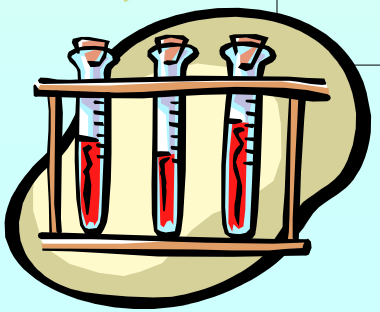
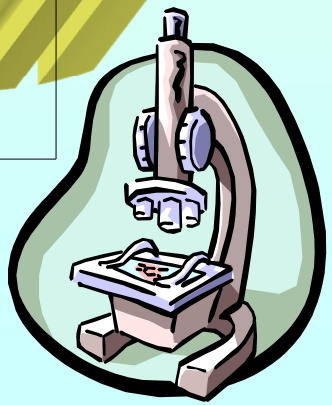
Dr. habil. Anna Salek

BIOTECHNOLOGY



Food Science



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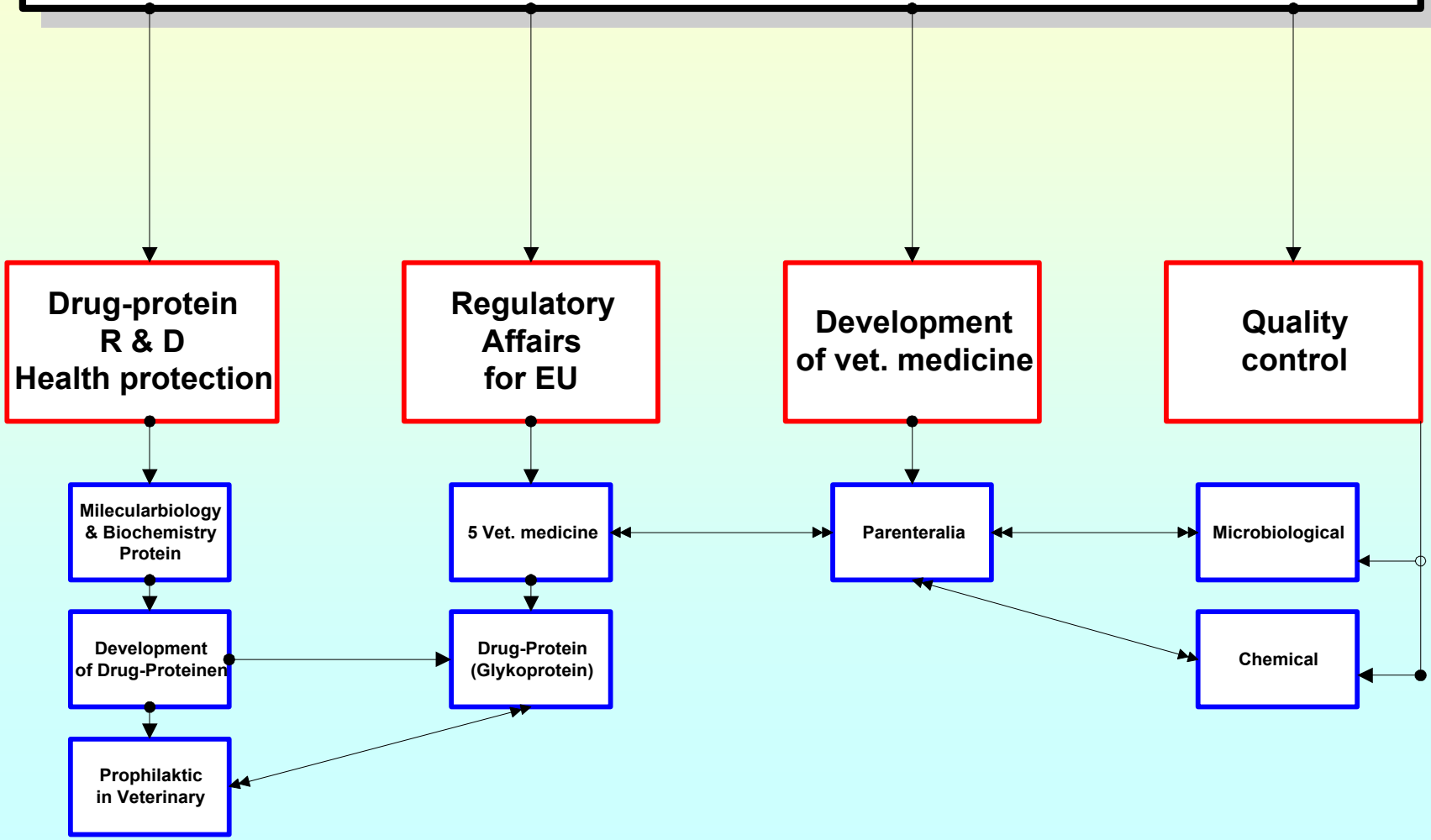
