IDENTIFICATION OF THE YEAST THAT CONTAMINATES THE INDUSTRIAL BAKING STRAINS OF SACCHAROMYCES CEREVISIAE AND DETERMINATION OF ITS KILLER ACTIVITY

A Master's Thesis

Presented by

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to

The Graduate School of Natural and Applied Sciences

of Middle East Technical University

in Partial Fulfillment for the Degree of

MASTER OF SCIENCE

in

SCIENCE EDUCATION

MIDDLE EAST TECHNICAL UNIVERSITY

ANKARA

September,1994

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ABSTRACT

IDENTIFICATION OF THE YEAST THAT CONTAMINATES THE INDUSTRIAL BAKING STRAINS OF SACCHAROMYCES CEREVISIAE AND DETERMINATION OF ITS KILLER ACTIVITY

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M.S. in Science Education

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September1994, 80 Pages

In this study the yeast that contaminates the industrial baking strains of S.cerevisiae (BSP1-4) and thus spoil the fermentation products was identified and its killer activity on those yeast strains along with the pathogenic bacteria was determined.

Biochemical test results that were interpreted with API LAB ID yeast identification computer programme showed that this contaminating yeast was Candida tropicalis (ID%=99.6). The killer activity tests on the killer sensitive S. cerevisiae NCYC 1006 and on the fermentation strains demonstrated that this contaminating C.tropicalis was a killer toxin producing strain. This toxin also inhibited the growth of some pathogenic bacteria that cause nosocomial infections as tested in a new method that was

applied in this study for the first time. Studies on the sensitivity determination of the contaminating yeast C.tropicalis to other yeast species of known killer phenotypes revealed that this killer toxin producing C.tropicalis was sensitive to K3 and K8 type killer toxins of the other yeast species, so introduction of the K3 and/or K8 killer trait to the fermentation strains would inhibit C.tropicalis contamination and thus prevent spoilage of the fermentation products.

Key words: Saccharomyces cerevisiae, Candida tropicalis, Killer character, Killer sensitivity

Science code: 401.02.02

ÖZ

ENDÜSTRİDE KULLANILAN SACCHAROMYCES CEREVISIAE HAMUR MAYASI SUŞLARINI KONTAMİNE EDEN MANTAR TÜRÜNÜN TANIMLANMASI VE ÖLDÜRÜCÜ ETKİSİNİN TAYİNİ

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Eylül 1994, 80 sahife

Bu çalışmada endüstride kullanılan Saccharomyces cerevisiae maya suşlarını (BSP1-4) kontamine ederek fermentasyonun verimini düşüren mantar türü tanımlanmış ve bu mantarın maya suşları ve bazı patojenik bakteriler üzerindeki öldürücü etkisi belirlenmiştir.

API LAB ID mantar tanımlama bilgisayar programı ile değerlendirilen biyokimyasal test sonuçları kontaminasyona sebep olan bu mantarın Candida tropicalis olduğunu göstermiştir.(Tanımlama doğruluk yüzdesi = 99.6)

S.cerevisiae NCYC 1006 standart test suşu ve diğer maya suşları üzerinde yapılan testler C.tropicalis 'in öldürücü toksin üreten bir suş olduğunu ortaya çıkarmıştır.

İlk defa bu çalışmada uygulanan bir metodla bu toksinin hastane enfeksiyonlarına sebep olan patojenik bakterileri de öldürdüğü belirlenmiştir.

Kontaminasyona sebep olan C.tropicalis 'in, öldürücü fenotipleri belirli diğer mantar türlerine karşı hassasiyetinin araştırılması için yapılan çalışmalar, öldürücü toksin üreten bu C.tropicalis 'in de diğer mantar suşları tarafından üretilen K3 ve K8 tipi toksinlere hassas olduğunu ortaya çıkarmıştır.

Böylece K3 ve/veya K8 tipi öldürücü özelliğin fermantasyon suşlarına aktarılması ile, fermantasyon ürünlerini bozan C.tropicalis kontaminasyonu önlenebilecektir.

Anahtar kelimeler: Saccharomyces cerevisiae, Candida tropicalis, öldürücü karakter, öldürücü karaktere karşı hassasiyet.

Anabilim Dali Sayisal Kodu: 401.02.02

ACKNOWLEDGEMENTS

I am very grateful to Assist. Prof. Dr. Fatih İzgü for valuable guidence, continued advice and critical discussion throughout this study.

I wish to thank Assoc. Prof. Dr. Hüseyin Gün and Dr. Mehmet Ali Saraçlı from GATA for their kindly helps during this study.

I also wish to thank Shahriyar Yadipour , Cemal Alper and Saeedeh Nafisi for their suggestions and their help during this study.And finally, I am very grateful to my family, for their support and encouragement.

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LIST OF SYMBOLS

API	Analytical Profile Index
ATCC	American Type Culture Collection
bp	Base pair
ds	Double stranded
dsRNA	Double stranded ribonucleic acids
K^+	Killer charecter
K^-	Non-killer charecter
kb	Kilo base
kd	Kilo dalton
LdsRNA	Large size double stranded ribonucleic acids
MdsRNA	Medium size double stranded ribonucleic acids
NCYC	National Collection of Yeast Cultures
Poly A	Polyadenylic acid
Poly U	Polyuridylic acid
R^+	Killer toxin immunity
R^-	Non-killer toxin immunity
ScV	Saccharomyces virus
TPCK	Tolylsulfonyl phenylalanyl chloromethyl ketone
VLP	Virus like plasmid

CHAPTER I

INTRODUCTION

Yeasts are unicellular fungi that are the most important and the most extensively used microorganisms in industry. They have been beneficial to human since the beginning of agriculture. They are cultured for the cell components and for the end products that they produce during alcoholic fermentation. Among 39 genera and 350 species especially strains of one species, Saccharomyces cerevisiae, are used in bread making and brewing. It is also looked on as a possible eukaryotic organism for the production of important pharmaceuticals from cloned genes [4,11,13,14]. (Table 1.1).

The yeasts currently used are descendants of the early S. cerevisiae. It is now possible to alter the yeast strains genetically in laboratories by hybridization and cloning methods to produce strains of desirable qualities. Rapid growth, a budding pattern resulting in desperst cells, the ease of mutant isolation, a well defined genetic system, a high versatile DNA transformation system and being non-pathogenic are some properties that make S. cerevisiae suitable for biological studies [23].

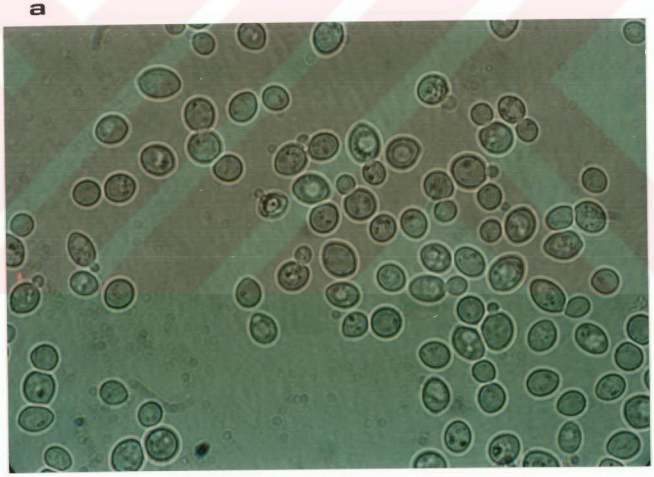
S. cerevisiae is a unicellular yeast with oval or spherical shaped cells (Fig.1.1). They can normally reproduce asexually by budding. As an alternative to vegetative (mitotic) proliferation the two different haploid cells (α and a) of S. cerevisiae are able to conjugate (mate) to form diploid a/α cells. The haploid state makes easy to follow recessive markers [17].

Table 1.1. Industrial Uses of Yeast and Yeast Products

Production of yeast cells
Baker's yeast, for bread making
Dried food yeast, for food supplements
Dried food yeast, for animal feeds
Yeast products
Yeast extract, for culture media
B vitamins, Vitamin D
Enzymes for food industry; invertase, galactosidase
Biochemicals for research; ATP, NAD, RNA
Pharmaceuticals; hormones, vaccines
Fermentation products from yeast
Ethanol, for industrial alcohol
Glycerol
Beverage alcohol
Beer
Wine
Whiskey
Brandy
Vodka
Rum

The nucleus of haploid S. cerevisiae cell contains approximately 14,000 kb of DNA subdivided into 16 linear chromosomal DNAs with sizes ranging from 250-2000 kb. In addition many strains carry 50 to 100 copies of 6.3 kb plasmid which is usually referred to as 2μm plasmid. The 75 to 80 kb of mitochondrial DNA is circular and each cell harbors 20-40 copies (Figure.1.2) [12,23].

a



b

Fig1.1 a. Vegetative propagation of Saccharomyces cerevisiae on YEPD medium.

b. Saccharomyces cerevisiae cells viewed under light microscope.

Fig.1.2 Genome of Diploid Saccharomyces cerevisiae Cell

	Mendelian		Non-Mendelian				
	Double-stranded DNA		Double-stranded RNA				
	Nuclear		RNA molecules				
Characteristic	Chromosomes	2-μm plasmid	Mito-chondrial	L-A	M	L-BC	T	W
Relative amount	85	5	10	80	10	9	0.5	0.5
Number of copies	2 sets of 16	60-100	~50(8-130)	1000	170	150	10	10
Size (kb)	14,000 (200-2200)	6.318	70-76	4.576	1.8	4.6	2.7	2.25
Deficiencies in mutants	All kinds	None	Cytochrome a a_3 b	Killer toxin		None		

Some yeast strains secrete polypeptide toxins that are lethal to sensitive cells of the members of the same species and frequently to those of other species and genus of yeast. These protein compounds are designated as killer factors or killer toxins and the yeast strains secreting these type of killer proteins are named as killer strains and killer yeasts [6].

The occurrance of a virus like particle in the cytoplasm and thus the secretion of a virally coded protein toxin, gives the cell its killer character. The strains lacking the virus are sensitive to the toxins produced by the harbouring strains which are immune to their own killer factors [51].

The killer phenotype (K^+) was first discovered in S. cerevisiae by Makomer and Bevan [15,27]. Other yeast species of different genera of killer types have also been isolated (Hansenula, Pichia, Candida. Trolopsis, Debaryomyces, Kloeckera, Crylococcus) [21,26,30,34,36,38,43,44].

Killer strains are of 10 types and so 10 toxin types (K1 - K10) were distinguished on the basis of pH optima, temperature stability, and sensitivity to proteases. K1 type was first recognized among laboratory strains of S. cerevisiae and widely distributed among wild type strains. K2 type killers were isolated from vini-culture yeasts and are capable of killing K1 killers. The third type designated as K3 was found in Saccharomyces capensis 761 and has killer activity on K1 and K2 type killers (Table 1.2). Other killer yeast species of different genera that have been isolated, differ in their toxic compounds in pH optima, temperature stability and sensitivity to proteolytic enzymes [6, 16, 33]

Table 1.2 Classification of the Genus Saccharomyces According to Killer Phenotypes

PHENOTYPE	***COMMENTS***
$K_1^+R_1^+$	Killer toxin producing strains that are immune to it but sensitive to K2 and K3 killer types
$K_2^+R_2^+$	Killer toxin producing strains that are immune to it but sensitive to K1 and K3 killer types.
$K_3^+R_3^+$	Killer toxin producing strains that are immune to it but sensitive to K1 and K2 killer types.
K^-R^+	Non-killer toxin producing strains but immune to killer proteins (Neutral strains).
$K_1^{++}R_1^+$	More active K1 killer protein producing strains (Super Killer Strains).
$K_1^+R_1^w$	Killer protein producing strains but exhibiting weak immunity to type K1 (Suicidal Strains).
K^-R^-	Non-killer toxin producing strains and sensitive to killer proteins.

The components of the killer systems are the cytoplasmic genetic elements on the dsRNA molecules and chromosomal genes that control the replication and expression of the cytoplasmic elements. The dsRNA molecules are closed in virus-like particles (VLPs) thus termed as ScV (Saccharomyces Virus). They are vertically transmitted and transmission occurs only by cytoplasmic mixing during budding, mating or by cell fusion. Their properties resembles to that of infectious RNA viruses and eukaryotic plasmids. As they depend on multiple genetic loci they are not autonomously replicating elements [49,50] .

S. cerevisiae strains carry as many as five distinct non-homologous dsRNAs, designated as M, L-A, L-(BC), T, and W and they show non-Mendelian inheritance. M, L-A and L-BC are packaged separately by a common major capsid protein into virus-like particle of the mycovirus. The virus is not infectious, it is vertically transmitted by vegetative cell division or through sexual fusion and thus behave as cytoplasmically genetic element [3, 51].

