mixed with 200µl Bradford reagent. 10µl protein sample was also diluted in 790µl 10 mM Na_2HPO_4- citric acid buffer and mixed with 200µl Bradford reagent. Blank sample was prepared by mixing 200µl Bradford reagent and 800µl 10 mM Na_2HPO_4- citric acid buffer. Hundred µl from blank, protein sample and standard protein solution were than pipetted into the wells of a 96-well microplate (Nunclon 167008, Nunc, Denmark). Absorbances were measured at 595 nm after incubation at room temperature for ten minutes following the addition of the bradford reagent by using UV visible spectrophotometer (model 1208, Shimadzu, Japan). Protein concentration in the sample was calculated using a standard curve of absorbance versus protein amount from the BSA standards.

2.2.8. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

A 5-20% linear gradient SDS polyacrylamide gel in a discontinuous buffer system was used for the molecular weight determination of the pure toxin [110]. A vertical slab gel electrophoresis unit SE 600 (Hoefer, USA) was used. The 5-20% linear gradient gel was prepared with a gradient maker (Hoefer, USA). The density of the gel was 20% at the bottom and decreased to 5% towards the top of the gel.

The seperating gel (gradient or 15%) was poured into the glass plate sandwiches and covered with water saturated n-butanol to avoid the contact of the gel with air. The gel was left for polimerization for 1 hour. N-butanol was washed with water and stacking gel respectively after the polimerization of the

gel. Than stacking gel solution was poured and left for polimerization for 1 hour. Separating and stacking gel components are given in Table 2.2. and 2.3.

Table 2.2. Stacking Gel Mixture (4%T).

Acrylamide-bisacrylamide (30:0.8)	1.33 ml
4X Stacking Gel Buffer (0.5M Tris-Cl , pH:6.8)	2.5 ml
10 % SDS	0.1 ml
ddH_2O	6 ml
10 % Ammonium persulfate*	50 µl
TEMED*	5 µl

*Ammonium Persulfate and TEMED were added after deaeration.

Table 2.3. Separating Gel Mixtures.

	5% Gel	15% Gel	20% Gel
Acrylamide-bisacrylamide (30:0.8)	3.34 ml	12.5 ml	13.2 ml
4X Seperating Gel Buffer (1.5M Tris-Cl , pH:8.8)	5 ml	7.5 ml	5 ml
10 % SDS	0.2 ml	0.3 ml	0.2 ml
ddH_2O	11.4 ml	9.6 ml	-
Sucrose	-	-	3 gr
10 % Ammonium persulfate*	66µl	150 µl	66µl
TEMED*	6.6µl	10 µl	6.6µl

*Ammonium Persulfate and TEMED were added after deaeration

Protein samples were combined with treatment buffer (125 mM Tris-Cl, 20% (v/v) glycerol, 4% (v/w) SDS, 0.02 % (v/w) bromophenol blue, pH 6.8) in 1:1 ratio. The sample was heated at 100 °C for 5 min. The samples were loaded onto the gel after the polymerization of the stacking gel.

Electrophoresis was done at 15 mA/0.75 mm gel (Power supply PP4000, Biometra, Germany) at 15 ^{o}C using a circulating water bath (Heto Holten, Denmark).

In order to prove the purity of the protein obtained from the gel permeation step the protein was subjected to non-denatured SDS polyacrylamide gel electrophoresis [110]. The sample was electrophoresed on a 15% linear, 0.75 mm thick native polyacrylamide gel in a discontinuous buffer system using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). The reducing agent 2-β-mercaptoethanol was left out the standard Laemmli protocol for non-denaturing conditions.

The gels were visualized by silver staining. For the molecular weight determination the SDS gel was scanned using a GT9500 Color Image Scanner (Epson, Japan) and the data were processed with Gelworks 1D software (UVP Products, UK).

2.2.9. Protein Detection in Gels

The gels were visualized by silver staining after electrophoresis.

In silver staining method, first the gel was placed in 100 ml Destain 1 solution and incubated for 30 minutes with gentle shaking. The solution was replaced with 100 ml Destain 2 solution for 30 minutes. Destain 2 was replaced with 100 ml of cross-linking solution. Than the gel was washed with several changes of water over 2 hours and DTT solution was added and incubated with slow shaking. After 30 minutes DTT solution was replaced with 100 ml of

Silver Nitrate Solution. Than the gel was washed with distilled water and developing solution respectively and 100 ml of fresh developing solution was added. After the appearence of the protein bands developing solution was replaced with Destain 2 solution [111].

2.2.10. Identification of the Clinical Isolates

All clinical isolates 19 in toto were identified by API test strips (Bio Merieux, France).

Rapid ID 32 Strep identification system based on standardized and miniaturized enzymatic tests was used for the identification of genera *Enterococcus*. The rapid ID 32 Strep strips consist of 32 test cupules which contain dehydrated reactive medium. After an incubation period of 4 hours, the reactions were read by mini API instrument.

Prior to testing the strains under examination must be checked that it belongs to the *Streptococcaceae* family with Gram staining or catalase testing. Than a subculture was made on blood agar and incubated for 18-24 hours at 35-37 ºC. After incubation period a suspension was prepared with turbidity adjusted to 4 McFarland by using a densitometer (Densimat, Bio Merieux, France) in sterile distilled water or in the suspension medium of the test system. 55 µl of the homogenized suspension was inoculated into each cupule and the lid was closed. The strip was incubated for 4 hours at 37 ºC in aerobic conditions. Prior to reading 1 drop of VP A and VP B reagent was added to the cupules for VP testing, 1 drop of FB reagent was added to the cupules from APPA to GTA and 1 drop of NIN was added to the cupule for HIP testing. After 5 minutes the reactions were read by mini API instrument.

The reader records the color of each cupule and transmits the information to the computer and the identification was obtained using the identification software.

ID 32 Staph identification system based on standardized and miniaturized biochemical tests was used for the identification of the genera *Staphylococcus*. The ID 32 Staph strips consist of 32 cupules, 26 of which are used as test cupules and contain dehydrated test substances. After 24 hours of incubation, the reactions were read by using mini API instrument (BioMerieux, France). Identification was obtained by using the identification software.

Prior to testing the strains under examination must be checked that it belongs to the genera *Staphylococcus* with Gram staining and the strains under examination must be subcultured on blood agar and incubated for 18-24 hours at 35-37 ºC. After incubation period a suspension was prepared with turbidity adjusted to 0.5 McFarland by using a densitometer (Densimat, Bio Merieux, France) in sterile distilled water or in the suspension medium of the test system. 55 µl of the homogenized suspension was inoculated into each cupule. Cupules for URE, ADH and ODC tests were overlaid with mineral oil and the lid was closed. The strip was incubated for 24 hours at 37 ºC in aerobic conditions. After incubation period 1 drop of NIT 1 and NIT 2 reagents were added to NIT test cupule, 1 drop of VP A and VP B reagent was added to the cupules for VP testing and 1 drop of FB reagent was added to the cupules from βGAL to PyrA tests. After 5 minutes the reactions were read by mini API instrument. The reader records the color of each cupule and transmits the information to the computer and the identification was obtained using the identification software.

The strains that showed 99.9% identification were selected for this research.

2.2.11. Optimization of Medium pH and Incubation Temperature for Antibacterial Susceptibility Testing

According to the reference document CLSI M7-A6, the pH value of the media is between 7.2-7.4 and the incubation temperature is 35 ºC for the antibacterial susceptibility tests [105, 106]. However the optimum pH value for

K5 type yeast killer protein is 4.5. Toxin is readily inactivated at pH values above 6.5. The optimum temperature for the toxin activity is 25 °C [81]. In order to eradicate the decrease in the activity of the K5 type yeast killer protein, the pH of the media and the incubation temperature would be decreased.

In an assay conducted in nutrient broth, the growth of 2 clinical isolates of *S. aureus* (A1, A6), *E. faecium* (A10, A11) and *E. faecalis* (A13, A14) and 1 quality control strain from *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212) were tested at pH's ranging from 4.5 to 5.5 at temperatures ranging from 25 to 30 ºC in a 96 well microtitre plate (Nunclon 167008, Nunc, Denmark) in order to determine the optimum pH and temperature for the maximum activity of K5 type yeast killer protein where acceptable bacterial growth (evident turbidity or growth with ≥ 2mm radius) was seen. Microtiter plates were incubated for 48 hours. With this assay the final pH, temperature and incubation time that would be used in antibacterial susceptibility tests were determined.

In order to control if pure K5 type yeast killer protein retained its activity at the pH that would be used in antibacterial susceptibility tests, the agar diffusion assay previously described was done with YEPD at the desired pH values according to the results of the studies [108].

2.2.12. Antibacterial Susceptibility Testing

Nineteen clinical isolates of human pathogenic gram-positive bacteria and two quality control strains were tested for their susceptibility to pure K5 type yeast killer protein according to the reference method CLSI (Clinical and Laboratory Standards Institue) (formerly NCCLS) M7-A6 (Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically) with modifications to ensure the stability and maximum activity of the K5 type yeast killer protein during testing [105].

Pathogenic bacteria were subcultured onto 7% blood agar plates to ensure purity and viability prior to antibacterial susceptibility testing.

Minimum inhibition concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by a broth microdilution test using 96 well microtitre plates (Nunclon 167008, Nunc, Denmark).

Two fold serial dilutions of the K5 type yeast killer protein were prepared in 10 mM Na_2HPO_4-citric acid buffer at pH 4.5, containing 10mM Na_2SO_4 ranging from 64-1024 µg/ml. Nutrient Broth was used as test media in order to eradicate the decrease in the activity of the K5 type yeast killer protein due to the binding of protein to carbonhydrates and the effects of cations in cation adjusted Mueller-Hinton broth recommended by CLSI M7-A6. Nutrient broth was prepared at twice its final concentration at pH 5.5.

Inoculum was prepared in sterile saline water and standardized to an optical density of 0.5 on the McFarland standard (Densimat, Bio Merieux, France) that yielded a stock suspension of bacteria that consists of approximately 1 x 10^8 cfu/ml. Twice the final inoculum size of 5 x 10^5 cfu/ml was achieved with 1:50 dilution followed by 1:2 dilution of the stock solution in nutrient broth.

Fifty µl of cell suspension was added into the wells of a microtitre plate containing 50 µl of protein solution. This step diluted the protein and medium concentrations and inoculum densities to the desired test concentrations as $5x10^5$ cfu/ml and 32-512 µg/ml and also adjusted the final pH to 5.0 for maximum activity of protein and acceptable bacterial growth.

Each row contained a growth control well. Growth control well was prepared by mixing 50 µl of cell suspension with 50 µl of 10 mM Na_2HPO_4-citric acid buffer pH 4.5, containing 10mM Na_2SO_4. Each row also contained a sterility control well including medium and toxin free buffer.

In order to be sure if the final inoculum concentration was approximately $5x10^5$ cfu/ml colony count was done from inoculum suspensions. After the inoculation, 0.01 mL was taken from growth control well and diluted in 10 mL of 0.9% NaCl. After shaking 0.1 mL from this suspension was taken and spread on 7% blood agar plates. The presence of 50 colonies after incubation proved that the inoculum concentration was $5x10^5$ cfu/ml.