Production of the killer toxin and immunity to the homologous protein are encoded by MdsRNAs. The variance of M called M_1, M_2, M_3, etc. encodes different extracellular toxins and immunity to the respective killer protein [10, 52]. M_1dsRNA, M_2dsRNA and M_3dsRNA encode toxin proteins for the K1, K2, and K3 killer systems respectively. Their copy numbers are high in the cell [10-100] (Table 1.3)
The replication of the MdsRNAs is dependent on a larger dsRNA called LdsRNA which is found in a higher copy number in the cell. Most Saccharomyces strains weather killers or not have this 4.5 kb linear dsRNA. It is commonly closed in a virus like particle and MdsRNA are only present in strains carrying LdsRNA. The large dsRNA comprises two unrelated families of molecules called L-A family and L-BC family. L-A encodes major code protein in which MdsRNA and itself are encapsulated. It is not clear whether L-BC family has any functional relation to the killer phenomena since some killer strains lack L-BC entirely [19, 20, 52] (Table 1.4).

Table 1.3 M Type Components of Saccharomyces cerevisiae dsRNAs

dsRNA	*DEFINITION-COMMENTS*	*PRODUCT IN VITRO*		*PRODUCT IN VIVO*	
		Name	*kd*	*kd*	*Mature*
M_1	1.9 kb dsRNA, determinant of $K_1^+R_1^+$ phenotype. Strains carrying M_1 have KIL-k_1 genotype.	M_1-P1	34.8 preprotoxin	42	Toxin, α 9.5 + β9 kd
M_2	1.7 kb dsRNA, determinant of $K_2^+R_2^+$ phenotype. Strains carrying M_2 have KIL-k_2 genotype.	M_2-P2	42 preprotoxin	Not determined	Toxin, 18 kd
M_3	1.5 kb dsRNA determinant of $K_3^+R_3^+$ phenotype. Strains carrying M_3 have KIL-k_3 genotype.	M_3	Not determined	Not determined	Not deter.

Table 1.4 L Typed Component of Saccharomyces cerevisiae dsRNA

dsRNA	*DEFINITION-COMMENTS*	*PRODUCT IN VITRO*		*PRODUCT IN VIVO*
		Name	kd	Mature
L-A	4.7kb dsRNA, maintanence of M_1 and M_2 dsRNA.			
L_{1A}	4.7 kb dsRNA, found in K1 type killers encodes for 88 kd capsid protein that encapsidates itself and M_1dsRNA.	L_{1A}-P1	88	VL_{1A}-P1 (ScV-P1): capsid for VLPs of L_{1A} and M_1dsRNA
L_{2A}	4.7 kb dsRNA, found in K2 type killers encodes for 84 kd capsid protein that encapsidates itself and M_2dsRNA.	L_{2A}-P1	84	VL_{2A}-P1 (ScV-P1): capsid for VLPs of L_{2A} and M_2dsRNA
L-B, L-C, L-BC	4.7 kb dsRNA, found in K1 and K2 type killers encodes for 82 kd capsid protein of their own VLPs. It is not required for maintanence of M_1 and M_2. No homology with L_{1A} and L_{2A}.	L_{BC}-P1	82	VL_{BC}-P1(ScV-P2): capsid for VLPs containing L_{BC}
		L_{BC}-P2	78	Identity not tested

L-A dsRNA variants carry various combination of the cytoplasmic genes ([EXL], [HOK], and [NEX]) that in different ways influence the killer systems (Table 1.5) [18].

Table 1.5 Varients of L-A dsRNA and the Cytoplasmic Genes

dsRNA	***DEFINITION AND COMMENTS***	***CYTOPLASMIC GENE***
L-A-E	L-A carrying [EXL] not [HOK] or [NEX]	[EXL] excluder of [KIL-k_2], gene excludes [KIL-k_2] in the absence of gene [NEX]
L-A-H	L-A carrying only [HOK], K_2 killer	[HOK] helper of killer, supplies helper function of M_1 replication
L-A-HN	L-A carrying [HOK] and [NEX], K_1 killer	[NEX] prevent exclusion [KIL-k_2] by [EXL].
L-A-HR	Mutant dsRNA derived from L-A-H that requires [EXL^R]	
L-A-HE	L-A carrying [HOK] and [EXL]	
L-A-null	Biochemically presence of [EXL], [HOK], [NEX], but lack the genetic activity	

Although the killer phenotypes are determined by the cytoplasmic genes, a series of other genes located on chromosomes are required for the replication and expression of MdsRNA and immunity to the killer toxins (Table 1.6) [49, 51]

Table 1.6 Chromosomal Genes Required for MdsRNA Replication, Expression and Immunity

GENES	*DEFINITION AND COMMENTS*
MAK	Needed for maintenance of M. MAK3, MAK10, are also required for the maintenance of (L-AdsRNA) [HOK], [EXL] and [NEX].
PET 18	Needed for cell growth at high temperature and for maintenance of L-A, MdsRNA, [HOK], [NEX], and [EXL].
MKT	Needed for the maintenance of [KIL-k_2]. M_2dsRNA in the presence of [NEX].
SKI	ski1 bypasses all MAK defects except mak16. ski2, ski3 and ski4 bypasses mkt1, and all mak mutations except mak16, pet18, mak10 and mak3. ski2, ski3, ski4, ski6, ski7, and ski8 mutants are cold sensitive only if MdsRNA is present.
REX	Needed for resistance expression of the cells on protein.
SEC	Needed for general protein secretion. Mutants in these genes are conditional lethal and effect killer toxin secretion.
KRE	Needed for normal toxin action on sensitive cells. kre1 effects the normal cell wall receptor β-(1-6) Glucan and receptor k2 toxin binding.
KEX	Needed for killer expression and processing of protoxin.

The killer toxin and the immunity to it are encoded in MdsRNA. M1dsRNA possesses a single open reading frame (ORF) that starts from AUC codon at the base 14 from 5'end of $M1^+$strand and terminates at UAC codon at base 963. The 3' region of the M1 genome lacks coding information but encompasses the sides for packaging and replication. Between the sides for packaging and replication (about 0.6 kb and the coding region about 1 kb) is a stretch of poly(A) / poly (U) of about 200 bp that is variable in generations. The 1.0 kb region codes for a precursor of the killer protein with a molar mass of 32 kg/M. This translation product consists of two polypeptides, α and β, 9.5 and 9.0 kd respectively that originate from the amino terminus and carboxyl terminal domains of the pre-protoxin. The subunit (α and β) domains flank a segment called γ which is not part of the toxin but assumed to be the immunity determinant (Figure 1.3) [10, 12, 48].

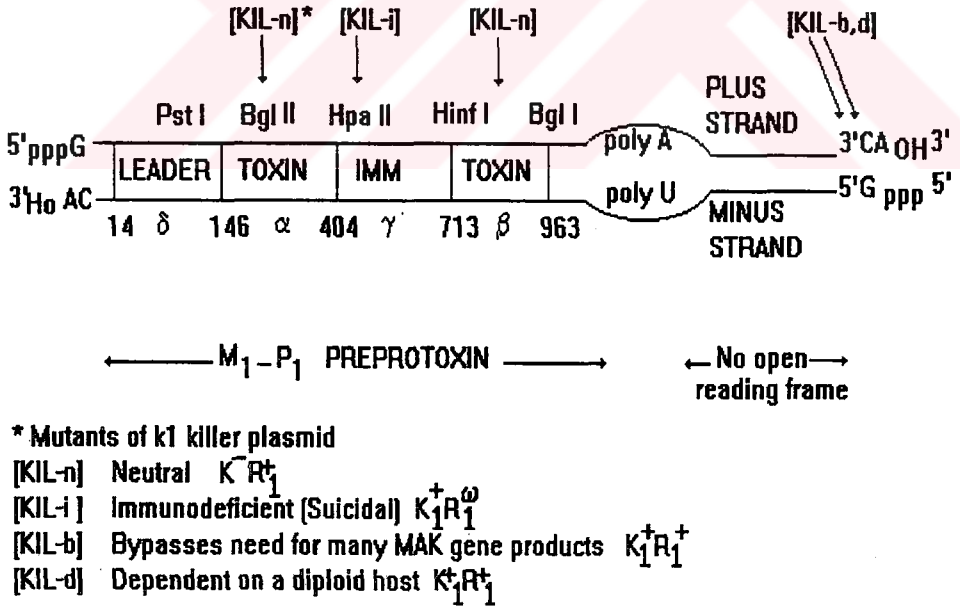


Figure 1. 3 Schematic Structure of M1dsRNA

The N terminal region δ is a hydrophobic leader sequence with high affinity for endoplasmic reticulum that permits the precursor to penetrate into the endoplasmic reticulum where the γ is glycosylated. No further modification occur during the passage through the golgi apparatus (sec18, sec7, kex1, and kex2 may be involved in the process) . In transport vesicles the precursor is fragmented by a specific protainase (TPCK- inhibital protease) and the leader sequence and the immunity region split out. In this way, the active killer protein is formed. By fusion of the transport vesicle by the cell membrane the killer toxin is secreted out of the cell with the immunity region but the γ remains bound to the outer side of the membrane to a specific receptor (Figure.1.4) [6, 49].

There exists two steps of toxin action: binding to the cell wall and action at the cell membrane. The killer protein binds to (1-6) β-D Glucan which is a component of surface receptor. Cells of sensitive strains and strains of the killer phenotype contain a large number of these receptors. The binding of protein to the receptor which is energy independent is not yet clear. It is not known whether proceeds via β component of the protein. The precise structure of the (1-6) β-D Glucan receptor and mediation of the toxin action are not known. It probably facilitates the passage of the toxin through the cell wall to the plasma membrane. The interaction of the killer protein with the plasma membrane by secondary receptors cause permeability changes of the membrane. The killer toxin decreases the ion gradient across the membrane and thus interrupts transport of protons (K) and amino acids [7, 25, 32, 37, 40, 41].

The action of killer proteins is pH and temperature dependent. They have low pH optima (4.2 - 4.9) and are inactivated generally above 24 °C.

The contamination of a starter strain in fermentation process with undesirable yeast species or the contamination during fermentation process, since

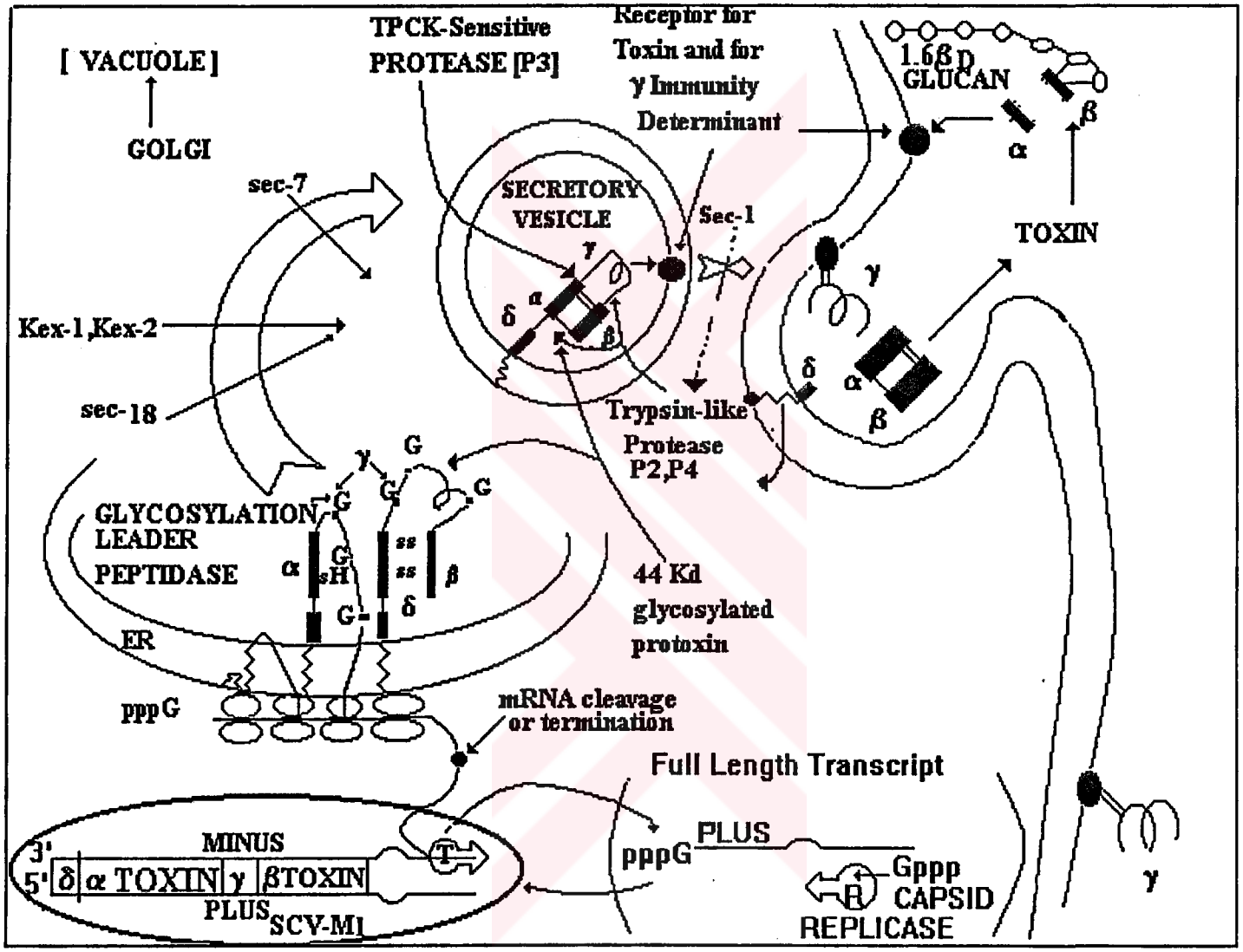


Fig 1.4. Scheme of the hypothetical course of synthesis and secretion pathway of the killer protein

fermentations are carried out in open systems (without sterilization) cause dramatically decrease in the quality of the product by the multiplication of the contaminating yeast and accumulation of their metabolites. In particular contamination with killer toxin producing yeast species of industrial baking and brewing strains is a potential problem in fermentation. If the starter strain is sensitive to the killer toxin, the killer phenomenon can be utilized for the protection of fermentation process against contaminating yeast. Introduction of killer trait into fermentation strains prevent the contaminating killers from spoiling the product. The solution to this problem is the construction of immune industrial strains by protoplast fusion or cytoduction that produce killer toxins and protect fermentation against contaminating killer yeasts [6, 8, 9, 27, 30, 42, 46].