Microtitre plates were incubated at 25 ^oC for 24-48 hours and the wells were mixed with a multi-channel pipette after first 2 hours. MIC values and MIC ranges were determined. MIC was defined as the lowest concentration of K5 type yeast killer protein that completely inhibited the visible growth of the organism after the incubation period of 48 hours determined by the unaided eye. When only less than nine isolates of a species were tested only MIC ranges of the antibacterial agents were tabulated so MIC_{50} (the MIC at which 50% of the isolates were inhibited) and MIC_{90} (the MIC at which 90% of the isolates were inhibited) values were not determined [112].

Minimum bactericidal concentration (MBC) was defined as the lowest protein concentration that killed 99.9% (with $\leq$ 5 colonies remaining) of the final inoculum. MBCs were determined by plating nonturbid wells at 48. hour. A 0.01 ml sample was plated onto 7% horse blood agar plates and incubated until growth was seen in the growth control subculture (usually 24 hours) at 35°C as described by Peterson [113].

The entire data is presented as the average of 3 independent experiments.

Concerning the possibility of toxin degradation by proteases produced by bacteria [114**,** 115] the antibacterial susceptibility testing of 2 clinical isolates of *S. aureus* (A1, A6)*, E. faecium* (A10, A11) and *E. faecalis* (A13, A14) and 1 quality control strain from *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212) were replicated by the addition of protease inhibitor. Complete Mini EDTA free protease inhibitor cocktail tablets (Roche, Germany) were used in order to eliminate the effect of bacterial proteases. One tablet was dissolved in

1,5ml water and 21 µl was used for 100µl inoculum and protein mixture. Concentration of the inoculum and protein was recalculated. The results of the tests performed with protease inhibitor and without protease inhibitor for the same strains were compared in order to determine if bacterial proteases produced by bacteria effects the results by reduced toxin activity.

2.2.13. Cell Killing Activity of the K5 Type Yeast Killer Protein

K5 type yeast killer toxin was tested on *Staphylococcus aureus* A1 and *Staphylococcus aureus* A3 with the MIC and MBC values of 32µg/ml and 256µg/ml for A1 and 32µg/ml and 64µg/ml for A3 for its cell killing activity in order to distinguish whether bacterial killing was concentration and/or time dependent: concentration-dependent bacterial killing occurs when the rate and extend of killing increases with progressively higher antibacterial concentrations, and time-dependent killing occurs when increasing antibacterial concentrations to more than the MIC do not result in proportional increases in killing. The rate and extend of killing were determined by plotting viable count ($\log_{10}$ cfu/ml) against time (h).

It was also determined if the toxin had bactericidal or bacteriostatic activity. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ decrease in cfu/ml, whereas bacterioststic activity was defined as a < 3 $\log_{10}$ decrease in cfu/ml [116-117].

Both strains were tested in its MIC and 2, 4 and 8 times the MIC.

Prior to analyses of cell killing kinetics, bacterial strains were freshly subcultured on 7% horse blood agar plates at 35 °C for 20 to 24 hours to ensure purity and viability.

Inoculum was prepared in sterile saline water and standardized to an optical density of 0.5 on the McFarland standard (Densimat, Bio Merieux,

France) that yielded a stock suspension of bacteria that consists of approximately 1 x 10^8 cfu/ml. Cell suspension was diluted 1:50 in 2 fold concentrated nutrient broth (pH 5.50) to achieve an inoculum of 2 x 10^6 cfu/ml.

Two fold serial dilutions of pure K5 type yeast killer protein was prepared in 10 mM Na_2HPO_4-citric acid buffer at pH 4.5, containing 10mM Na_2SO_4 with the range of 64-512 µg/ml were prepared in 10mM Na_2HPO_4-citric acid buffer pH 4.5 containing 10mM Na_2SO_4**.**

Threehundred µl of cell suspension was mixed with 300 µl of protein solutions with the range of 64-512 µg/ml in a sterile tube to obtain the desired cell density of approximately 10^6 cfu/ml, final toxin concentration of 256 (8 x MIC) to 32µg/ml (MIC), the final pH of 5.0 and the desired medium concentration. Growth control tube included bacterial suspension with toxin free buffer**.** Test solution tubes were incubated at 25°C without agitation for 48 hours.

Fifty µl sample was aseptically removed from growth control and test tubes at predetermined time points (0, 4, 8, 12, 24, 28, 32, 36 and 48 hours) and samples were 2 x 10^4 fold diluted in cold 0.9% NaCl. 50µl of the samples were spread on to 7% horse blood agar plates at pH 7.2 for colony count determination. Deactivation of the toxin was achieved by this step as the toxin was readily inactivated at pH values above 6.5 [81]. Colony counts were determined after the incubation period of 24 hours at 35 ^oC.

If the colony counts were suspected to be less than 2 x 10^4 CFU/ml samples were diluted 1000 fold in cold 0.9% NaCl. If the colony counts were suspected to be more than 10^6 CFU/ml samples were diluted 4x10^5 fold in cold 0.9% NaCl. If the colony counts were suspected to be less than 1000 CFU/ml samples were diluted 100 fold in cold 0.9% NaCl. The limit of detection for this metod was 100 CFU/plate, corresponding to 2 $\log_{10}$ CFU/ml.

All time-kill curve experiments were performed in triplicate.

CHAPTER III

RESULTS

3.1. Production of the K5 Type Yeast Killer Toxin and Crude Toxin Preparation

Pichia anomala NCYC 434 (K5) cells were grown until stationary phase in 1L of YEPD broth medium buffered to pH 4.5 with citrate-phosphate buffer and supplemented with 5% glycerol as toxin stabilizer at 20 ^{o}C for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA) to maintain the optimum conditions for highest degree of killer toxin activity since the production of yeast killer toxins are highly dependent on the pH of the cultivating medium and incubation temperature [81]. Concentration of the protein mixture was 3,2µg/ml.

Pichia anomala NCYC 434 (K5) cells were removed from the culture liquid by centrifugation (KR 22i, Jouan, France) at 5000 rpm for 10 min. at 4 ^{o}C to obtain the cell free culture medium and filtered through 0.45µm and 0.2µm cellulose acetate membranes (Sartorius, AG, Germany) respectively for the sterilization of the medium [81].

The filtrate containing the K5 type yeast killer protein was further concentrated 400-fold by using a centrifugal 30kDa-cutoff ultrafilter (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 rpm, 4 ^{o}C (MR23i, Jouan, France)

to yield a final protein concentration of 1280µg/ml. In order to obtain the YEPD free sample the concentrated crude protein was buffer-exchanged to 30mM N-methylpiperazine-HCl at pH 4.8 by using a centrifugal 5kDa-cutoff ultrafilter (Vivaspin VS2012, Sartorius ,AG, Germany) at 4200 rpm, 4 ^{o}C (MR23i, Jouan, France). This step was performed three times. The samples were than filtered by 0.2µm syringe filter (Sartorius, AG, Germany) prior to to injection onto the HPLC and tested for its killing activity with an agar diffusion assay [81].

3.2. Assesment of Killer Toxin Activity

Killer toxin activity was tested with agar diffusion assay before every stage of the study. Thirty µl of the concentrated and buffer-exchanged protein was spotted onto YEPD pH 4.5 agar seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells. After 48 h incubation at 22 ^{o}C a clear growth inhibition zone of 25 mm which corresponds to 2.5 AU killer toxin was observed (Figure 3.1) [81, 108].

3.3. K5 Type Yeast Killer Toxin Purification

Toxin purification steps were done on a a fully automated HPLC system (Biocad 700E Perseptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at 20 ^{o}C [81].

K5 type yeast killer protein was purified by anion exchange chromatography using POROS HQ/M column for the optimal resolution (Figure 3.2). Toxin was eluted in the fraction (indicated by arrow) corresponding to 120 mM of NaCl. Killer protein obtained from anion exchange chromatography was concentrated and buffer exchanged to 100mM Na_2HPO_{4-} citric acid buffer, pH 4.5 by using 5 kDa molecular cut-off ultrafilter (Vivaspin VS2012, Sartorius ,AG, Germany) at 4200 rpm, 4 ^{o}C (MR23i, Jouan, France) and then put on a gel

permeation column TSK G 2000SW 7.5 mmD/300mmL (TosoHaas, Japan) (Figure 3.3). Active fraction was eluted at 8.5 ml (indicated by arrow) [81].

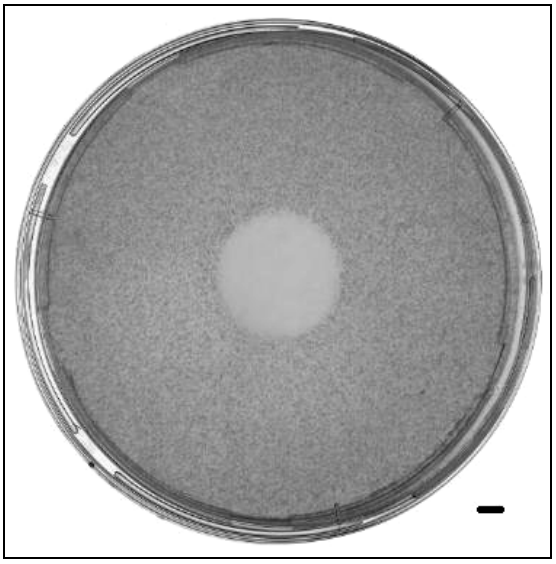

Figure 3.1. Killer Activity of the Crude K5 Type Toxin on Killer Toxin Sensitive *S. cerevisiae* NCYC 1006 Cells Determined by Agar Diffusion Assay. Thirty µl of the concentrated and buffer-exchanged protein (38,4 µg/ml) gave a clear growth inhibition zone of 25 mm which corresponds to 2.5 AU killer toxin. Bar scale represents 5 mm.

These active fractions are then concentrated (totally 500-fold concentrated toxin was obtained) and buffer exchanged to the 10mM Na_2HPO_4-citric acid buffer, pH 4.5 containing 10mM Na_2SO_4 but the salt by using 5 kDa molecular cut-off ultrafilters (Vivaspin VS2012, Sartorius, AG, Germany). Thirty µl of the purified protein obtained from gel permeation chromatography was spotted on to YEPD (pH 4.5) agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells for the assessment of the killer toxin activity and gave a clear inhibition zone of 14 mm which corresponds to 1.4 AU killer toxin (Figure 3.4.)[81].

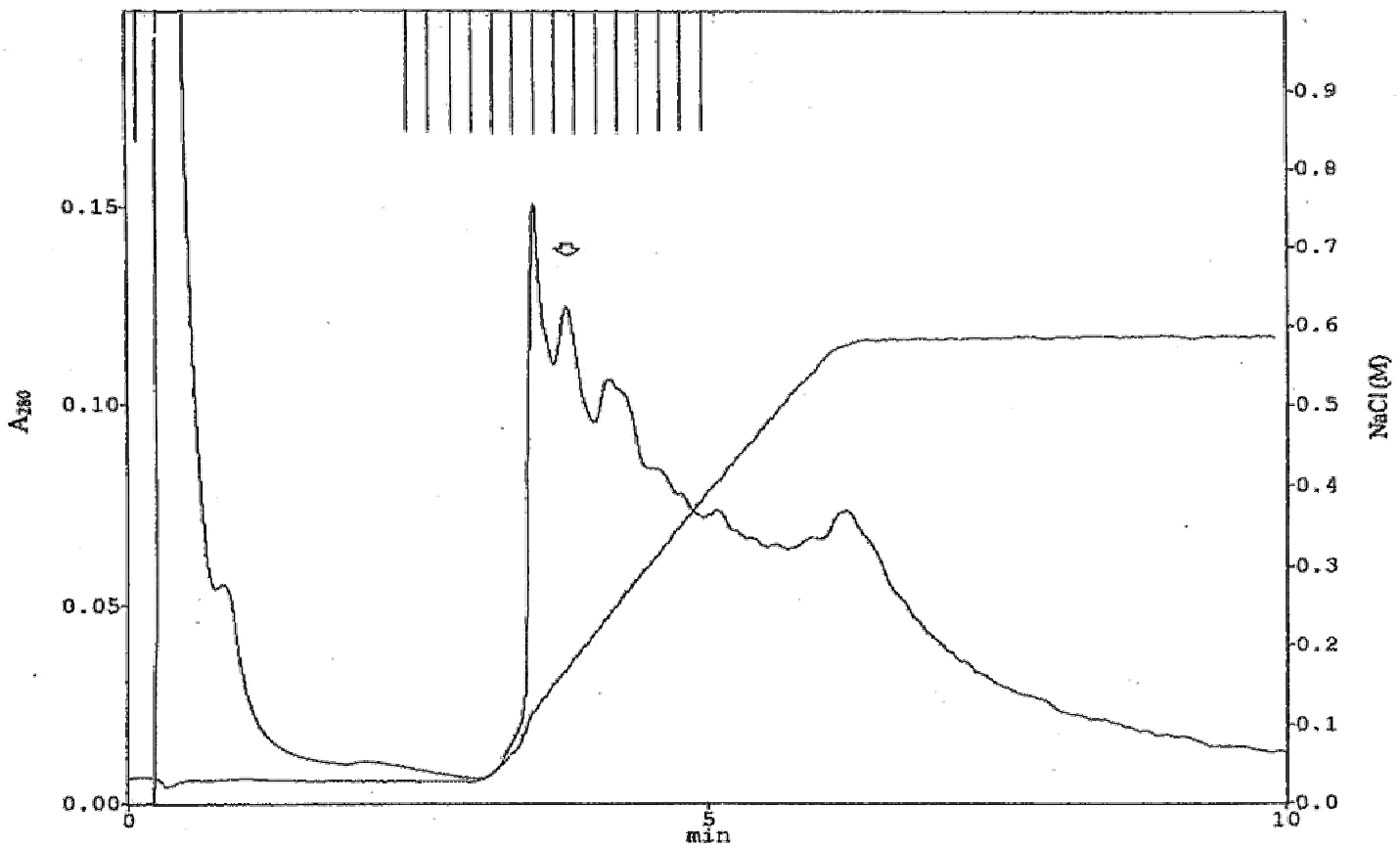

Figure 3.2. Elution Profile of the K5 Type Toxin on a POROS HQ/M Column.

Column size: 4,6 mmD/100mmL; Sample: 750 µl; Starting buffer 30 mM N-methyl piperazine- HCl pH:4,8; Gradient: 0-500 mM NaCl in the starting buffer in 20 CV; Flow rate: 10 ml/min; Detection: UV 280 nm.; Fraction volume: 1600 µl. Fraction indicated by arrow contains the K5 type killer toxin corresponding to 120 mM NaCl.

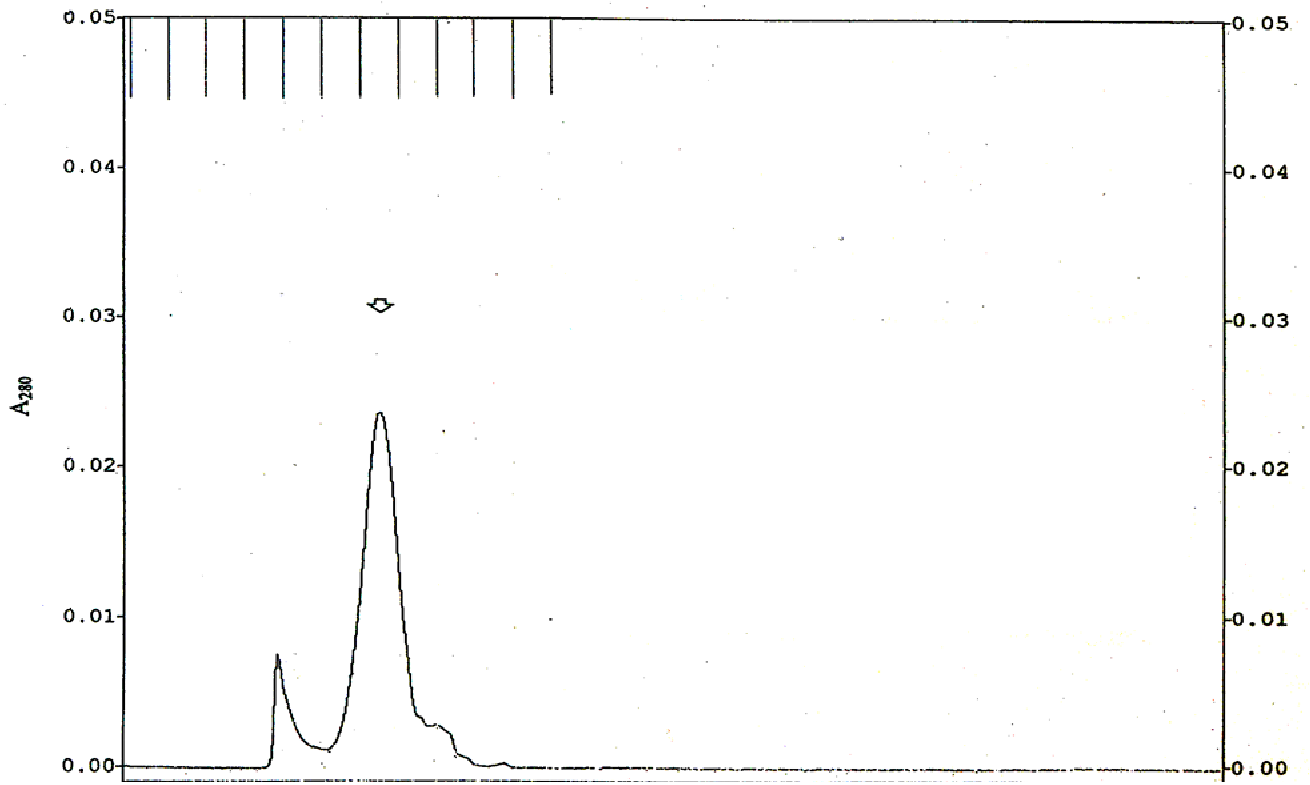

Figure 3.3. Elution Profile of the K5 Type Toxin on a TSK G2000SW Column.

Column size: 7.5 mmD / 300 mmL; Sample: 40 µl; Elution buffer: 0.1M Na_2HPO4 pH 4.5 + 0.1 M Na_2SO4 ; flow rate 1 ml/min; detection 280 nm UV Fraction volume 1300 µl. Fraction containing killer protein is eluted at 8,5 ml and indicated by arrow.

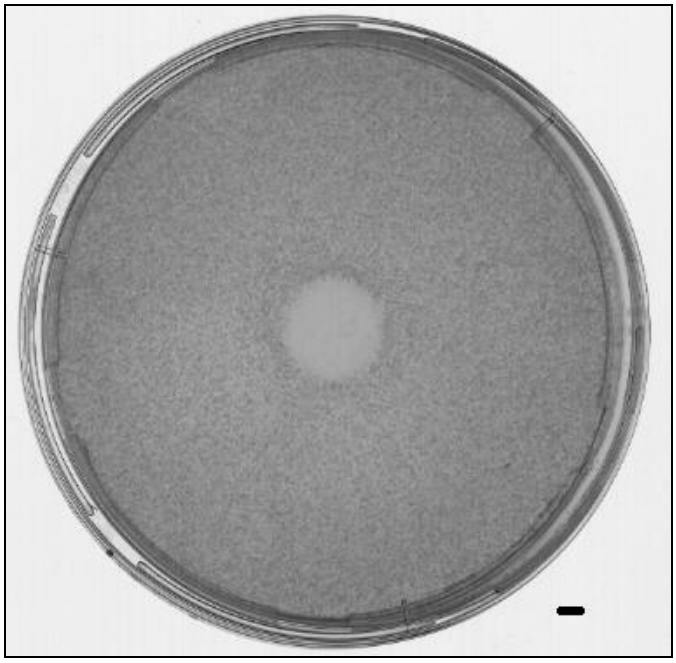

Figure 3.4. Killer Activity of the Pure K5 Type Yeast Killer Protein on Killer Toxin Sensitive *S. cerevisiae* NCYC 1006 Cells Determined by Agar Diffusion Assay. Thirty µl of the purified protein obtained from gel permeation chromatography (30,75 µg/ml) gave a clear growth inhibition zone of 14 mm which corresponds to 1.4 AU killer toxin. Bar scale represents 5 mm.

3.4. Assessment of Protein Concentration

Protein concentration was determined as described previously by Bradford [109]. Protein concentration in the sample was calculated using a standard curve of absorbance versus protein amount from the bovine serum albumin standards (Figure 3.5.).

10 µl of the purified K5 type yeast killer protein gave an UV absorbance of 0,0185 at 595 nm which corresponds to 10.25 µg and approximately equal to 1024 µg/ml for antibacterial susceptibility testing.

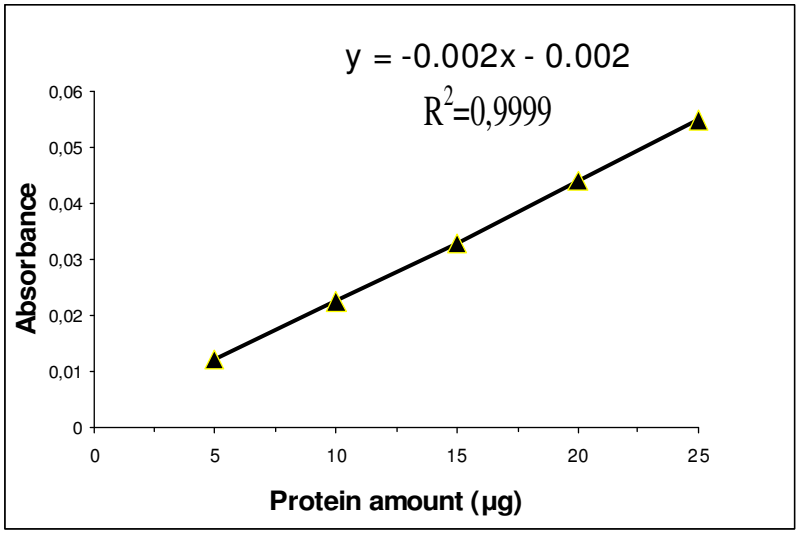

Figure 3.5. Standard Curve of Protein Amount versus Absorbance. Bovine serum albumin solutions were used as standards. 10 µl of the purified K5 type yeast killer protein gave an UV absorbance of 0,0185 at 595 nm which corresponds to 10.25 µg.

3.5. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

Purified killer protein (0.8µg) was electrophoresed on a 15% linear SDS-PAGE gel in a discontinuous buffer system under non-denaturing conditions to check its purity. Observation of single protein band on the silver stained gel indicates the absence of any contamination (Figure 3.6.).