The Turkish baking industry is confronted with drastic problems of undesirable yeast contamination of the starter industrial yeasts during fermentation that prevent multiplication of the baking yeasts and thus spoil the product quality.

In this study, we have aimed to identify the contaminating yeast strain that causes severe fermentation problems in Turkish baking industry and to determine the reason of its growth inhibition of the starter baking strains. The purpose of this study was also to reveal the methods of immunizing industrial starter strains against the undesirable contaminating yeasts. As there is very little evidence in the literature about the bactericidal effects of the yeast killer proteins, we have also examined the killer toxin activities of various yeast species belonging to different genus on bacteria that are the major cause of nosocomial infections.

CHAPTER II

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Yeast Strains:

2.1.1.1. Industrial baking strains:

S. cerevisiae BSP-1

S. cerevisiae BSP-2

S. cerevisiae BSP-3

S. cerevisiae BSP-4

The above mentioned S. cerevisiae industrial strains utilized in this study were provided as slab cultures from PAK GIDA A.S.

2.1.1.2. Contaminating yeast strain (unidentified) was obtained from PAK GIDA A.S.

2.1.1.3. Killer yeast strains and killer sensitive strain:

Species	Strain	Killer Systems
Saccharomyces cerevisiae	NCYC 232	K1
Saccharomyces cerevisiae	NCYC 738	K2

Species	Strain	Killer Systems
Saccharomyces cerevisiae	NCYC 761	K3
Hansenula anomala	NCYC 432	K4
Pichia membranaefaciens	NCYC 333	K7
Hansenula anomala	NCYC 435	K8
Hansenula mrakii	NCYC 500	K9
Kluyveromyces drosophilarum	NCYC 575	K10
Kluyveromyces lactis	NCYC 1368	dsDNA
Saccharomyces cerevisiae	NCYC 1006	Killer sensitive

Yeast strains with NCYC numbers were all provided as freeze-dried cultures from National Collection of Yeast Culture (U.K.)

2.1.2. Bacterial Strains:

Species	Strain
Bacillus subtilis	ATCC 6051
Sarcina lutea	ATCC 9341
Klebsiella pneumonia	ATCC 10081
Escherchia coli	ATCC 25922
Staphylococcus aureus	ATCC 25923
Pseudomonas aeruginosa	ATCC 27853
Proteus vulgaris	A 232
Streptococcus pyogenes	AA 3

The bacterial strains with ATCC numbers are all provided as freeze-dried cultures from American Typed Culture Collection (USA) and the others from Pasteur Institute (FR).

2.1.3. Culture Media:

2.1.3.1. Culture Media for Yeast Cells:

For maintanence and routine growth of the cells Malt Extract, YM, and YEP-glucose, for the determination of killer activities YEPD and YEPD-MB mediums were used.

2.1.3.2. Culture Media for Bacterial Cells

For maintanence of the bacteria Muller Hinton Broth and for routine growth tryptic soy agar (blood agar) were used.

The composition of these different culture media are given in AppendixA

2.1.4. Buffers:

The preparation and composition of the buffers that were used are given in Appendix B.

2.1.5. Chemicals

The chemicals and the suppliers that were used in our study are listed in Appendix C.

2.1.6. Strains:

Staining solutions and their preprations are given in Appendix D.

2.2. METHODS

2.2.1. Maintenance of Yeast Cultures:

The glass ampoules that contain freeze-dried yeast cultures were opened in sterile conditions and about 0.5 ml of YM broth or Malt Extract were added to the dried material by using sterile pasteur pipette. After incubation at 25 ° C for o/n the cells were plated on YEP-glucose medium (pH 5.5).

After colony formation, stock cultures of the yeast strains were prepared as slab cultures containing 8 ml of YEP-glucose + 1.0 % agar (pH 5.5) in screw capped glass tubes (shott) and stored at 4 °C [23,35].

2.2.2. Maintenance of Bacterial Cultures :

The bacterial strains were obtained from the supplier also as freeze-dried cultures. For the growth of the freezed-dried bacterial cells a procedure similar to that of freezed-dried yeast cells were applied. Except, for the Muller Hinton Broth (pH 6.9) that was used for the growth of freeze-dried bacterial cultures and blood agar (pH 6.9) for the slab cultures [5].

2.2.3. Sterilizations :

The glassware were sterilized on dry-cycle at 200 °C for 2 hours. The media for the maintenance or for propagation of the yeast and bacterial cells were sterilized at 15 lb/sq inc. for 20 min on liquid-cycle [39].

2.2.4. Identification of Contaminating Yeast Strain :

The identification of the yeast strain that contaminates industrial starter baking strains of S.cerrvsiae was done with API LAB ID 32C system (Bio Merieux-FR), by using standardized and miniaturized assimilation tests with a special adapted data base (ID 32C Strip).

A yeast colony from YEP-glucose medium plate was removed and a suspention having a turbidity equal to 2 McFarland units, measured with the aid of the ATB 1550 Densitometer (FR) was prepared. A 250 ml of the suspension was added to C medium with a ATB pipette and homogenized. The cupules of the ID 32C strip, each containig a dehydrated carbohydrate substrate were filled with 135 µl of the C Medium and the strip was incubated at 30 °C for 48 hours. After 48 hours incubation, the growth in each cupule was detected by the ATB reader and identification was obtained by referring to the API LAB ID 32 computer programme [1,17].

2.2.5. Screening of Strains for Killer Activity and Killer Sensitivity:

2.2.5.1. Killer Activity Determination of the Yeast that Contaminates Industrial Baking Strains of S.cerevisiae :

The killer activity was determined by killer zone assay in a plate test with YEPD-glucose and YEPD-MB medium that were both buffered to pH 4.5 with 0.1 M citrate-phosphate buffer, at 20 °C. The cells of killer sensitive S. cerevisiae NCYC 1006, standardized to an optical density of 1 on the Mc Farland standard

(ATB densitometer) were suspended in 15 ml of molten (45°) YEPD and YEPD-MB then the suspention was poured in to sterile petri dishes.

The seeded YEPD-MB plates were incubated for 24-42 hours until colonies were formed and the strain to be tested for its killer activity was streak inoculated on the surface of the agar and subjected to further incubation at 20 °C for 24-72 hours.

The YEPD plates were also streak inoculated with the strain to be tested for the killer property, but just after the seeded agar was solidified.

For the YEPD-MB plates a second procedure was applied for testing killer charecters. The killer sensitive S. cerevisiae 1006 (Mc Farland 2) was spread on to the hardened agar and incubated for 24-48 hours. After colony formation the plates were streak inoculated with the strain to be tested and incubated at 20 °C for 24-72 hours.

In both procedures mentioned above the killer activity was recognized by the inhibition of growth of the seeded strain surrounding the growth of the killer strain [15,24,27,29,46].

2.2.5.2. Killer Sensitivity Determination of the S. cerevisiae Industrial Baking Strains to Contaminating Yeast Strain:

The YEPD-MB and YEPD plates were seeded with the industrial baking strains of S.cerevisiae BSP-1, BSP-2, BSP-3, and BSP4. The plates were streak inoculated with the contamination yeast strain.

2.2.5.3. Killer Sensitivity Determination of the Contaminating Yeast Strain to Marked Killer Yeast Strains:

The YEPD-MB and YEPD plates were seeded with the contaminating yeast strain. The plates were then streak inoculated with the marked killer yeasts (S.cerevisiae NCYC 232, S.cerevisiae NCYC 738, S.cerevisiae NCYC 761, H.anomala NCYC 432, P.membranaefaciens NCYC 333, H.anomala NCYC 435, H.mrakii NCYC 500, K.drosophilarum NCYC 575, K.lactis NCYC 1368).

2.2.5.4. Determination of Sensitivity to yeast killer toxins of pathogenic bacteria (applied in this study for the first time):

2.2.5.4.1. The cells of bacterial strains to be tested for their sensitivities to killer toxins were standardized to an optical density of 0.5 on the Mc Farland standard (ATB densitometer) and spread on blood agar medium pH 7.2 with a sterile cotton swab. The plates were then left to dry at room temperature for 30 minutes. The wells of 10 mm in diameter were cut in the bacterial medium and filled with YEPD buffered to pH 4.5 with 0.1 M citrate-phosphate buffer. Then the YEPD medium was inoculated with a loopfull of killer yeast strains and the plates were incubated at 20 °C for 24-48 hours. Bacteriocidal effects of the killer toxins were determined by examining the growth inhibition zones of the bacterial cells on the blood agar, surrounding the growth of the killer strain.

2.2.5.4.2. We have applied a second method for determination of bacterial sensitivity to killer yeast toxins that was applied in a very few laboratories. The killer yeast cells were incubated in liquid YEPD-glucose medium buffered to pH 4.5 with 0.1 M citrate-phosphate buffer, at 20 °C to log phase (2-3 days), with aeration (200 rpm-Nuve Rotary Shaker). Samples were centrifuged at 3000 rpm for 5 min and the supernatent fluids were filter sterilized through 0.22 µm pore size cellulose acetate membrane filters (Sartorius). 60 µl samples of each filtrate were inoculated into the wells that were cut in the bacterial medium. The plates were then incubated for 3 days at 20 °C and the growth inhibition zones were examined.

2.2.6. Gram-Staining :

Heat fixed smears of the bacterial cultures were covered for 1 minute with crystal violet and were rinsed prior to application of iodine solution for 1 minute. The slides were then decolorized with ethanol (95%) for 15 seconds and covered with carbol fuchsin for 1 minute, rinsed again and allowed to dry. Finally, the cells were observed under the light microscope (Olympus BH2) by oil immersion lens [5].

CHAPTER III

RESULTS

Identification of the contaminating yeast that was obtained from PAK GIDA A.Ş. was done by API LAB ID 32 yeast identification system. The growth of the organism after 48 hours incubation in the cupules of ID 32 C strip was detected by ATB reader (Fig. 3.1). After automatic reading, results were interpreted with API LAB ID 32C computer programme (Table 3.1) and the contaminating yeast was identified as Candida tropicalis (% ID = 99.6)

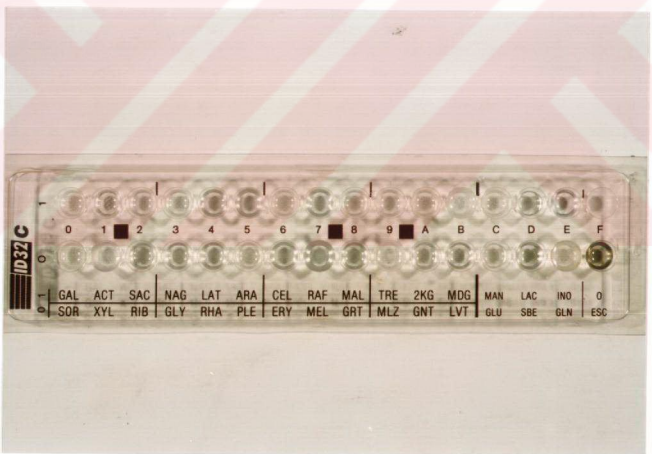


Figure 3.1. The growth of the yeast cells in the cupules of ID 32C strip. Each cupule contained a dehydrated carbohydrate substrate.

Table 3.1. Biochemical Test Results interpreted with

API LAB ID 32 Computer Programme.