Molecular weight of the K5 type yeast killer protein was determined with a 5%-20% linear gradient SDS-PAGE gel in a discontinuous buffer system under denaturing conditions. Molecular weight markers were used to determine the molecular weight of K5 type yeast killer protein. SDS gel was scanned using a GT9500 Color Image Scanner (Epson, Japan) and the data were processed with Gelworks 1D software (UVP Products, UK). K5 type yeast killer protein migrated between glutamate dehydrogenase (55,562) and aldolase (39,212) and its molecular weight was found to be 49 kDa (Figure 3.6.).

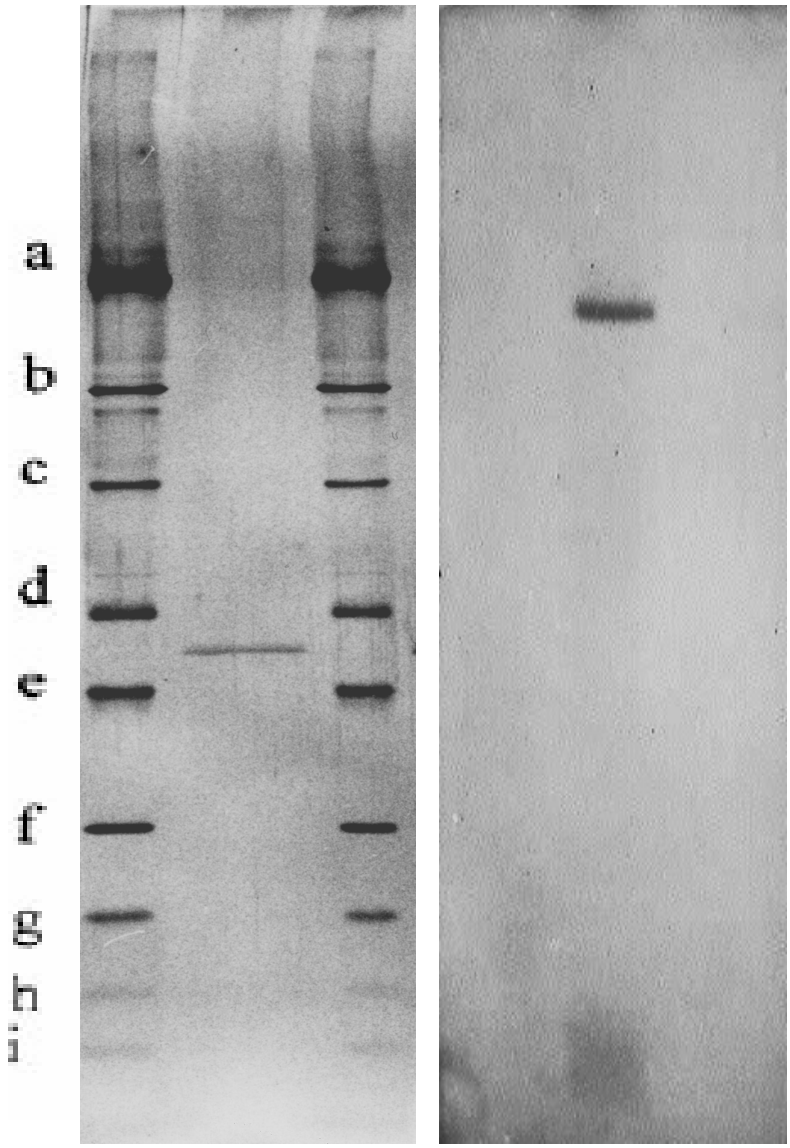

Figure 3.6. Gradient SDS-PAGE (**A**) and Non-denatured SDS-PAGE (**B**) Profiles of the K5 Type Yeast Killer Protein. Lanes 2-4 are K5 type yeast killer protein. Lanes 1-3 are molecular mass markers (Da). a) α_2-macroglobulin (170,000), b) β-galactosidase (116,353), c) fructose-6-phosphate kinase (85,204), d) glutamate dehydrogenase (55,562), e) aldolase (39,212), f) triose phosphate isomerase (26,626), g) trypsin-inhibitor (20,100), h) lysozyme (14,307), i) aprotinin (6,500).

3.6. Identification of the Clinical Isolates

All clinical isolates 19 in toto were identified by API test strips (Bio Merieux, France).

ID 32 Staph identification system was used for the identification of the genera *Staphylococcus* and rapid ID 32 Strep identification system was used for the identification of genera *Enterococcus*. The reactions were read by mini API instrument. Identification was obtained by using the identification software.

Only the strains that showed 99.9% identification were selected for this research.

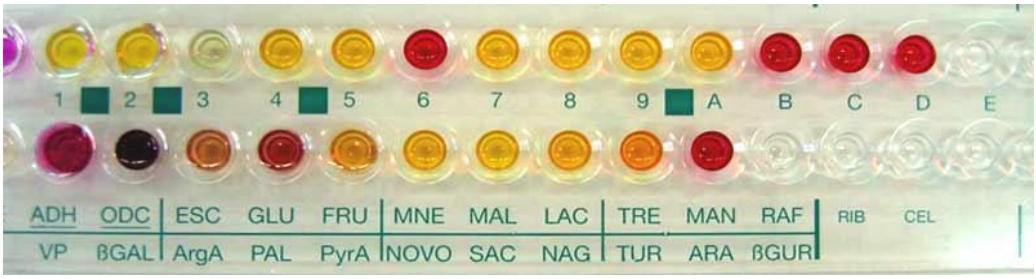

Figure 3.7. Test Strip for API ID 32 Staph Identification System [119]

In this study; identification of 19 clinical isolates of gram-positive bacteria isolated from skin and wound infections was done including 9 clinical isolates of *Staphylococcus aureus*, 7 clinical isolates of *Enterococcus faecalis* and 3 clinical isolates of *Enterococcus faecium* (Figure 3.8.).

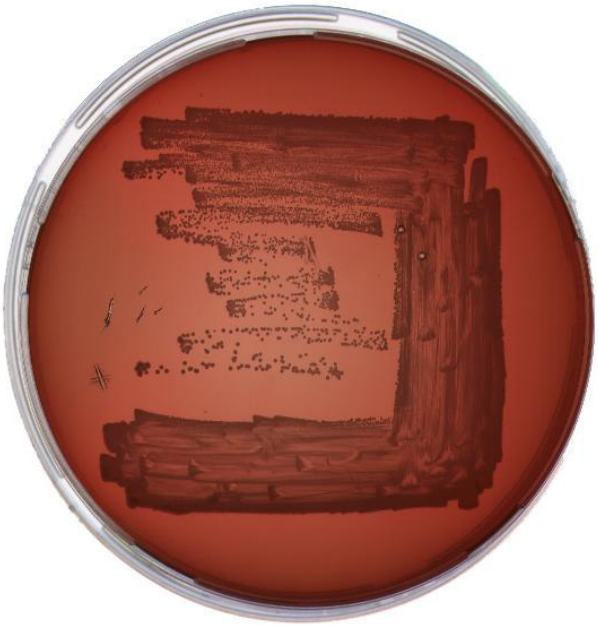

Figure 3.8. *Staphylococcus aureus* (MSSA) A5 (99.9% Identification with API System) Cells on 7% Horse Blood Agar Medium.

3.7. Optimization of Medium pH and Incubation Temperature for Antibacterial Susceptibility Testing

The optimum pH and temperature for the maximum activity of K5 type yeast killer protein where acceptable bacterial growth (evident turbidity or growth with ≥ 2mm radius) was seen were determined. Acceptable bacterial growth was seen at pH's ranging from 5.0 to 5.5 at all temperatures from 25 to 30 ºC in nutrient broth for 2 clinical isolates of *S. aureus* (A1, A6), *E. faecium* (A10, A11) and *E. faecalis* (A13, A14) and 1 quality control strain from *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212). According to the results it was determined that the final pH of the inoculum and protein suspension must be 5.0 and the incubation temperature must be 25 ºC and incubated for 48 hours for acceptable bacterial growth and maximum activity of K5 type yeast killer protein in antibacterial susceptibility tests.

The agar diffusion assay previously described was done with YEPD at pH 5.0 and it was shown that pure K5 type yeast killer protein retained its activity at pH 5.0 [108].

3.8. Antibacterial Susceptibilty Testing

Nineteen clinical isolates of human pathogenic gram-positive bacteria isolated from skin and wound infections and 2 quality control strains were tested for their susceptibility to pure K5 type yeast killer protein in different concentrations ranging from 32 to 512 µg/ml with a broth microdilution test using 96 well microtitre plates according to the reference method Clinical and Laboratory Standards Institue M7-A6 with modifications to ensure the stability and maximum activity of the K5 type yeast killer protein during testing [105]. MICs were determined visually (Figure 3.9.) and for MBC determination a 0.01 ml sample from all visually clear wells was plated onto 7% horse blood agar plates and incubated for 24 hours at 35°C (Figure 3.10.) [113]. Concerning the possibility of toxin degradation by proteases produced by bacteria [114, 115] the antibacterial susceptibility testing of 2 clinical isolates of *S. aureus* (A1, A6), *E. faecium* (A10, A11) and *E. faecalis* (A13, A14) and 1 quality control strain from *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212) were replicated by the addition of protease inhibitor and MIC and MBC values performed with protease inhibitor and without protease inhibitor for the same strains were compared in order to determine if bacterial proteases produced by bacteria effects the results by reduced toxin activity. The results reveal that there were no changes in the MIC and MBC values of the strains tested with the addition of protease inhibitor thus indicating that the proteases produced by bacteria had no effect on the results of the susceptibility testing.

The entire data is presented as the average of 3 independent experiments.

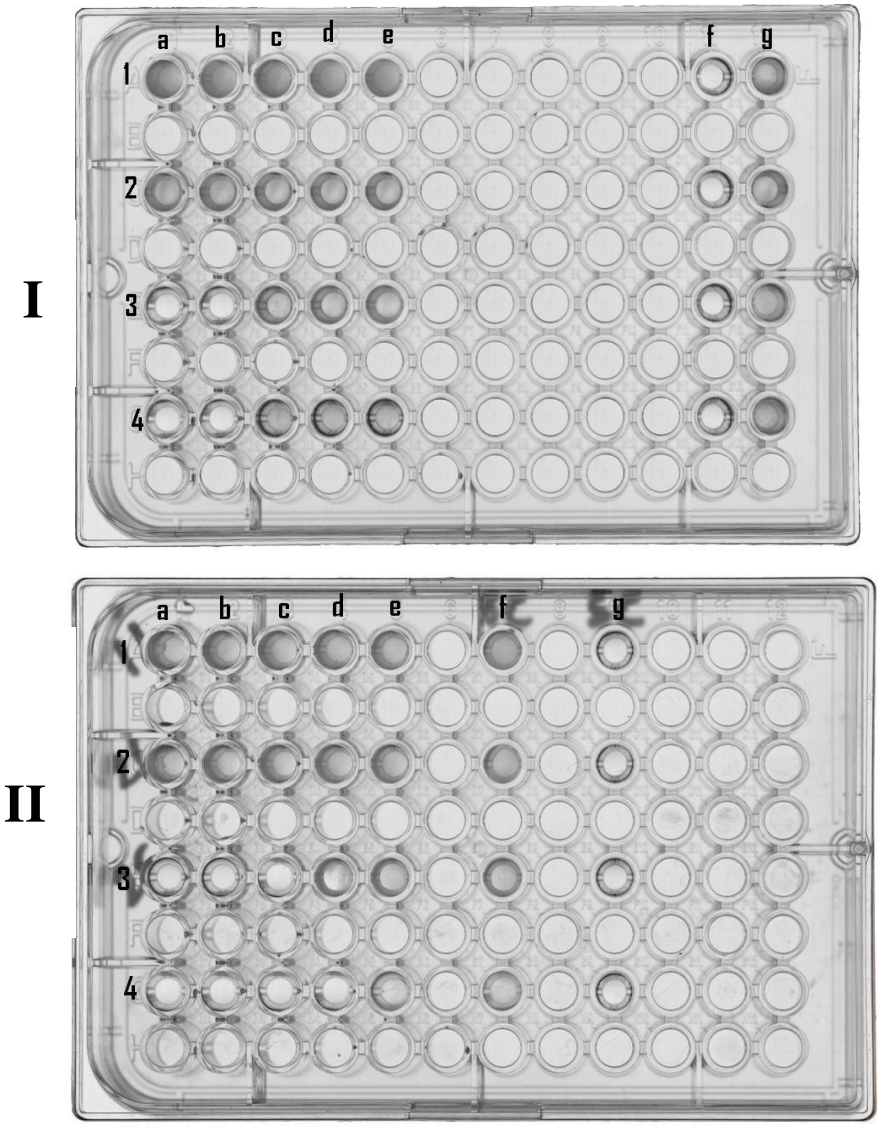

Figure 3.9. Antibacterial Susceptibility Studies of the K5 Type Yeast Killer Toxin on a Microtitre Plate. Arrows indicate the MICs (visually clear wells). I) Protein concentrations; a) 512 µg/ml b) 256 µg/ml c) 128 µg/ml d) 64 µg/ml e) 32 µg/ml; f) Sterility Control, g) Growth Control 1) *Enterococcus* faecalis ATCC 29212 MIC: - 2) *Enterococcus* faecalis A15 MIC: - 3) *Enterococcus faecium* A10 MIC: 256µg/ml 4) *Enterococcus faecium* A11 MIC: 256 µg/ml II) Protein concentrations; a) 512 µg/ml b) 256 µg/ml c) 128 µg/ml d) 64 µg/ml e) 32 µg/ml; f)Growth Control, g) Sterility Control; 1) *Staphylococcus aureus* (MRSA) A6 MIC: - 2) *Staphylococcus aureus* (MRSA) A7 MIC: - 3) *Staphylococcus aureus* (MSSA) ATCC 29213 MIC: 128 µg/ml 4) *Staphylococcus aureus* (MSSA)A4 MIC: 64µg/ml 34) ("-" indicates no inhibition)

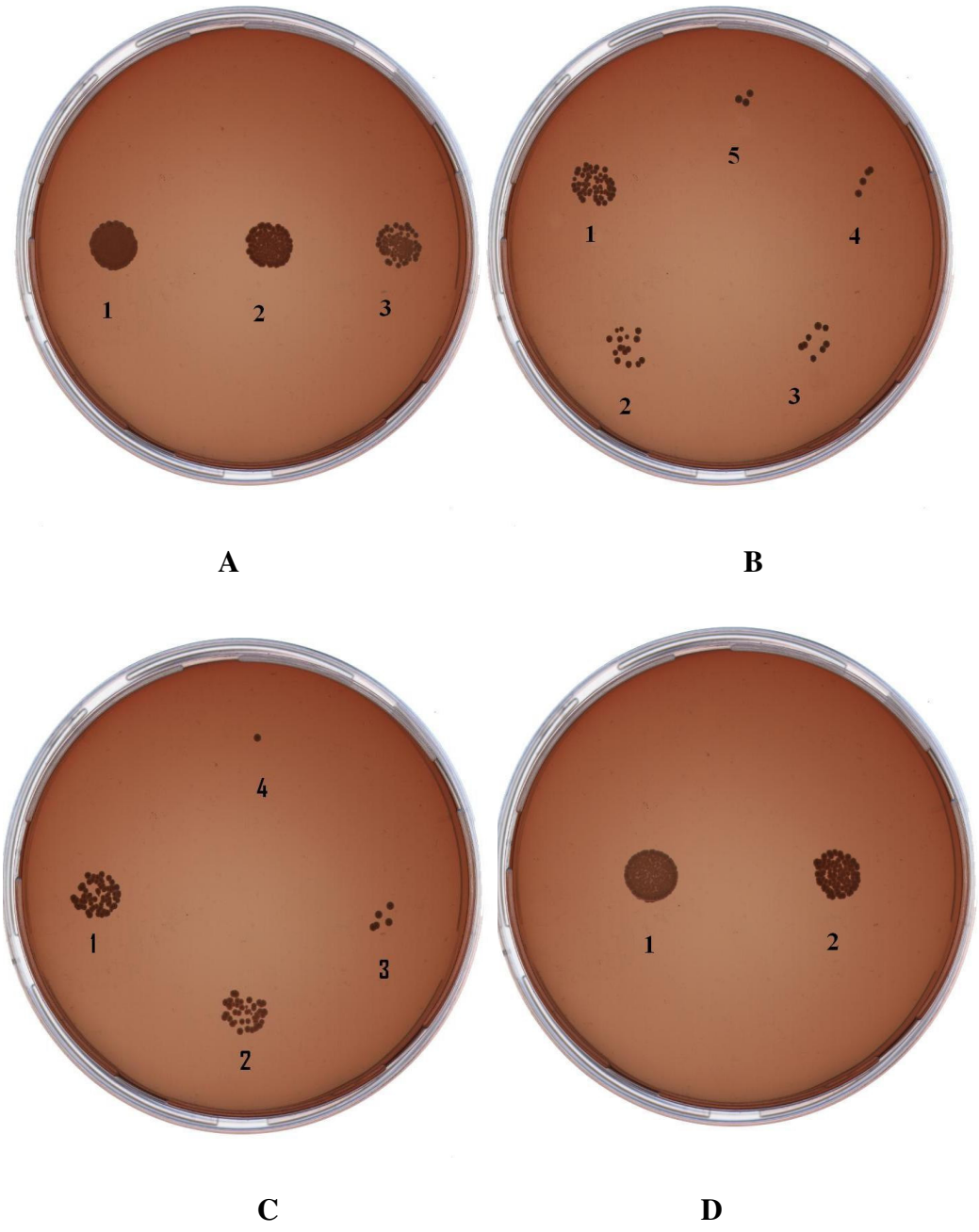

Figure 3.10. MBC Determination on 7% Horse Blood Agar Plates. A) *Staphylococcus aureus* (MSSA) ATCC 29213 MIC: 128µg/ml MBC: >512µg/ml 1-128µg/ml 2- 256µg/ml 3- 512µg/ml B) *Staphylococcus aureus* (MSSA) A3 MIC: 32µg/ml MBC : 256µg/ml 1- 32µg/ml 2- 64µg/ml 3- 128µg/ml 4- 256µg/ml 5- 512µg/ml C) *Staphylococcus aureus* (MSSA) A4 MIC : 64µg/ml MBC : 256µg/ml 1- 64µg/ml 2-128µg/ml 3- 256µg/ml 4- 512µg/ml D) *Enterococcus faecium* A11 MIC: 256µg/ml MBC : >512 1- 256µg/ml 2- 512µg/ml

The results of susceptibility testing of the 19 pathogenic gram-positive bacteria isolated from skin and wound infections and 2 quality control strains to the purified K5 type yeast killer protein (MICs and MBCs) are represented in Table 3.1. K5 type yeast killer protein showed a slight inhibitory activity against clinical isolates of MSSA (5) and quality control strain *Staphylococcus aureus* (MSSA) ATCC 29213. All clinical isolates of MRSA (4) were found to be not susceptible to the toxin. Three clinical isolates of *Enterococcus faecium* were slightly susceptible to the purified K5 type yeast killer protein. All clinical isolates of *Enterococcus faecalis* (7) and the quality control strain *Enterococcus faecalis* ATCC 29212 were found to be not susceptible to the toxin. MIC range of the K5 type yeast killer protein against *Staphylococus aureus* (MSSA) and *Enterococcus faecium* are represented in Table 3.2.

3.9. Cell Killing Activity of the K5 Type Yeast Killer Protein

Time-kill curve of the K5 type yeast killer toxin was determined for *Staphylococcus aureus* (MSSA) A1 and *Staphylococcus aureus* (MSSA) A3 with the MIC and MBC values of 32µg/ml and 64µg/ml for A1 and 32µg/ml and 256µg/ml for A3 in order to distinguish whether bacterial killing was concentration and/or time dependent and if the toxin had bactericidal or bacteriostatic activity. Prior to analyses of cell killing kinetics, bacterial strains were freshly subcultured on 7% horse blood agar plates at 37 °C for 20 to 24 hours to ensure purity and viability.

Cell killing activity studies were done in 2 fold concentrated Nutrient broth (final pH 5.0) with concentrations of protein equal to 1, 2, 4 and 8 times the MIC.

The plot of the killing activity of the K5 type yeast killer toxin as the $\log_{10}$ CFU/ml versus time is presented as time- kill curve in Figure 3.11. and Figure 3.12.

Table 3.1. MICs and MBCs of the K5 Type Yeast Killer Protein Against 19 Pathogenic Gram-Positive Bacteria Isolated From Skin and Wound Infections and 2 Quality Control Strains ("-" represents no inhibition).

STRAIN	**STRAIN NUMBER\ SOURCE**	**MIC µg/ml**	**MBC µg/ml**
Staphylococus aureus (MSSA)	ATCC 29213	128	>512
S. aureus (MSSA)	A1	32	64
S. aureus (MSSA)	A2	64	512
S. aureus (MSSA)	A3	32	256
S. aureus (MSSA)	A4	64	256
*S. aureus (MSS*A)	A5	32	>512
S. aureus (MRSA)	A6	_	_
S.aureus (MRSA)	A7	_	_
S.aureus (MRSA)	A8	_	_
S.aureus (MRSA)	A9	_	_
E. faecium	A10	256	>512
E. faecium	A11	256	>512
E. faecium	A12	256	>512
Enterococcus faecalis	ATCC 29212	_	_
E. faecalis	A13	_	_
E. faecalis	A14	_	_
E. faecalis	A15	_	_
E. faecalis	A16	_	_
E. faecalis	A17	_	_
E. faecalis	A18	_	_
E. faecalis	A19	_	_

Table 3.2. MIC range of the K5 type yeast killer protein against MSSA.