ABBREVIATIONS	SUBSTRATES	RESULTS	ABBREVIATIONS	SUBSTRATES	RESULTS
SOR	Sorbitol	+	GAL	GALactose	+
XYL	D -XYLose	+	ACT	ACTidione	-
RIB	RIBose	-	SAC	SACcharose	+
GLY	GLYcerol	-	NAG	N-Acetyl-Glucosamine	+
RHA	RHAmnose	-	LAT	DL-LAcTate	-
PLE	PaLatinosE	+	ARA	L-ARAbinose	-
ERY	ERYthritol	-	CEL	CELlobiose	+
MEL	MELibiose	-	RAF	RAFfinose	-
GRT	GulucuRonaTe	-	MAL	MALtose	+
MLZ	MeLeZitose	+	TRE	TREhalose	+
GNT	GlucoNaTe	-	2KG	2-Keto-Gluconate	+
LVT	LeVulinaTe	-	MDG	α-Methyl-D-Glucoside	+
GLU	GLUcose	+	MAN	MANnitol	+
SBE	SorBosE	-	LAC	LACtose	-
GLN	GLucosamiNe	+	INO	INOsitol	-

The killer character of the Candida tropicalis strain that contaminates industrial baking strains of S. cerevisiae was tested against killer sensitive S. cerevisiae NCYC 1006 in both YEPD and YEPD-MB mediums. In YEPD medium, after incubation for 48 hours a clear growth inhibition zone of the killer sensitive S.cerevisiae 1006 surrounding the growth of the C.tropicalis was observed (Figures 3.2, 3.3).

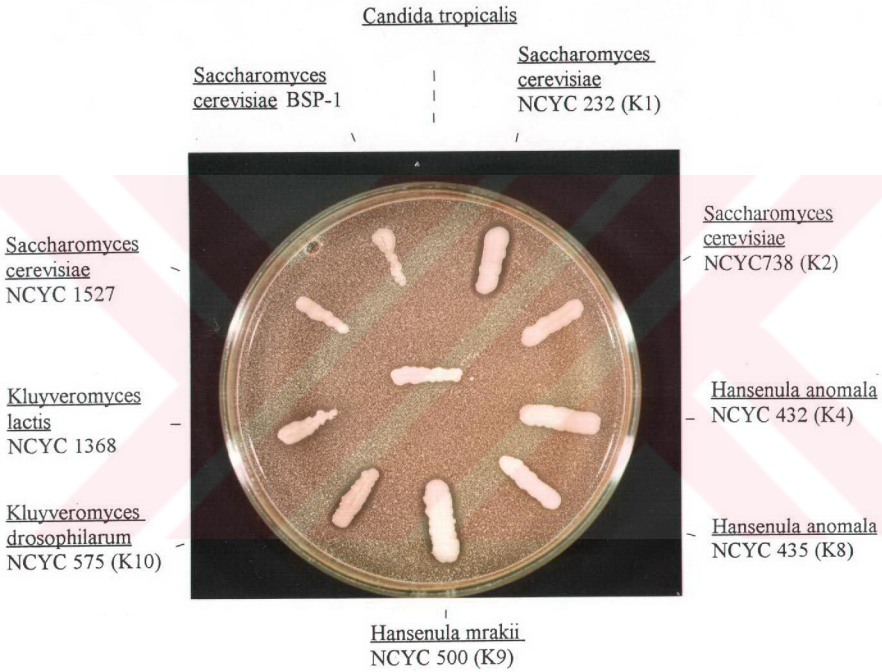


Fig. 3.2. Growth inhibiton zones of the killer sensitive S. cerevisiae 1006 on YEPD medium. The yeast strains other then C. tropicalis were used as control strains (K^+ killer, K^- non killer character).

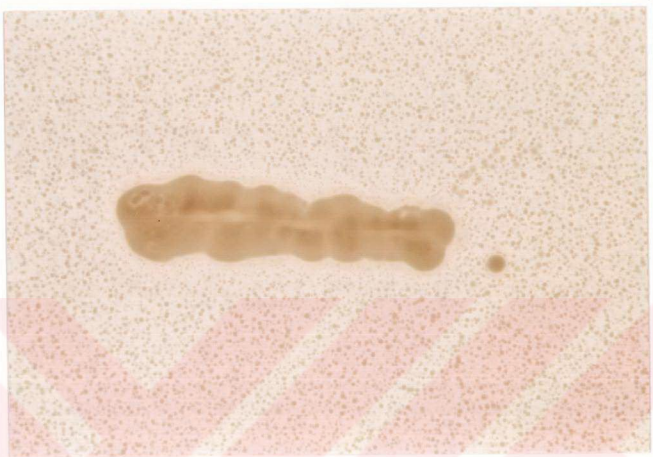


Fig. 3.3. Growth inhibition zone of the killer sensitive S. cerevisiae NCYC 1006 on YEPD medium surrounding C. tropicalis strain that showed killer activity.

In the YEPD-MB medium that was seeded with the killer sensitive S.cerevisiae 1006, dark blue zones of dead cells appeared surrounding the C.tropicalis strain as the cells were effected by the killer toxin and thus stained more with methylene blue (Figures 3.4, 3.5)

Fig. 3.4. Appearance of the dead cells of the killer sensitive S. cerevisiae NCYC 1006 that were stained with methylene blue, surrounding the growth of C.tropicalis and other control strains.

Fig. 3.5. Light blue inhibition zone with heavily methylene blue stained dead cells in YEPD-MB medium of killer sensitive S. cerevisiae 1006 surrounding the growth of the killer toxin producing C.tropicalis.

Also on the YEPD-MB medium that killer sensitive S. cerevisiae 1006 was spread by sterilized cottan swab, a light inhibition zone and dark blue dead cells, stained with methylene blue were observed surrounding the growth of C. tropicalis and the other control strains (Figures 3.6, 3.7).

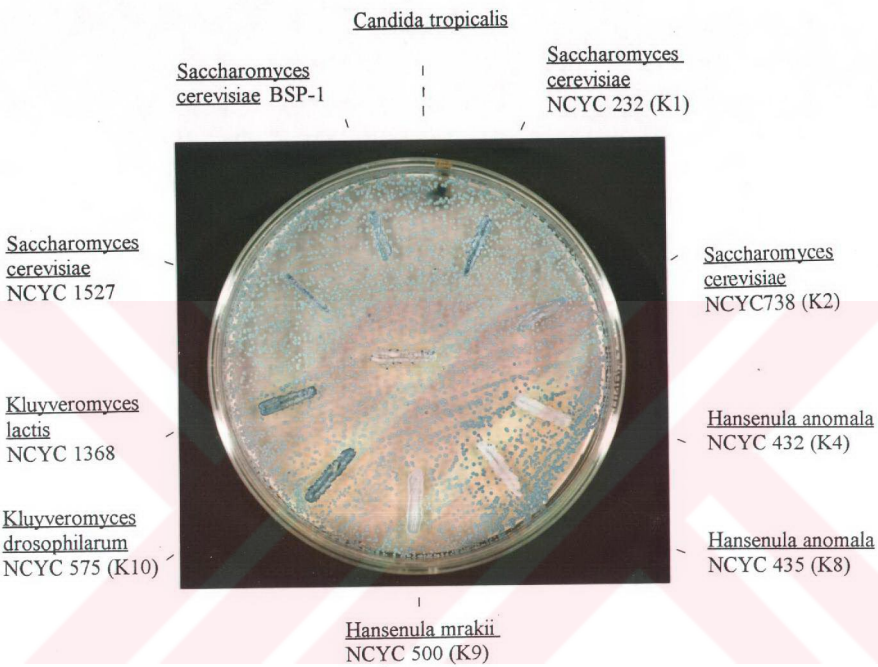


Fig. 3.6. Appearence of the growth inhibition zones and the methylene blue stained dead cells of the killer sensitive strain on YEPD-MB medium, surrounding the growth of C.tropicalis and other control strains.

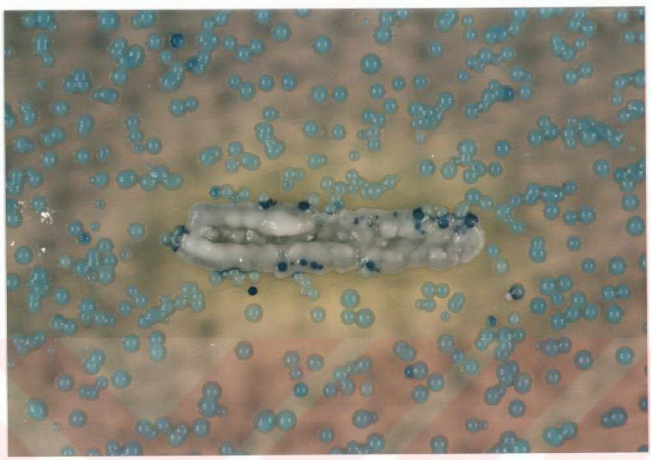


Fig. 3.7. Dark blue dead cells of the killer sensitive strain of S.cerevisiae 1006 touching to the streak-inoculated C.tropicalis. As the cells were killed by the toxins of C.tropicalis, they were heavily stained with methylene blue on YEPD-MB medium.

The killer activity of the contaminating yeast C.tropicalis on the industrial baking strains of S.cerevisiae (BSP1-4) was tested by suspending the cells of each strain in both YEPD and YEPD-MB mediums in seperate petri-dishes. The S.cerevisiae strains used as starters in baking industry were all found to be sensitive to C.tropicalis as clear growth inhibition zones in YEPD medium and dark blue zones of dead cells in YEPD-MB medium appered surrounding the growth of streak inoculated C.tropicalis (Figures 3.8-3.17).

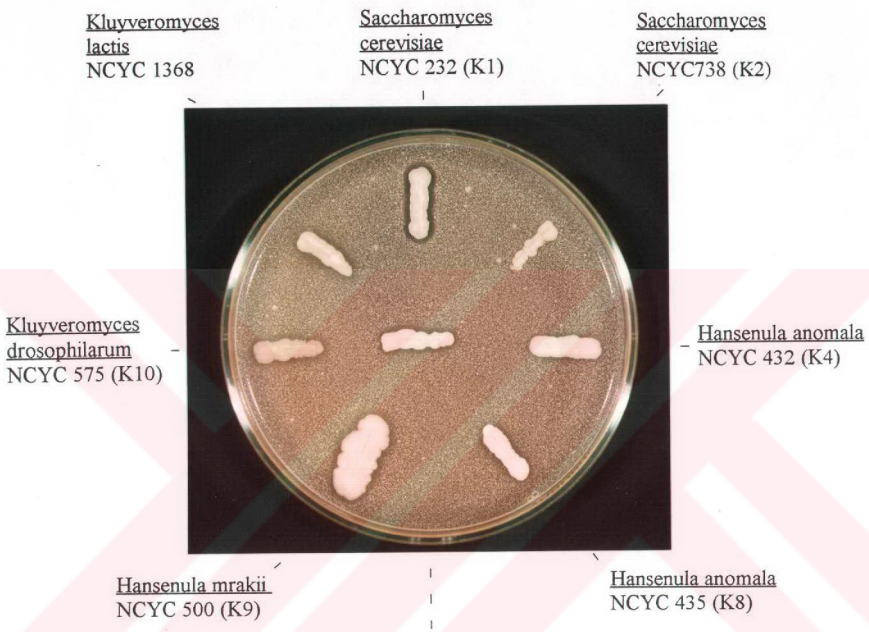


Fig.3.8. Sensitivity of S.cerevisiae BSP1 to the contaminating yeast C.tropicalis and to control strains(K^+) in YEPD medium.

Fig.3.9. Sensitivity of S.cerevisiae BSP2 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD medium.

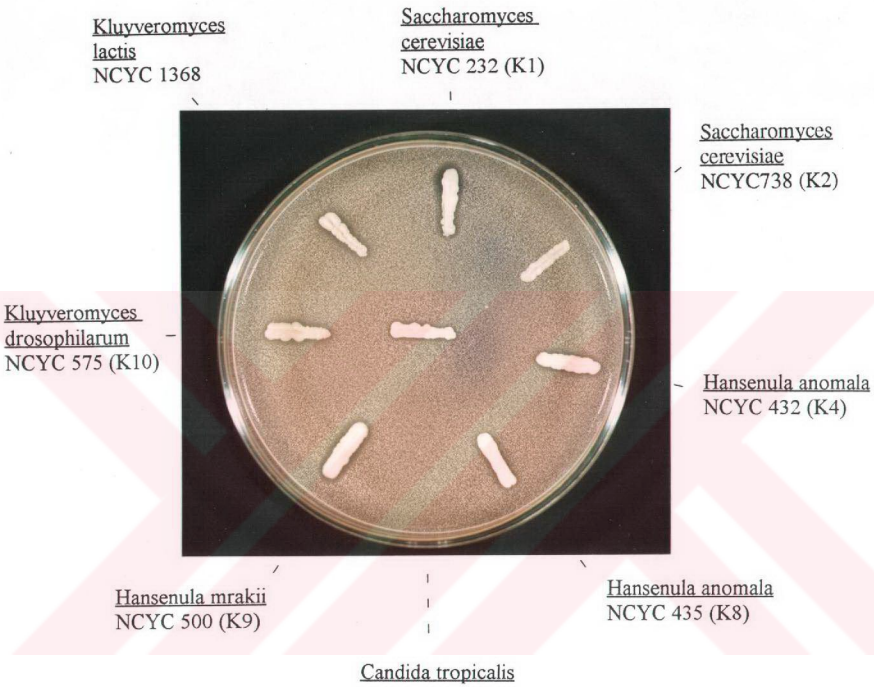


Fig.3.10. Sensitivity of S.cerevisiae BSP3 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD medium.