Species (Number of isolates)	MIC Range µg/ml
Staphylococus aureus (MSSA) (6)	32-128

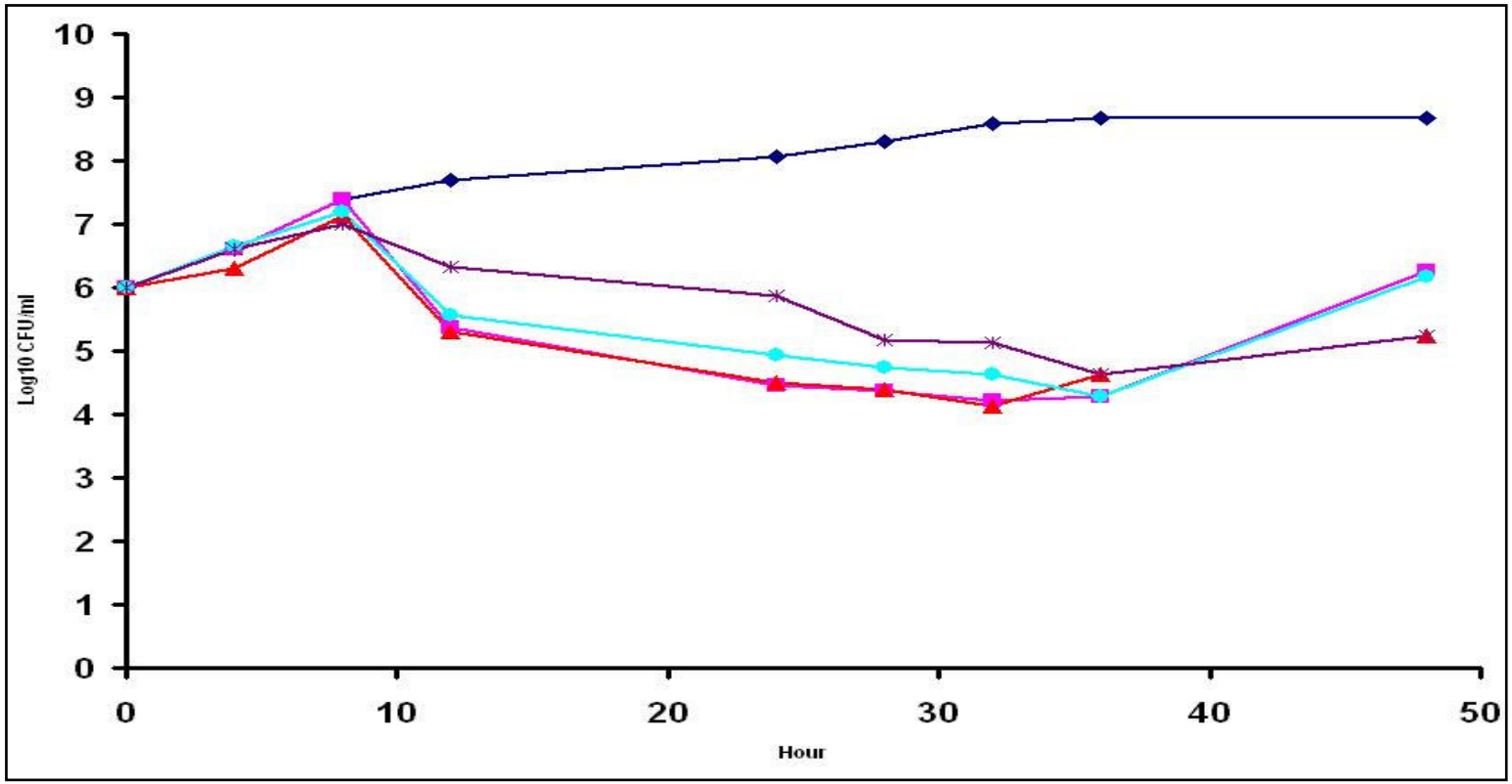

Figure 3.11.Time-kill Curve Plot for *Staphylococcus aureus* (MSSA) A1. Protein concentrations: the MIC: 32 µg/ml (■), 2 times the MIC: 64 µg/ml (▲), 4 times the MIC: 128 µg/ml (●) and 8 times the MIC: 256 µg/ml (*). (♦ represents the growth control).

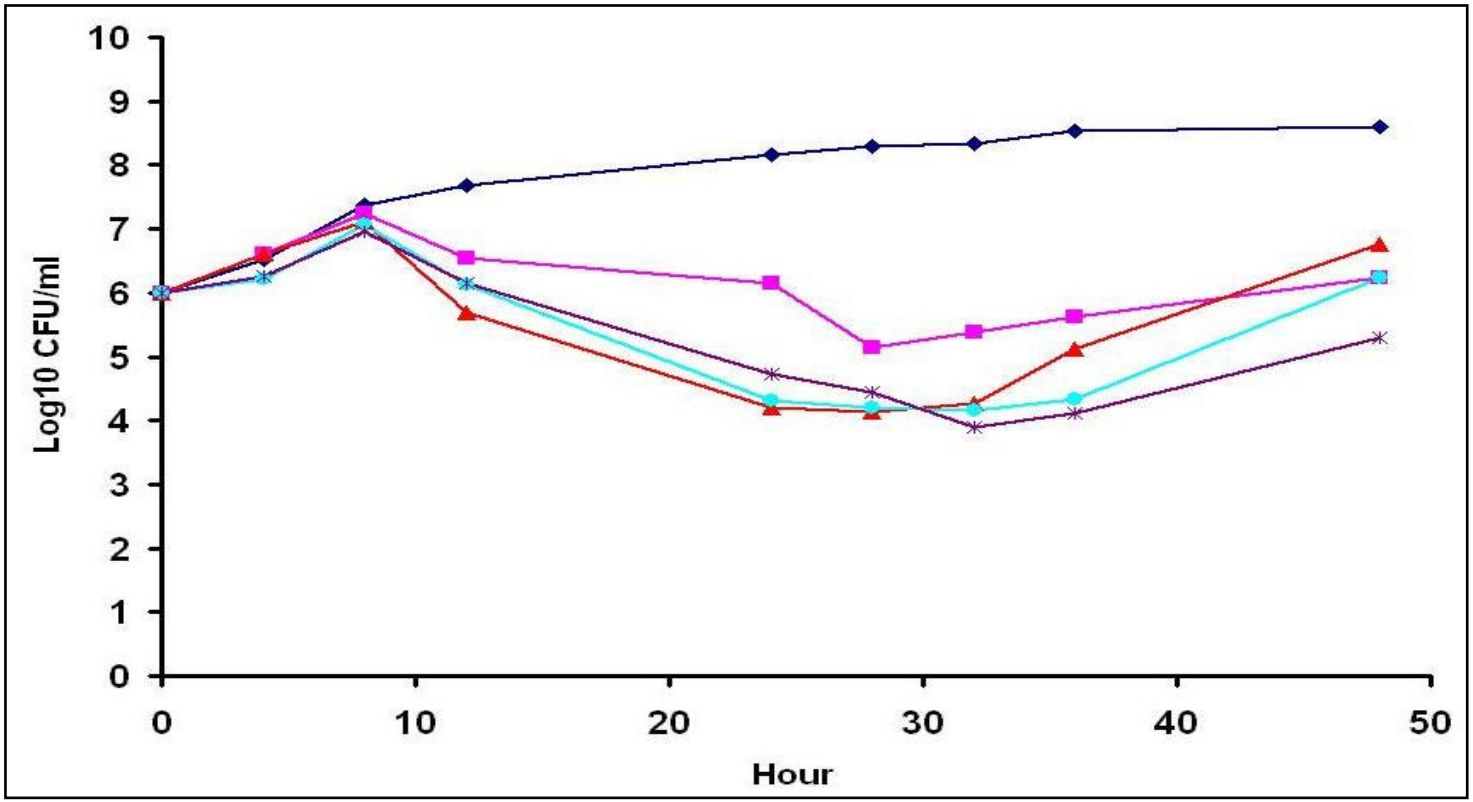

Figure 3.11.Time-kill Curve Plot for *Staphylococcus aureus* (MSSA) A3. Protein concentrations: the MIC: 32 µg/ml (■), 2 times the MIC: 64 µg/ml (▲), 4 times the MIC: 128 µg/ml (●) and 8 times the MIC: 256 µg/ml (*). (♦ represents the growth control).

CHAPTER IV

DISCUSSION

Despite a century of often successful prevention and control efforts, infectious diseases remain an important global problem in public health, causing over 13 million deaths each year. The emergence and rapid dissemination of infectious agents resistant to currently used antibiotics continue to be matters of concern for the medical community and have emphasized the need for new alternatives for the treatment of diseases [28, 29, 97].

In skin and soft tissue infections, the incidence of infections caused by multidrug-resistant gram-positive organisms, which are the major pathogens in these infections as *Staphylococcus aureus*, has been increasing over the last 20 years [31]. Methicillin-resistant *S. aureus* (MRSA), first reported in 1961 following the introduction of the semisynthetic lactamase-resistant penicillins, has increased in prevalence, the proportion of isolates resistant to methicillin in the USA rising from 2.4% in 1975 to 29% in 1991 [29]. *Staphylococcus aureus* where methicillin resistance, which encompasses resistance to all currently available β-lactams, macrolides, lincosamides and aminoglycosids is a common phenotype among multiresistant nosocomial isolates [32, 33]. A recent survey of more than 7000 isolates of *S. aureus* from ten western European countries indicated that 12.8% were methicillin-resistant [29]. Today MRSA is recognized as a major nosocomial pathogen, accounting for 28% of surgical wound infections and 21% of skin infections in the USA [29]. Vancomycin has been widely used to treat MRSA infections, and unsurprisingly, this has led to

the identification of vancomycin-intermediate *S. aureus* in 1997 and fully vancomycin-resistant *S. aureus* in 2002. *S. aureus* continues to be a significant health problem in the hospital and other settings, such as nursing homes . Nasal carriage of *S. aureus* is an important risk factor in the hospital setting, particularly in patients requiring surgery, implanted devices, or hemodialysis [32]. Community-acquired MRSA is also becoming a problem, causing infective endocarditis in intravenous drug users and invasive infections in immunocompromised patients [29].

Enterococcus has been declared the pathogen of the 1990s. Enterococci is the second leading cause of nosocomial infections and an increase in the emergence of multidrug-resistant *Enterococcus faecium* has been described [34]. Since the first report of vancomycin-resistant enterococci (VRE) in 1988, VRE have spread worldwide with unanticipated rapidity [28]. Especially *Enterococcus faecium*, are intrinsically resistant to β-lactams and aminoglycosides and have acquired high-level resistance to many other antibacterials [35]. Vancomycin has been used to treat such resistant gram-positive bacterial infections for some time. However, the wide spread of vancomycin-resistant enterococci (VRE) reduce the clinical efficacy of vancomycin, and the lack of effective drugs against such multiresistant pathogens is also a serious medical problem [35]. Unfortunately, effective therapy against VRE is presently lacking [34].

For wound infections, the current standard of care involves using systemic antibiotics or topical antimicrobial agents, such as silver sulfadiazine, mafenide acetate, and gentamicin sulfate, all initially introduced in the 1960s. These products have various limitations, including a limited ability to penetrate partial-and full-thickness burns, limited efficacy against both gram-positive and gram-negative bacteria, potential toxicity to host cells and the emergence of antibiotic-resistant bacteria. Recent reports that mortality remains significantly higher in patients who receive inadequate antimicrobial therapy support the need for novel strategies to prevent and treat wound infections [28, 30]. In skin and soft tissue infections, the incidence of infections caused by multidrug-

resistant gram-positive organisms, which are the major pathogens in these infections as *Staphylococcus aureus*, has been increasing over the last 20 years [31]. Enterococci is the second leading cause of nosocomial infections and an increase in the emergence of multidrug-resistant *Enterococcus faecium* has been described [34].

Consequently, the priority for the next decades should be focused on the development of alternative drugs and/or the recovery of natural molecules that would allow the consistent and proper control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance [36]. The new generation of native peptide and proteins, also known as antimicrobial peptides and proteins, isolated from a full range of organisms and species from bacteria to man are suggested as a new alternative for the treatment of pathogen-caused diseases. They have been termed "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria and filamentous fungi in addition to protozoan and metazoan parasites [36].

Yeast killer proteins which are produced and secreted into the environment by certain yeast strains with a killer phenotype (K^+) are highly suggested as antimicrobial agents in the medical field and may be used as a new and natural alternative to chemical antibiotics after further studies [37]. Secreted yeast killer toxins show a broad spectrum of killing activity against a great number of human pathogens. Since yeast cell wall contains mannoproteins, chitin, (1-3)-β-D-linked glucan and (1-6)-β-D-linked glucans and these are the primary binding sites for yeast killer toxins antifungal research is currently focused on the possible use of yeast killer proteins as novel antifungals. Especially toxins of the genus *Hansenula*, *Pichia*, and *Kluyveromyces* have a wide range of killing spectrum on human and animal fungal pathogens [55, 59, 94]. The killer toxins from *Williopsis subsuffciens* , *Hanseniaspora uvarum* , *W. beijerinckii* , *W. mrakii* and *Hansenula anomala* are active against *Candida spp*. [95, 96].

There are several studies reporting the wide-range intergeneric killing spectrum of *Pichia anomala* NCYC 434. In several studies *P. anomala* toxins have been reported to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts in several studies [95, 99-101]. Among *P. anomala*, the strain NCYC 434 especially has been extensively studied for various applications [55, 101].

Certain killer yeast strains are known to have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Bacillus subtilis, Streptococcus pyogenes* and *Staphylococcus aureus* besides certain pathogenic yeast and fungal strains [54]. However, previous studies comprising the effect of killer proteins against human pathogenic bacteria are limited to the interactions of the killer protein producing strains with human pathogenic bacteria or performed with crude toxin preparations [54, 94]. Further studies with the purified killer proteins are required for the potential use of these agents as novel antibacterial agents.

Yeast killer toxins are probably not suitable for oral and/or intravenous administration because they are antigenic, the ones that are stable at wide pH and temperature ranges might be used as a new and natural alternative in the topical treatment of superficial infections caused by gram-positive pathogenic bacteria thus it is known that certain killer yeast strains are known to have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Bacillus subtilis, Streptococcus pyogenes and Staphylococcus aureus* [59, 62]. Due to the high stability of the K5 type yeast killer toxin at pH values between 2.5-6.5 and temperatures up to 37 °C, it might be possible to use this protein as a topical antibacterial agent in the treatment pathogenic gram-positive bacteria associated skin and soft tissue infections and wound infections, after in vitro susceptibility tests and approppriate formulation studies if further studies confirm the inhibitory effect determined by the interactions of the killer toxin producer cells and pathogenic microorganisms.

Recently, we have purified and characterized the killer protein of *P. anomala* NCYC 434 and determined its mode of action in our laboratory [81, 102]. In vitro susceptibility studies of *P. anomala* NCYC 434 killer protein were done in our laboratory against 32 clinical isolates and 9 standart strains of dermathophytes and found to be affective on all of the tested strains with the MIC-2 values ranged from 0.25 to 2 µg ml and MIC-0 values ranged from 1 to 8 µg ml [103]. Antifungal activity of the K5 type yeast killer protein was tested against 21 *Candida* isolates including 9 quality control strains and found to be affective on all of the tested strains with the MIC range of 0.5-8 µg/ml. *C. albicans* strains that were resistant to azole derivatives such as flucanazole or itracanazole, was susceptible to the toxin in the MIC range of 1-2 µg/ml. Our studies showed that both the clinical isolates and the standard strains of *C. krusei* were susceptible to the K5 type yeast killer toxin in the MIC range of 2-8 µg/ml [104]. The results of the in vitro susceptibility studies of *P. anomala* NCYC 434 killer protein on human pathogenic fungi highlights the use of the K5 type yeast killer protein against human fungal infections with appropriate formulations [103,104].

In vitro susceptibility testing of pure *P. anomala* NCYC 434 killer protein against human pathogenic bacteria remained to be explored and now it became possible to determine the effect of pure K5 type yeast killer protein on human pathogenic bacteria.

Therefore we first determined the susceptibility of the 19 clinical isolates of gram-positive bacteria including drug-resistant strains isolated from skin and wound infections to purified K5 type yeast killer protein by determining MIC and MBC values. Nineteen human pathogenic clinical isolates of gram-positive bacteria and two quality control strains were tested for their susceptibility to pure K5 type yeast killer protein with a concentration ranging from 32 to 512 µg/ml. In this study CLSI M7-A6 was used as a reference document and its recommendations were modified in order to provide toxin stability and maximum toxin activity. The modifications include a change in the incubation temperature from 35 to 25° and a decrease in pH to 5.0.

K5 type yeast killer protein was found to have inhibitory activity against tested 5 cilinical isolates and 1 quality control strain of Methicillin-sensitive *Staphylococcus aureus*. K5 type yeast killer protein was found to have no inhibitory activity against tested 4 clinical isolates of Methicillin-resistant *Staphylococcus aureus.* The toxin was found to have inhibitory activity against tested 3 clinical isolates of *Enterococcus faecium* and it was found to have no inhibitory activity on 7 clinical isolates and 1 quality control strain of *Enterococcus fecalis*. All of the tested clinical isolates were obtained from skin and wound infections.

K5 type yeast killer protein produced by *Pichia anomala* NCYC 434 uses (1-3)-β-D-linked glucans as a target and inhibits the growth of sensitive microbial cells by hydrolyzing this cell wall component which causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure resulting in cell burst [81]. (1-3)-β-D-linked glucan is an integral cell wall component of a variety of fungi, plants, and bacteria [118-120]. Susceptibility of the tested strains, that belongs to the different species, to the K5 type yeast killer protein may be varied due to different cell wall compositions and β-1,3-glucan content. Also minor differences were observed in different strains of the same species. These differences is probably due to the minor modifications of the β-1,3-glucan content of the cell wall.

K5 type yeast killer protein was tested against 5 cilinical and 1 quality control strain of Methicillin-sensitive *Staphylococcus aureus* isolated from skin and wound infections. MIC and MBC values were between 32-128µg/ml and 64- >512µg/ml respectively.

Cell killing activity analysis were done for two clinical isolates of Methicillin-sensitive *Staphylococcus aureus* and the results reveal that the toxin has a bacteriostatic activity on both of the tested strains. Cell killing activity started between 8. and 12.hours and regrowth occurred for the toxin at MIC and 2, 4 and 8 times the MIC.

Cell killing activity analysis results for *Staphylococcus aureus* (MSSA) A1 indicated that the toxin reduced the initial inoculum from 6 $\log_{10}$ CFU/ml to 4,21 $\log_{10}$ at MIC, 4,14 $\log_{10}$ at 2 times the MIC, 4,28 $\log_{10}$ at 4 times the MIC and 4,63 $\log_{10}$ at 8 times the MIC. Maximum cell killing activity was seen at 2 times the MIC (4,14 $\log_{10}$). Increased concentrations of the toxin retard the regrowth of bacteria.

Cell killing activity analysis results for *Staphylococcus aureus* (MSSA) A3 indicated that the toxin reduced the initial inoculum from 6 $\log_{10}$ CFU/ml to 5,142 $\log_{10}$ at MIC, 4,14 $\log_{10}$ at 2 times the MIC, 4,15 $\log_{10}$ at 4 times the MIC and 3.9 $\log_{10}$ at 8 times the MIC. Maximum cell killing activity was seen at 8 times the MIC (3.9 $\log_{10}$). Increased concentrations of the toxin retard the regrowth of bacteria.

For both strains, maximum cell killing activity was seen at the protein concentration of MBC. The MIC value of the toxin was 32µg/ml for *Staphylococcus aureus* (MSSA) A3 and MBC value was 256µg/ml and maximum cell killing activity was achieved at 256µg/ml (8 times the MIC) for this strain. MIC and MBC values for *Staphylococcus aureus* (MSSA) A1 was 32µg/ml and 64µg/ml respectively and maximum cell killing activity was achieved at 64µg/ml (2 times the MIC) for this strain.

In a previous study, it was shown that certain killer yeasts, including the K5 type yeast killer toxin producing strain have a killer activity against *Staphylococcus auresus* [94]. The results of this research confirm the results of the previous study as purified K5 type yeast killer toxin has growth inhibitory activity against MSSA. Also in another study done in our laboratory, it was shown that K4 and K9 type yeast killer toxins have potential growth inhibitory activity on *Staphylococcus aureus* (MSSA) ATCC 25923 [54]. The results of this study also confirm the results of that research thus mode of action of the K4 and K9 type yeast killer protein was also studied previously in our laboratory and it was shown that K4 and K9 type yeast killer proteins exert their lethal

affect by hydrolyzing β-1,3-glucan residues of the cell wall [123, 124]. K5 type yeast killer protein also shows its lethal effect by hydrolyzing β-1,3- glucan residues of the cell wall like K4 and K9 type yeast killer proteins [81]. Like K4 and K9 type yeast killer toxins with exo β-1,3- glucanase activity K5 type yeast killer toxin has growth inhibitory activity on Methicillin-sensitive *Staphylococcus aureus*.

REP8839 is an entirely synthetic compound that has potent activity against a variety of pathogens including drug-resistant strains and is currently in preclinical development as a topical antibiotic for the treatment of skin infections and for the eradication of nasal carriage of *S. aureus.* REP8839 was found to have a bacteriostatic activity like mupirocin against *S. aureus* when tested at its MIC and 4 times the MIC. Reduction in colony count was observed after 24 hours but it was not bactericidal according to conventional criteria. When maximum decline for the number of bacteria were compared for REP8839 (>5 log10 at 4 times MIC at 24. hour) and K5 type yeast killer toxin (4.14 $\log_{10}$ at 2 times the MIC at 24. hour and 3.9 $\log_{10}$ at 8 times the MIC at 32 hour), it indicates that the bacteriostatic activity of K5 type yeast killer protein was more potent when compared to REP8839 [32].

Indolmycin was discovered in 1960 with its anti-staphylococcal activity. It has a bacteriostatic activity on *S. aureus* (both methicillin sensitive and resistant strains). In a study that was done in order to demonstrate the anti-staphylococcal activity of indolmycin against clinical isolates of *S. aureus* comparable to fusidic acid and mupirocin, the maximum decline of bacteria in number was seen at 16 times the MIC with >7,5$\log_{10}$ at 2. hour and regrowth started after 2 hours. The number of reduced bacteria (maximum decrease) was 3,9 $\log_{10}$ at 8 times the MIC after 32 hours for K5 type yeast killer toxin. When these two results were compared it can be clearly seen that although it has a slow activity (inhibition starts after 8 hours and the maximum decline of bacteria in number was achieved at 32. hour) the toxin has more potent bacteriostatic activity when compared to the bacteriostatic activity of indolmycin [125].

K5 type yeast killer protein was tested against 4 clinical isolates of Methicillin-resistant *Staphylococcus aureus* isolated form skin and wound infections. No inhibition of growth was seen for all tested strains of MRSA and all of the MIC values for tested MRSA strains were found to be >512µg/ml. K5 type yeast killer protein may not be the agent of choice for infections caused by MRSA.

K5 type yeast killer protein was tested against 7 clinical isolates and 1 quality control strain of *Enterococcus fecalis* isolated from skin and wound infections. No inhibition of growth was seen for all tested strains of *Enterococcus fecalis* and all of the MIC values for tested *Enterococcus fecalis* were found to be >512µg/ml. K5 type yeast killer protein may not be the agent of choice for infections caused by *Enterococcus fecalis*.

K5 type yeast killer protein was tested against 3 clinical isolates of *Enterococcus faecium* isolated from skin and wound infections. MIC and MBC values were found to be 256 and >512 µg/ml respectively.