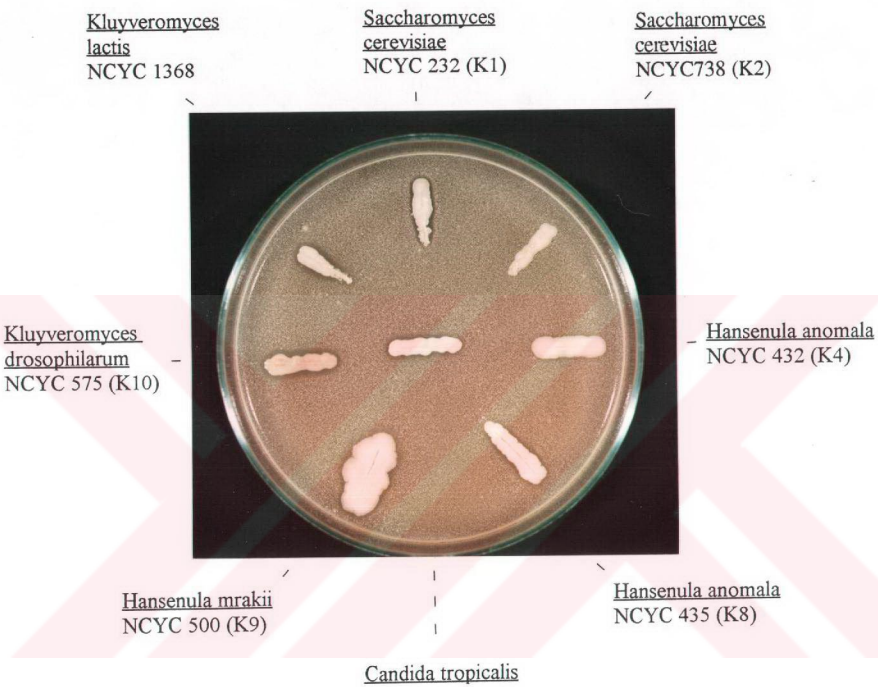


Fig.3.11. Sensitivity of S.cerevisiae BSP4 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD medium.

Fig.3.12. Growth inhibition zone of S.cerevisiae BSP1 surrounding the contaminating yeast C.tropicalis in YEPD medium (As similar growth inhibition zones were appeared for all S.cerevisiae strains only the zone for BSP1 was shown).

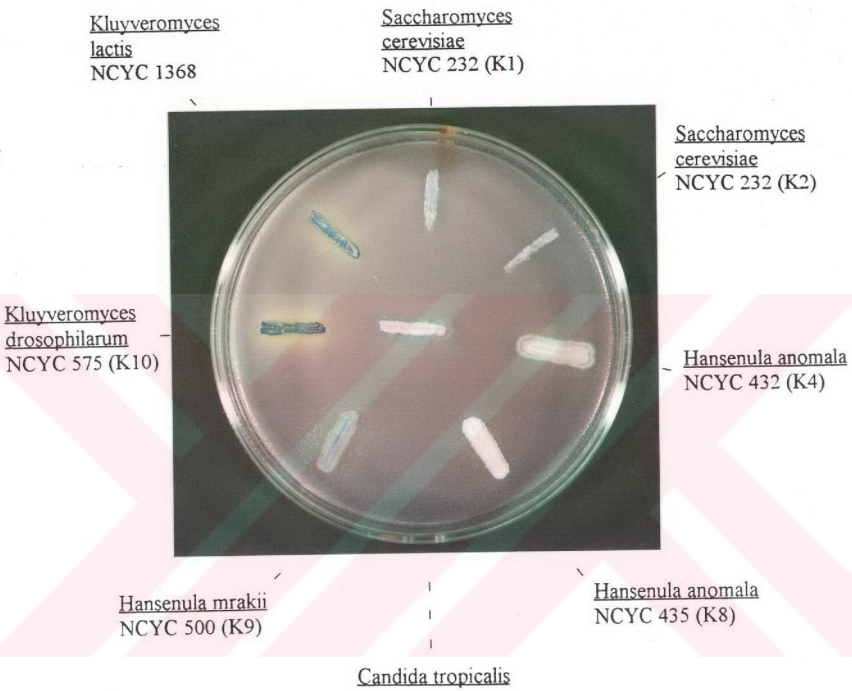


Fig.3.13. Sensitivity of S.cerevisiae BSP1 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD-MB medium.

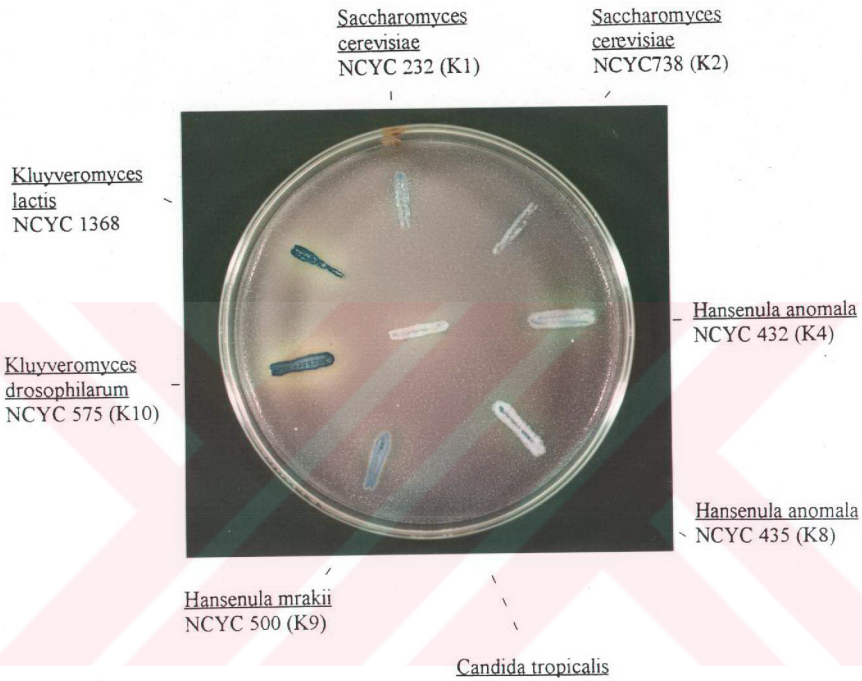


Fig.3.14. Sensitivity of S.cerevisiae BSP2 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD-MB medium.

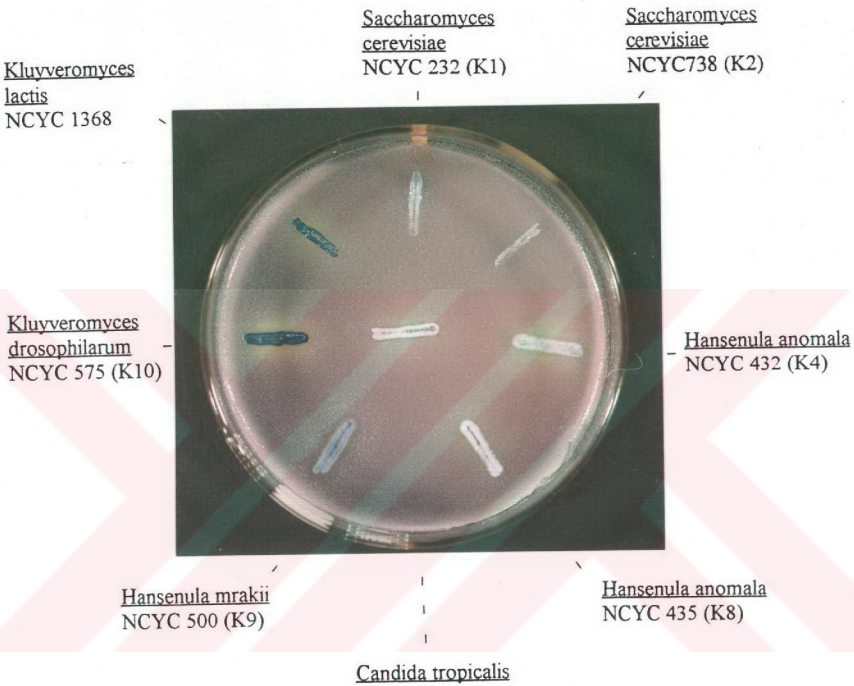


Fig.3.15. Sensitivity of S.cerevisiae BSP3 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD-MB medium.

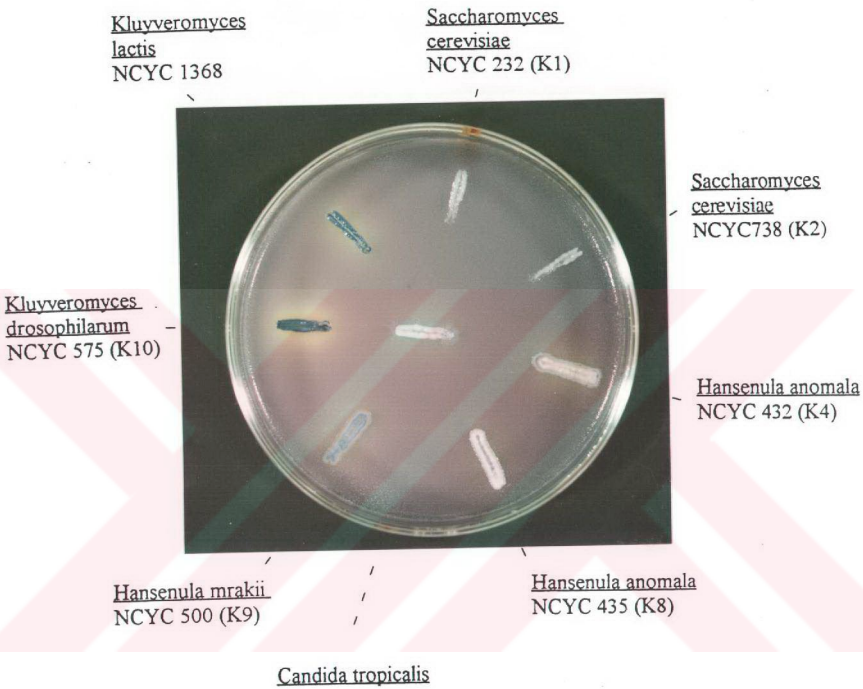


Fig.3.16. Sensitivity of S.cerevisiae BSP4 to the contaminating yeast C.tropicalis and to control strains (K^+) in YEPD-MB medium.

Fig.3.17. Occurance of dark blue zone with methylene blue stained dead cells of S.cerevisiae BSP1 surrounding C.tropicalis in YEPD-MB medium. The cells were stained heavily with methylene blue as they were killed with killer toxin action of C.tropicalis (As similar zones were appeared for each of the S.cerevisiae strains only the zone for BSP1 was shown).

The killer toxin producing C.tropicalis was tested for its sensitivity to other killer yeast strains of known killer types in YEPD medium. The C.tropicalis which itself is killer toxin producing was found to have sensitivity to S.cerevisiae NCYC 761(K3) and Hansenula anomala NCYC 435(K8) (Figures 3.18-3.20).

Fig.3.18. The activities of killer toxin producing strains on C.tropicalis. Among nine killer strains two of them, S.cerevisiae NCYC 761 and H.anomala NCYC 434 had potential killer activity on C.tropicalis.

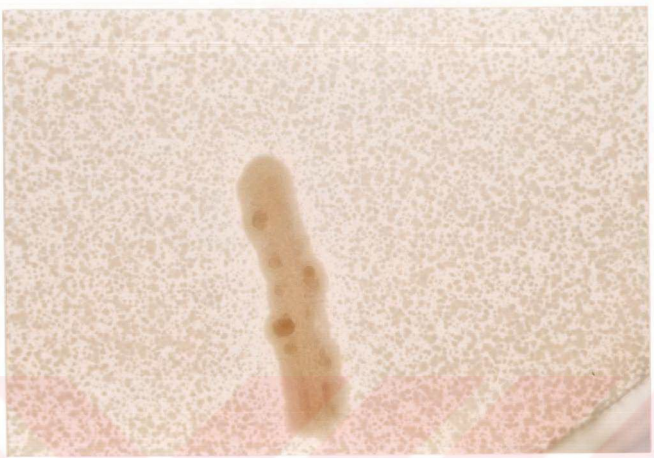


Fig.3.19. The growth inhibition zone of C.tropicalis in YEPD medium surrounding the S.cerevisiae NCYC 761 which has killer phenotype K3.