Bacteriostatic activity was also defined as a ratio of MBC to MIC of >4 so it can be said that K5 type yeast killer toxin has a bacteriostatic effect on the tested 3 cilinical isolates of *Enterococcus faecium* [117].

Bacteriostatic activity is defined as any decrease in viable count from the starting inoculum or a $< 3\log_{10}$ decrease in CFU per milliliter. A treatment is said to be bacteriostatic when it stops bacterial cell growth but does not kill the cells [126, 127]. Tetracyclines, lincomycin, clindamycin, erythromycin, clarithromycin, azithromycin**,** mupirocine, indolmycine are the topical antibiotics with bacteriostatic effect and these are all used in the treatment of skin and wound infections. Topical antibiotics and medications with bacteriostatic and anti-inflammatory properties are effective for treating mild to moderate inflammatory acne. Chlortetracycline is a topical ocular antibiotic with bacteriostatic activity. The antibiotic with bacteriostatic activity against

gram-positive microorganisms found on normal skin is very important thus the agent prevents a small number of bacteria from rapidly proliferating. In the treatment of skin and wound infections a combination therapy including both the bacteriostatic and bacteriocidal agents prevent emergence of resistant strains, provide temporary treatment until diagnosis is made [125, 128-130].

Enterococcus spp. is responsible for about 10% of all nosocomial infections. The enterococci have become the second most common bacterium isolated from nosocomial urinary and wound infections, and the third most common cause of nosocomial bacteremia. Each year in the U.S., in fact, enterococci account for approximately 110,000 urinary tract infections, 40,000 wound infections, 25,000 cases of nosocomial bacteremia, and 1100 cases of endocarditis. Infections commonly caused by enterococci include urinary tract infections, endocarditis, bacteremia, wound infection, and intra-abdominal and pelvic infections. Many infecting strains originate from the patient's intestinal flora. From here, they can spread and cause urinary tract, intra-abdominal, and surgical wound infections. Bacteremia may result with subsequent seeding of more distant sites. For example, genitourinary tract infection or instrumentation often precedes the onset of enterococcal endocarditis. Meningitis, pleural space, and skin or soft tissue infections have also been reported. *E. faecium* is known to have a resistance to several types of antibiotics including quinolones and aminoglycosides. Resistance to penicillin was first observed in *E. faecium* in 1983, and in 1988 the first cases of resistance to the, vancomycin, were detected in Europe. Vancomycin-resistant strains of *E. faecium* were reported in the US in 1989. Resistance to several antibiotics and tolerance for adverse conditions makes *E. faecium* a major concern for the medical community [6, 131, 132]. According to the results of this study K5 type yeast killer protein might be used as a topical antibacterial agent with its bacteriostatic activity for the treatment of *E. faecium* caused infections including wound infections and skin-soft tissue infections and for the treatment of secondary infections caused by *E. faecium* after the appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study.

Bacteraemia, osteomyelitis, septic arthritis, epidural abscess, prosthetic joint infection, endocarditis, vascular graft infection, wound infections, burn wound cellulitis, postoperative wound infections, pyomyositis, cholangitis, angular chelitis and furunclosis are the infections caused by MSSA [133-138]. Nosocomial infections result in considerable morbidity and mortality among neonates, especially those in neonatal intensive care units. The most frequently found microorganism involved in these infections is Gram-positive *Staphylococcus aureus*; it is considered the most important agent of endemic infections as well as outbreaks. The clinical forms of staphylococcal infections were divided into invasive infections (31%), including cases of sepsis, and non-invasive (69%), including conjunctivitis and cutaneous infection, which were the most prevalent forms. MSSA infections are more prevalent in neonates, especially those with the following risk factors: premature birth, low weight, breathing syndromes, immunodeficiency, antimicrobial use, prolonged hospital stay, invasive methods and surgical interventions. *Staphylococcus aureus* is the second most common microorganism isolated from infections in neonatal intensive care units; methicillin-susceptible samples being the most common. Such infections predominantly involve sepsis, pneumonia, cutaneous infection and conjunctivitis [139]. Also the majority of *Staphylococcus aureus* isolated from ocular infections as keratitis and conjunctivitis are MSSA [140].

According to the results of this study K5 type yeast killer protein might be used as a topical antibacterial agent with its bacteriostatic activity for the treatment of MSSA caused infections including skin-soft tissue infections and ocular infections after the appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study.

One of the common causes of skin and soft-tissue infections is the occurrence of secondary bacterial infection that complicates skin lesions. The skin lesions that can be secondarily infected with bacteria are scabies, psoriasis, poison ivy, atopic dermatitis, eczema herpeticum and kerion. The predominant aerobic and facultative bacteria were *Staphylococcus aureus*, group A streptococci, and *Pseudomonas aeruginosa* and *S. aureus* is the most frequently

dominating bacteria among them in secondary bacterial infections. The commonest secondary bacterial infection in eczema is due to *S. aureus*. Secondary bacterial infections also seen after HIV, influenza, cutaneous leishmaniasis and mycetoma and *S. aureus* infections are the most frequently seen secondary bacterial infections after most of the disease given above [141-143]. After appropriate formulation studies, due to its bacteriostatic activity K5 type yeast killer toxin might be used with the antimicrobial agents in the treatment of bacterial, fungal or viral diseases in order to prevent secondary infections that could be caused by the bacteria *Staphylococcus aureus*.

Damaged areas of skin are more prone to cellulitis with micro-organisms e.g. Methicillin sensitive *Staphylococcus aureus* (MSSA) and Methicillin resistant *Staphylococcus aureus* (MRSA), therefore the appropriate management of all forms of dermatitis is essential for the healthcare workers and also the patients in their care [144]. K5 type yeast killer protein might be used as a disinfectant after appropriate formulation studies in order to prevent healthcare workers and also the patients in their care from the infections of *S. aureus* and *E. faecium*.

Disinfectants are also used on non-living surfaces and objects to decrease the number of microbes on that surface and many disinfectants have bacteriostatic effect on bacteria. Disinfection of hospital instruments and materials from the bacteria *S. aureus* and *E. faecium* might be achieved by the use of the toxin as a disinfectant after appropriate formulation studies.

After appropriate formulation studies, K5 type yeast killer protein might be added to cosmetics thus many cosmetics can cause allergic skin disorders that can be further contaminated by *S. aureus* and *E. faecium* resulting in secondary bacterial infections.

Antibacterial susceptibility testing and cell killing activity analyses of K5 type yeast killer protein on 5 clinical isolates and 1 quality control strain of MSSA and 3 clinical isolates of *Enterococcus faecium* all isolated from skin and

wound infections reveal that the toxin might be used as a topical antibacterial agent with its bacteriostatic activity for skin and wound infections caused by MSSA and *Enterococcus faecium* with appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study. Also the toxin might be used with the other antimicrobial agents in the treatment of bacterial, fungal or viral diseases in order to prevent secondary infections that could be caused by the bacteria *Staphylococcus aureus* and *E. faecium*. The application field of the toxin might be broadened by the use of the toxin in ocular infections of *Staphylococcus aureus*, by the use as a disinfectant and by the use in cosmetics in order to prevent secondary bacterial infections that can be caused by *Staphylococcus aureus* and *E. faecium.*

CHAPTER V

CONCLUSION

1. K5 type yeast killer toxin was found to be affective against all tested isolates of Methicillin-sensitive *Staphylococcus aureus* within the MIC and MBC ranges of 32-128µg/ml and 64- >512µg/ml respectively.

2. K5 type yeast killer toxin was found to have a bacteriostatic effect on MSSA according to the results of cell killing activity studies.

3. K5 type yeast killer toxin exerts its bacteriostatic effect between 8. and 12. hour, regrowth occurs for the toxin at MIC and 2, 4 and 8 times the MIC and increased concentrations of the toxin retard the regrowth of bacteria but it has little effect on the killing activity.

4. K5 type yeast killer protein was found to be affective against all tested isolates of *Enterococcus faecium* within the MIC and MBC ranges of 256 and >512 µg/ml respectively.

5. K5 type yeast killer toxin also has a bacteriostatic effect on the isolates of *Enterococcus faecium* as bacteriostatic activity was also defined as a ratio of MBC to MIC of >4.

6. K5 type yeast killer toxin was found not to be effective against all tested isolates of MRSA and *Enterococcus fecalis*.

7. K5 type yeast killer protein might be used as a topical antibacterial agent with its bacteriostatic activity for skin and wound infections caused by MSSA and *Enterococcus faecium* and might be used with the other antimicrobial agents in the treatment of bacterial, fungal or viral diseases in order to prevent secondary infections caused by MSSA and *Enterococcus faecium* with appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study.

8. The toxin might be also used as a topical agent in the treatment of ocular infections, might be used as a disinfectant, might be used in cosmetics against the bacteria *S. aureus* and *E.faecium* with its bacteriostatic activity after appropriate formulation studies upon the antibacterial spectrum determination of the toxin in this study.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)

Aceton (Merck, Germany)

Acrylamide (Boehringer Mannheim, Germany)

Ammoniumpersulphate (Pharmacia Biotech, Sweden)

Bacto-agar (Difco, USA)

Bacto-peptone (Difco, USA)

Bacto-trptone (Difco, USA)

Bacto- yeast extract (Difco, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bovine Serum Albumine Fraction V (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA)

Butanol (Merck, Germany)

β-mercaptoethanol (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Coomassie Brilliant Blue G-250 (ICN, USA)

Dithiothreitol (DTT) (Boehringer Mannheim, Germany)

D-Glucose (Merck, Germany)

Di-sodium Hydrogen Phosphate (Merck, Germany)

Di-potassium Hydrogen Phosphate (Merck, Germany)

Ethanol (Merck, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Methanol (Merck, Germany)

N-methylpiperazine (Merck, Germany)

Nutrient Broth (Merck, Germany)

Phosphoric acid (Merck, Germany)

Potassium Dihydrogen Phosphate (Merck, Germany)

Silver Nitrate (Merck, Germany)

Sodium Carbonate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium Dodecyl Sulfate (Merck, Germany)

Sodium Hydroxide (Merck, Germany)

Sodium Sulfate (Merck, Germany)

TEMED (Pharmacia Biotech, Sweden)

Trichloroacetic Acid (Merck, Germany)

Trypticase Soy Broth (Sigma, USA)

Tris (Merck, Germany)

APPENDIX B

BUFFERS AND SOLUTIONS

1.SDS-PAGE

Buffers and solutions used in SDS-PAGE are; monomer solution (30.8% T, 2.7% C_{bis}), 4X running gel buffer (1.5 M Tris-Cl , pH 8.8), 4X stacking gel buffer (0.5 M Tris-Cl , pH 6.8), SDS (10%), initiator (10% ammonium persulfate), 2X treatment buffer (0.125 M Tris-Cl , 4% SDS , 20% glycerol , 10% β-mercaptoethanol , 0.020% bromophenol blue , pH 6.8) and tank buffer (0.025 M Tris , 0.192 M glycine , 0.1% SDS , pH 8.3.).

2.Silver Stain

Buffers and solutions used in silver stain are; destain solution I (40% methanol , 7% acetic acid), destain solution II (5% methanol , 7% acetic acid), cross-linking solution (10% glutaraldehyde), DTT solution (5 µg/ml), silver nitrate solution (0.1% w/v), sodium carbonate (3% w/v) and developing solution (3% sodium carbonate, 0.019% formaldehyde).