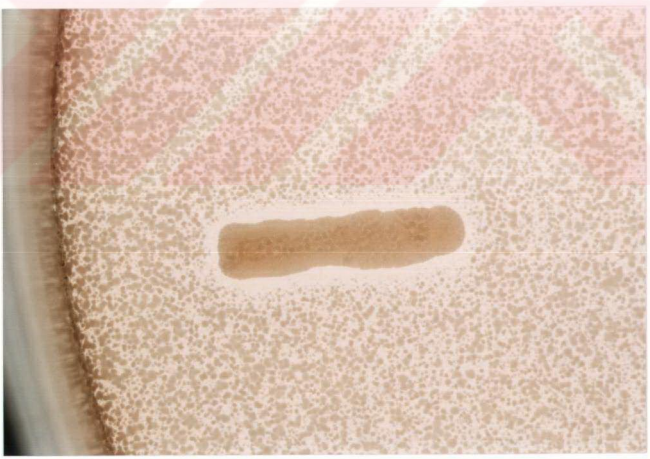


Fig.3.20. The growth inhibition zone of C.tropicalis in YEPD medium surrounding the H.anomala NCYC 434 which has killer phenotype K8.

The effects of the killer toxin producing yeast strains of known phenotypes along with the contaminating Candida tropicalis on bacteria that cause nosocomial infections, were tested on blood agar plates in which wells of 10 mm diameter were filled with YEPD medium that was inoculated with the yeast strains (Figures 3.21, 3.28)

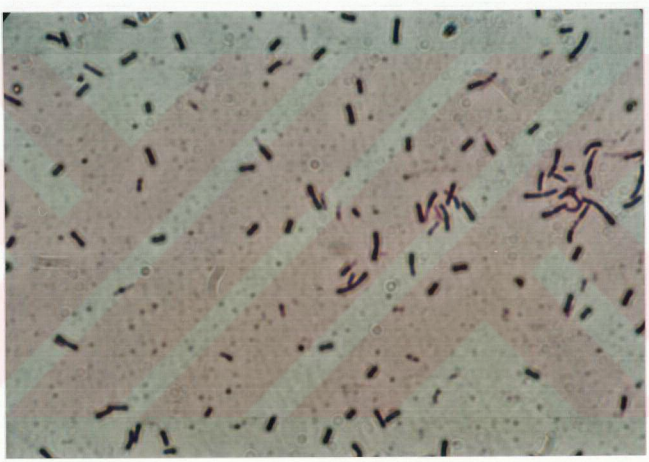


Fig.3.21.a. Gram stained Bacillus subtilis ATCC 6051 viewed under light microscope (Olympus BH2)

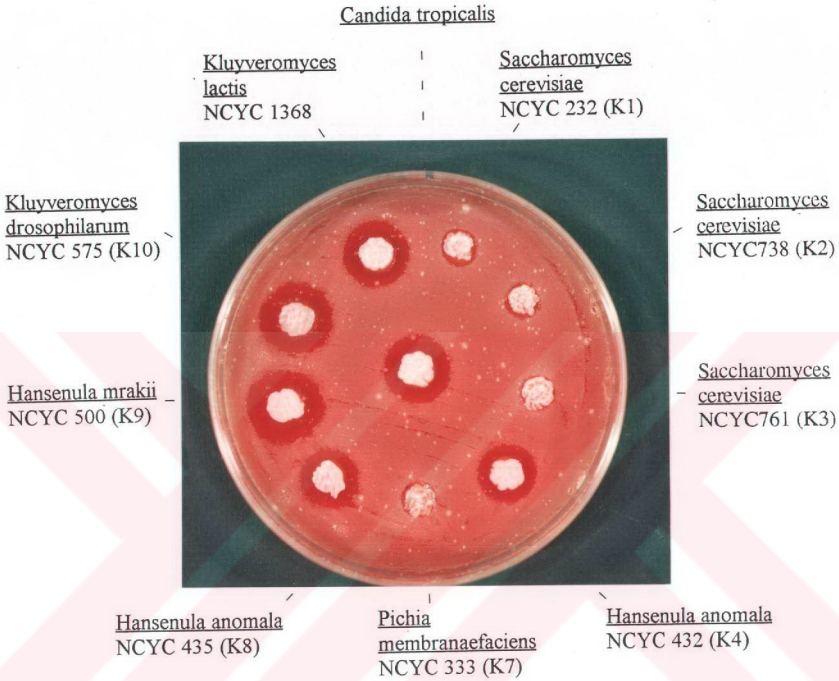


Fig.3.21.b. Growth inhibition zones of Bacillus subtilis ATCC 6051 on blood agar surrounding the growth of yeast cells on YEPD medium.

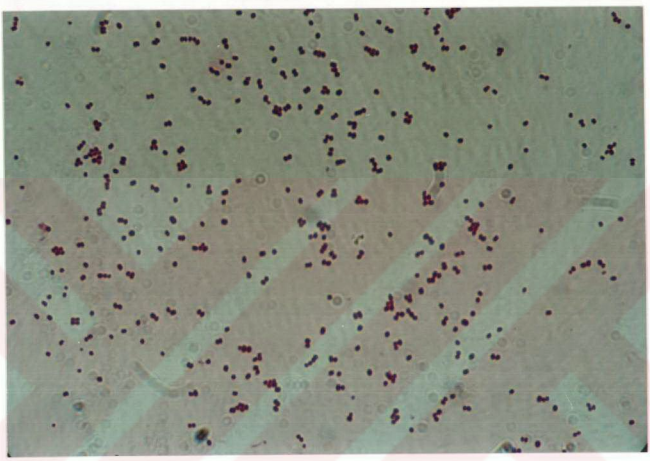


Fig.3.22.a.Gram stained Sarcina lutea ATCC 9341 viewed under light microscope(Olympus BH2).

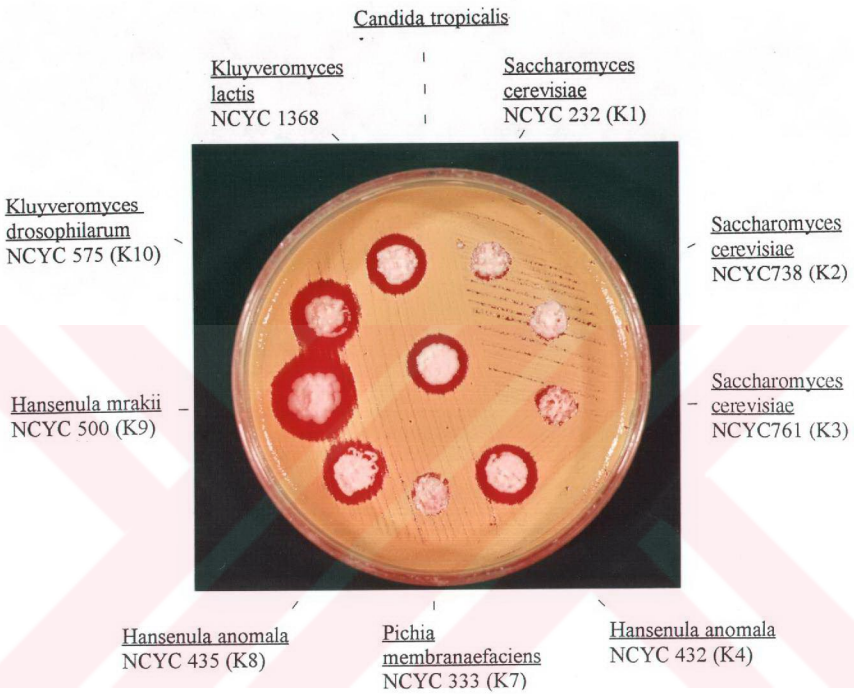


Fig.3.22.b. Growth inhibition zones of Sarcina lutea ATCC 9341 on blood agar surrounding the growth of yeast cells on YEPD medium.

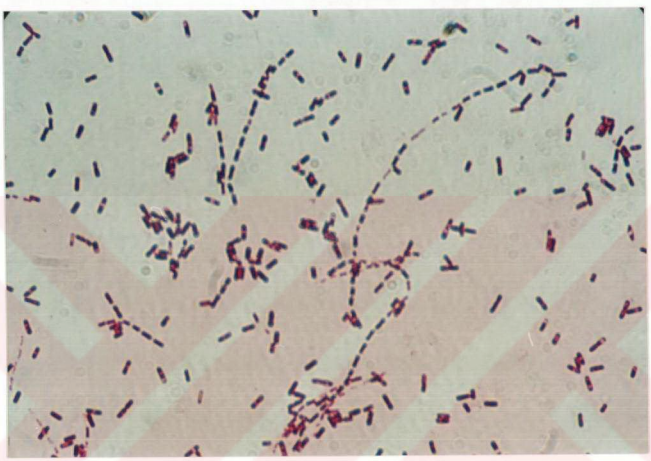


Fig.3.23.a. Gram stained Klebsiella pneumonia ATCC 10081 viewed under light microscope(Olympus BH2).

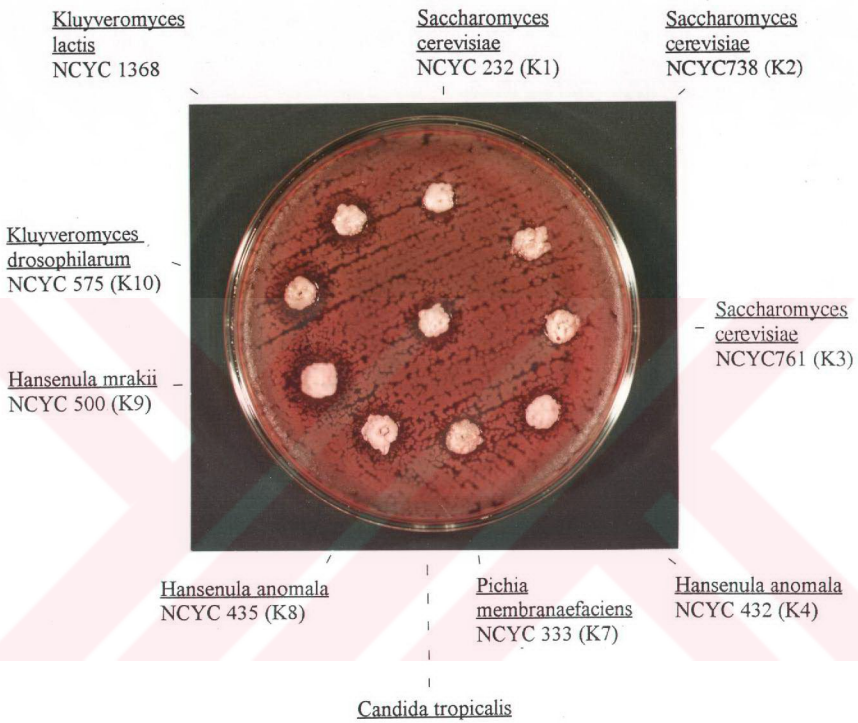


Fig.3.23.b. Growth inhibition zones of Klebsiella pneumonia ATCC 10081 on blood agar surrounding the growth of yeast cells on YEPD medium.

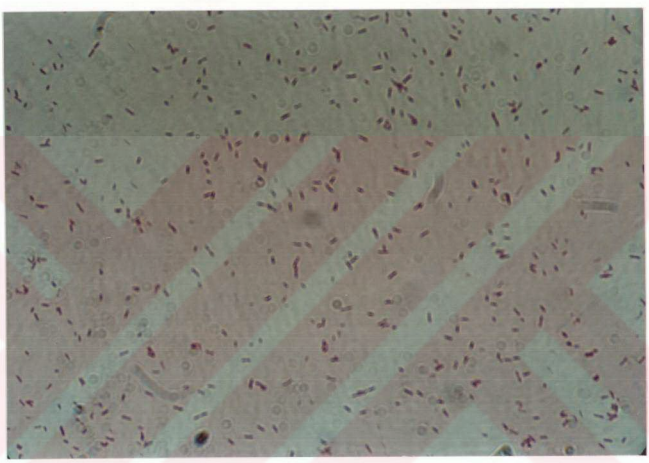


Fig.3.24.a. Gram stained Escherichia coli ATCC 25922 viewed under light microscope(Olympus BH2).

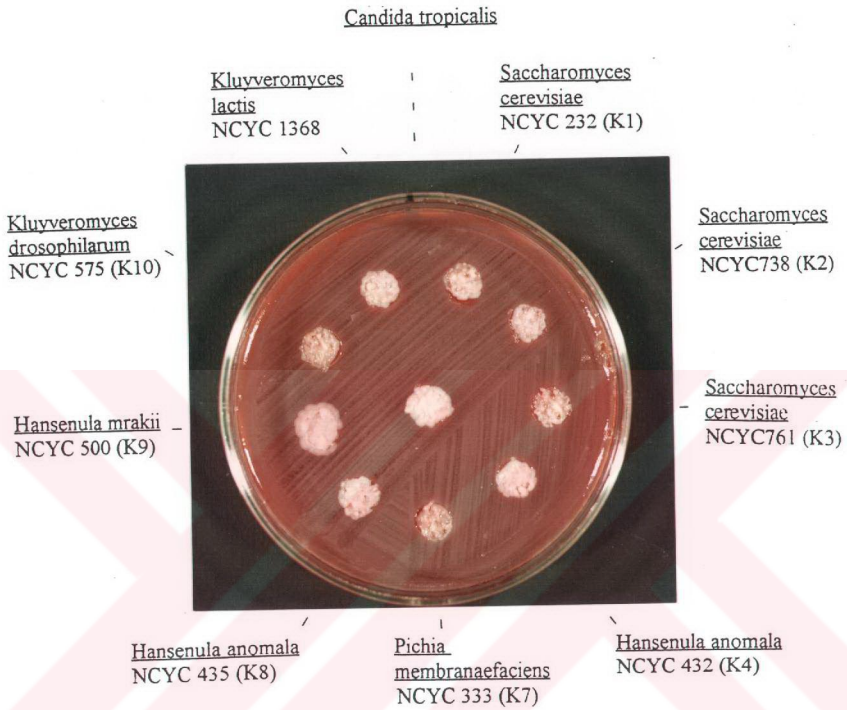


Fig.3.24.b. Growth inhibition zones of Escherichia coli ATCC 25922 on blood agar surrounding the growth of yeast cells on YEPD medium.

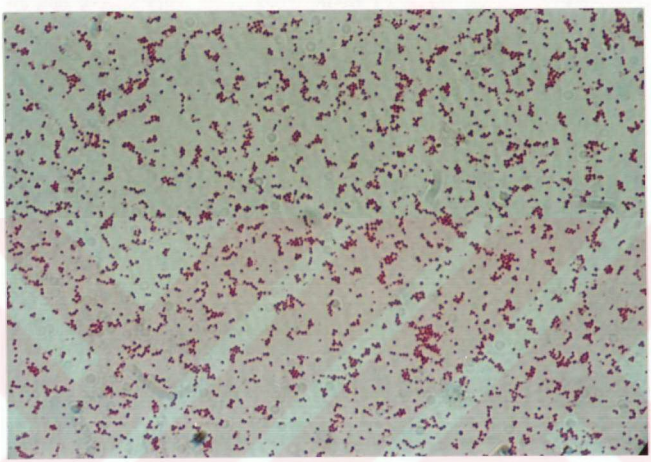


Fig.3.25.a. Gram stained Staphylococcus aureus ATCC 25923 viewed under light microscope(Olympus BH2).

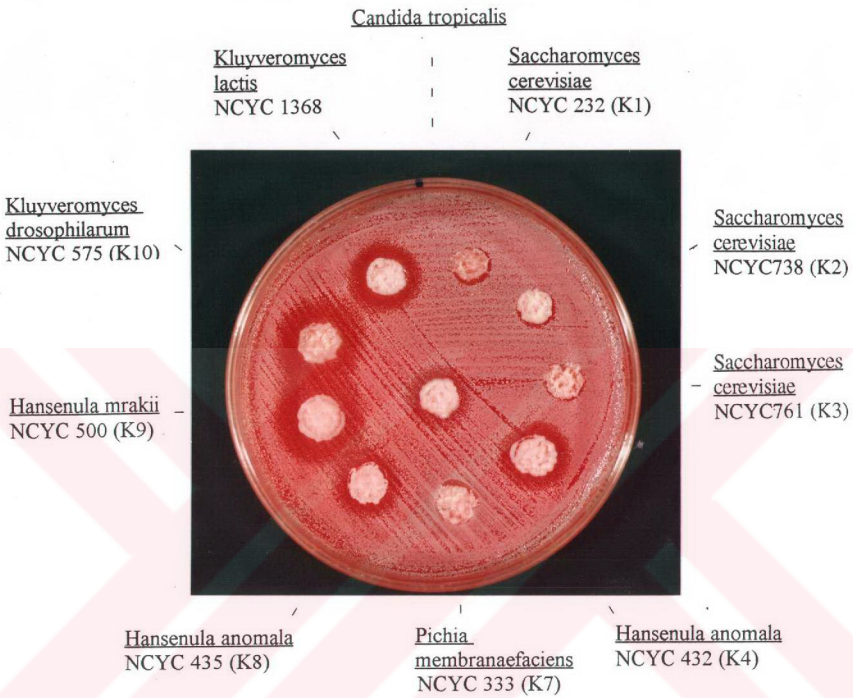


Fig.3.25.b. Growth inhibition zones of Staphylococcus aureus ATCC 25923 on blood agar surrounding the growth of yeast cells on YEPD medium.

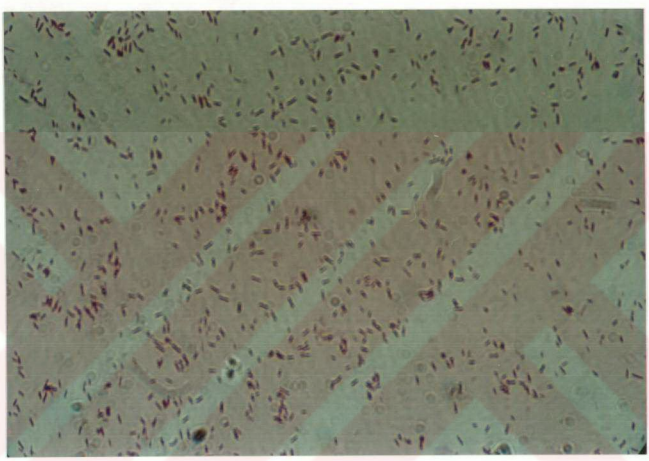


Fig.3.26.a. Gram stained Pseudomonas aeruginosa ATCC 27853 viewed under light microscope.

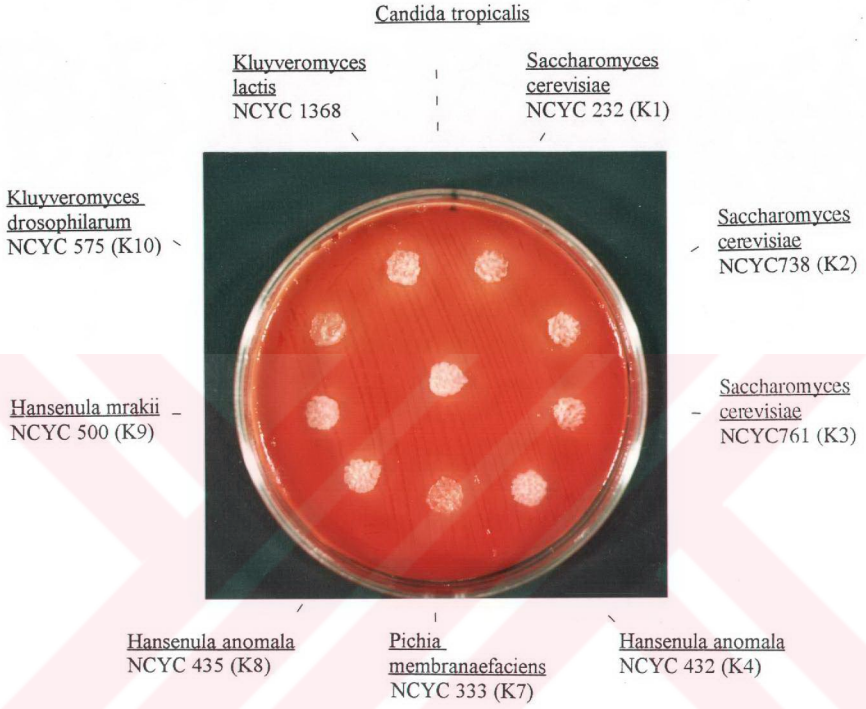


Fig.3.26.b. Growth inhibition zones of Pseudomonas aeruginosa ATCC 27853 on blood agar surrounding the growth of yeast cells on YEPD medium.

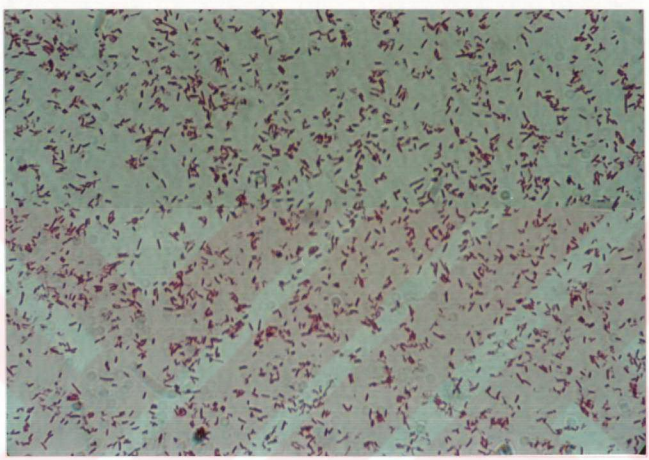


Fig.3.27.a. Gram stained Proteus vulgaris A 232 viewed under light microscope.

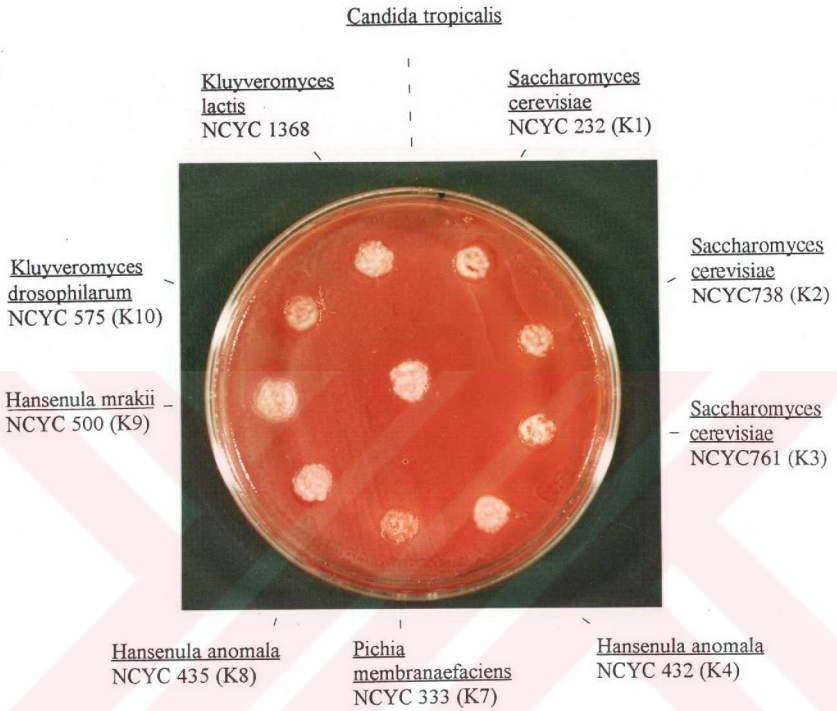


Fig.3.27.b. Growth inhibition zones of Proteus vulgaris A 232 on blood agar surrounding the growth of yeast cells on YEPD medium.

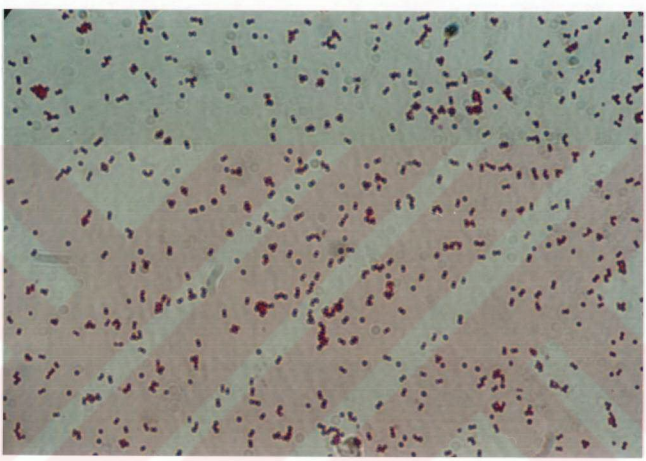


Fig.3.28.a. Gram stained Streptococcus pyogenes AA 3 viewed under light microscope.

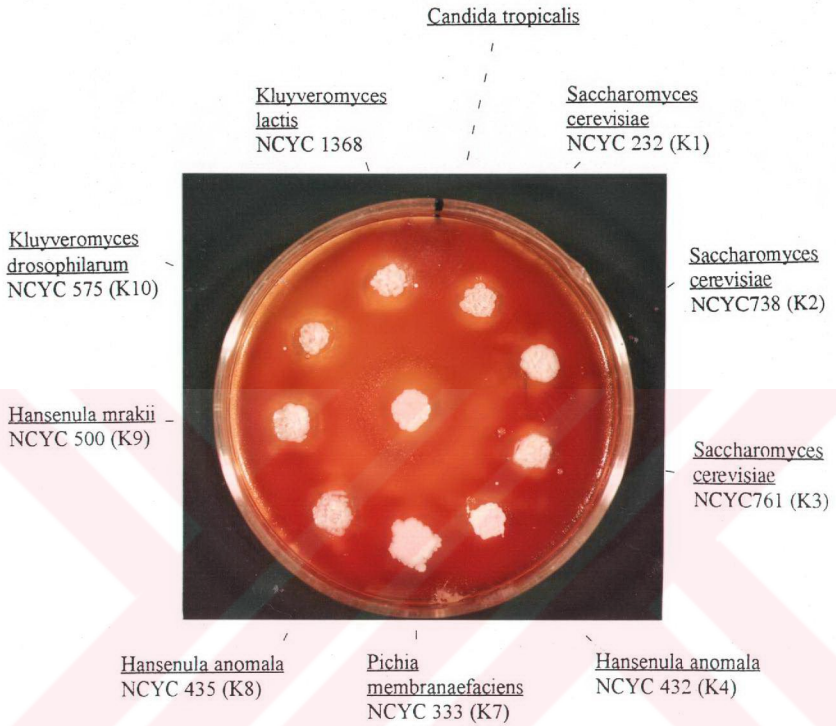


Fig.3.28.b. Growth inhibition zones of Streptococcus pyogenes AA 3 on blood agar surrounding the growth of yeast cells on YEPD medium.

CHAPTER IV

CONCLUSION

Killer yeasts extrude proteins which are lethal to sensitive species of yeasts. Some killer strains invade cultures of industrial yeast and spoil their products. In this study we have identified the yeast that contaminates the starters of industrial baking strain of S.cerevisiae and determined its killer activity.

According to the biochemical test results (Table 3.1), this contaminating yeast was identified as C.tropicalis with 99.6 % accuracy (API LAB ID Yeast Identification System).

C.tropicalis is a unicellular yeast that belongs to genus Candida, the family Cryptococcaceae and the group Deuteromyces (Fungi Imperfecti). It grows well at 20-30 °C in aerobic conditions forming smooth white colonies with round, oval or oblong budding cells (Figure 4.1) [45].

C.tropicalis has emerged as a potentially dangerous opportunistic fungus which is common both in environment and as a human commensal organism. Under certain conditions, it has been shown to cause significant morbidity and mortality [21].

C.tropicalis has been identified as the etiologic agent in a variety of infections including plyelonephritis, lower urinary tract infections, thrombophlebitis, arthritis, bursitis, meningitis, pericarditis and candidal vulvaginatis. It is a major hazard to immuno comprimised patients [21, 45, 47].

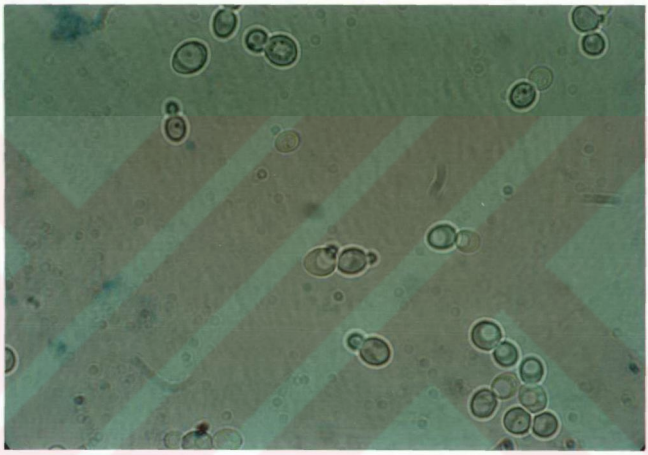


Fig 4.1 Appearance of Candida tropicalis cells under the light microscope (Olympus BH2)

Besides its pathogenicity to humans C.tropicalis contamination inhibits the growth of the fermentation strains and spoil the product. Our study on the reason of the growth inhibition of the industrial S.cerevisiae strains that are contaminated with C.tropicalis demonstrated that this contaminating yeast strain was a killer toxin producing strain as it was tested on killer sensitive S.cerevisiae NCYC 1006 (Figure.3.2)(Table 4.1).

Table 4.1. Killer Activities of C.tropicalis and the other Control Strains on Killer Sensitive S.cerevisiae NCYC 1006.

Species	Strain	Killer Phenotypes /genotypes	Killer Activity on Killer Sensitive S.cerevisiae NCYC 1006
S. cerevisiae *	NCYC 232	K1	+
S. cerevisiae *	NCYC 738	K2	+
H.anomala *	NCYC 432	K4	+
H.anomala *	NCYC 435	K8	+
H. mrakii *	NCYC 500	K9	+
K.drosophilarum*	NCYC 575	K10	+
K.lactis *	NCYC1368	PGKL1, PGKL2	+
S. cerevisiae *	NCYC1527	α leu 2-3 leu112 his3-11 his3-15 ura2 trp1 CAN S	-
S. cerevisiae	BSP1	Polyploid	-
C.tropicalis			+

*Control Strains

The four strains of S.cerevisiae (BSP1-4) were all killed by this killer yeast strain when they were both cultivated in the same medium (Figures. 3.8, 3.9, 3.10, 3.11) (Table 4.2).

Table 4.2 Killer sensitivity of the industrial baking strain of S.cerevisiae to the contaminating yeast C.tropicalis and to other control strains of known phenotypes.

Species	Strain	Killer Phenotypes /genotypes	Killer Activity on S.cerevisiae Industrial Baking Strains (BSP1-BSP4)
S. cerevisiae *	NCYC 232	K1	+
S. cerevisiae *	NCYC 738	K2	+
H.anomala *	NCYC 432	K4	+
H.anomala *	NCYC 435	K8	+
H.mrakii *	NCYC 500	K9	+
K.drosophilarum*	NCYC 575	K10	+
K.lactis *	NCYC1368	PGKL1, PGKL2	+
C.tropicalis		K^+	+

*Control Strains

Although the killer strains are immune to their own toxins, they are susceptible to the effects of the toxins of another immunity groups. Connected with this fact, we have determined the sensitivity of the killer toxin producing C.tropicalis to other marked killer toxin producing yeasts of different killer phenotypes. Among the tested different killer phenotypes, C.tropicalis was found to be immune to K1, K2, K4, K7, K9, K10 but sensitive to K3 and K8 type toxins (Figure 3.3) (Table 4.3).

Table 4.3. The activities of killer yeast strains of known phenotypes on C.tropicalis.

Species	Strain	Killer phenotypes /genotype	Killer activity on C.tropicalis
S.cerevisiae	NCYC 232	K1	-
S.cerevisiae	NCYC 738	K2	-
S.cerevisiae	NCYC 761	K3	+
H.anomala	NCYC 432	K4	-
P.membranaefaciens	NCYC 333	K7	-
H.anomala	NCYC 435	K8	+
H.mrakii	NCYC 500	K9	-
K.drosophilarum	NCYC 575	K10	-
K.lactis	NCYC1368	PGKL1, PGKL2	-

The killer phenomenon can be utilized for the protection of fermentation process against contaminating yeast. Introduction of killer trait into fermentation strains and thus constructing of immune strains which produce killer toxins, can be used to prevent killer strains from spoiling the product. Fermentation strains with killer toxin producing properties can be constructed by induced protoplast fusion or most efficiently by cytoduction. We have found that the contaminating yeast C.tropicalis inhibited both by S.cerevisiae of K3 and Hansenula anomala of K8 toxin types (Figure 3.18). As the later inhibited the growth of C.tropicalis more potentially (Figure 3.20), the introduction of the K8 type killer determinant to industrial baking strains of S.cerevisiae (BSP1-4) will establish immunity to C.tropicalis killer toxin and prevent product spoilage.

In this study we have also examined the effect of the toxin of the contaminating killer yeast C.tropicalis on bacteria that cause nosocomial infections. As there is little evidence on the bacteriocidal effects of the yeast killer toxins; along with the C.tropicalis we have determined the activities of the killer yeasts of known phenotypes on those bacteria (Table 4.4) and developed a new method (2.2.5.4.1) which we found more efficient than the one (2.2.5.4.2) used in a few previous studies for killer toxin activity on bacteria.

Table 4.4 Inhibitory Effects of the Killer Yeast Toxins on some Pathogenic Bacteria of Clinical Importance

Species	Strain	Gram Staining	Clinical Disease /Syndrom (5,11,31)	Killer Yeasts with inhibitory activities
Bacillus subtilis	ATCC 6051	+	Respiratorytract infections (pneumonitis) Endocarditis, Bacteremia Menengitis	K.lactis(dsDNA), H.anomala(4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Sarcina lutea	ATCC 9341	+	Gastro intestinal tract infections	K.lactis(dsDNA), H.anomala(K4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Klebsiella pneumonia	ATCC 10081	+	Urinary tract infections Respiratorytract infections (pneumonitis)	K.lactis(dsDNA), H.anomala(K4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis

continued on the following page

Escherichia coli	ATCC 25922	-	Gastroenteritis Urinary tract infections Meningitis	K.lactis(dsDNA), H.anomala(K4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Staphylococcus aureus	ATCC 25923	+	S. skin syndrome(SSSS) Toxic shock syndrome Staphylococcal food poisoning	K.lactis(dsDNA), H.anomala(K4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Pseudomonas aeruginosa	ATCC 27853	-	Pulmonary, ear, burn, eye, urinary tracts, gastrointestinal tracts infections,hematologic and malignant deseases	S.cerevisiae(K1), S.cerevisiae(K2), S.cerevisiae(K3), H.anomala(K4), K.lactis(dsDNA), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Proteus vulgaris	A 232	-	Meningitis Urinary tract infections Gastroenteritis	K.lactis(dsDNA), H.anomala(K4), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis
Streptococcus pyogenes	AA 3	+	Skin infections (impetigo, pyoderma	S.cerevisiae(K1), S.cerevisiae(K2), S.cerevisiae(K3), H.anomala(K4), K.lactis(dsDNA), H.anomala(K8), H.mrakii(K9), K.drosophilarum(10), C.tropicalis

We have concluded from this work that the yeast which contaminates the baking strains of S.cerevisiae in Turkish baking industry is a killer toxin producing Candida tropicalis that has also inhibitory effect on pathogenic bacteria.

To avoid the contamination of the S.cerevisiae industrial baking strains with C.tropicalis instantly, will be of great importance from the point of both public health and fermentation efficiency. Therefore we have planned and started to a new project concerning the immunization of the industrial baking strains of S.cerevisiae (BSP1-4) to the contaminating C.tropicalis. Also purification studies of the killer proteins of this killer strain is underway in our laboratory, as killer toxins of yeasts would be used as potential antibiotics in the near future.

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APPENDICES

APPENDIX A

COMPOSITION AND PREPRATIONS OF CULTURE MEDIAE

1. Blood Agar (pH 7,2)

Components:	g/lt
Tryptic soy agar(Gibco BRL)	40

After sterilization at 15 Ibs (121 °C), base medium was cooled to approximately 45°C and 50 cc of blood (sheep) was added aseptically and then poured into sterile petridishes. These plates may be stored for a week at 4 °C without deterioration.

2. Muller Hinton Broth(Gibco BRL)

Components:	g/lt
Peptone	21.5
Beef infusion	2.0
Soluble starch	1.5

3. YM mediae(pH 5.5)

Components:	g/lt
Yeast extract	3
Malt extract	3
Peptone	5
Glucose	10

4. YEP-glucose(pH 5.5)

Components:	g/lt
Yeast extract	5
Peptone	5
Glucose	10

5. YEPD medium

Components:	g/lt
Yeast extract	10
Peptone	20
Glucose	20

6. Malt Extract

Components:	g/lt
Malt Extract	3
Peptone	5

7. YEPD-MB

Components:	g/lt
Yeast extract	10
Peptone	20
Glucose	20
Methylene blue	

All the components were resuspended in dH_20 and sterilized in liquid cycle for 20 min. at 121°C.

APPENDIX B

BUFFERS AND SOLUTIONS

1. Citrate Buffer (pH 4.5)

   0.1 M Citric Acid

   0.1 M Di Potassium Hydrogen Phosphate

2. C medium (pH 6.5)

   5.0 g amonium sulphate

   0.31g monopotassium phosphate

   0.45g dipotassium phophate

   0.92g disodium phosphate

   0.1g sodium chloride

   0.05g calcium chloride

   0.2g magnesium sulphate

   0.005g histidine

   0.02g tryptophan

   0.02g methionine

   0.5g agar

   1ml vitamine solution

   10ml trace elements

1000ml dd water

3. Suspension Medium

   2ml sterile dd water

All the buffers were sterilized in liquid cycle for 20 min. at 121 °C.

APPENDIX C

CHEMICALS AND SUPPLIERS

Chemicals	Suppliers
Yeast Extract	DIFCO-USA
Malt Extract	MERCK-GER
Bacto Peptone	DIFCO-USA
Bacto Agar	DIFCO-USA
Methylene Blue	SIGMA-USA
Glucose	MERCK-GER
Citric Acid	MERCK-GER
Dipotassium Hydrogen Phosphate	MERCK-GER
Muller Hinton Agar	GIBCO-BRL
Tryptic Soy Agar	GIBCO-BRL
C Medium	BIOMERUX-FR
Suspension Medium	BIOMERUX-FR

APPENDIX D

STAINING SOLUTIONS

1. Crystal Violet

   Components:

Crystal violet	5 g
Ethanol (95 %)	50 ml
Ammonium oxalate	4 g
Distilled H_20	400 ml

2. Iodine Solution

   Components:

Crystal Iodine	1 g
KI	2 g
Distilled H_20	300 ml

3. Carbol Fuchsine

Components:

Carbol fuchsine	30 ml
Distilled H_20	270 ml

4. Methyl-Red Indicator

Components:

Methyl-red	20 mg
Ethanol	60 ml
Distilled H_20	40 ml

All solutions may be kept at room temperature.