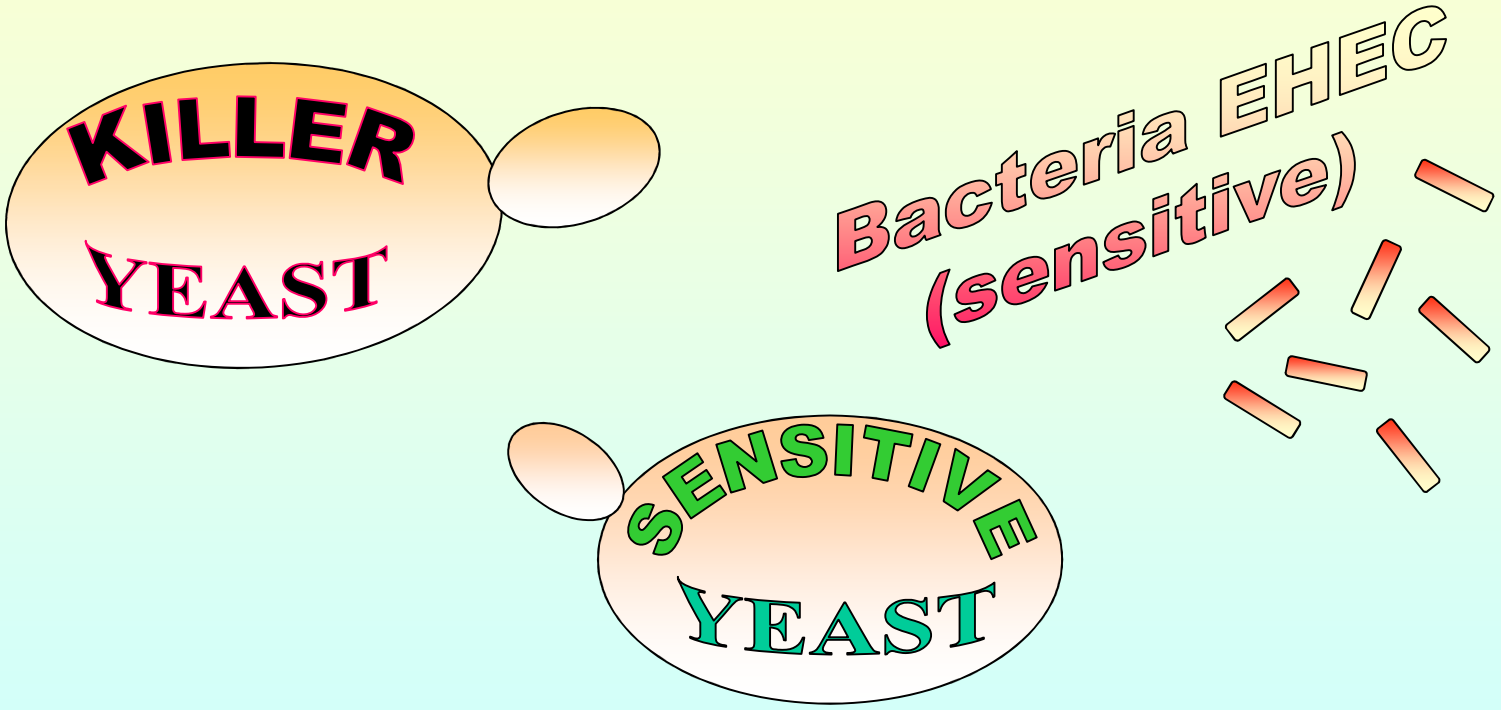
BIOTECHNOLOGY of Pharmaceutics



Yeast Antimicrobial Proteins



Dr. habil. Anna Salek

Yeast Antimicrobial Proteins

I. BIOTHERAPEUTIC EFFECT OF KILLER TOXIN

II. KILLER PHENOMENON

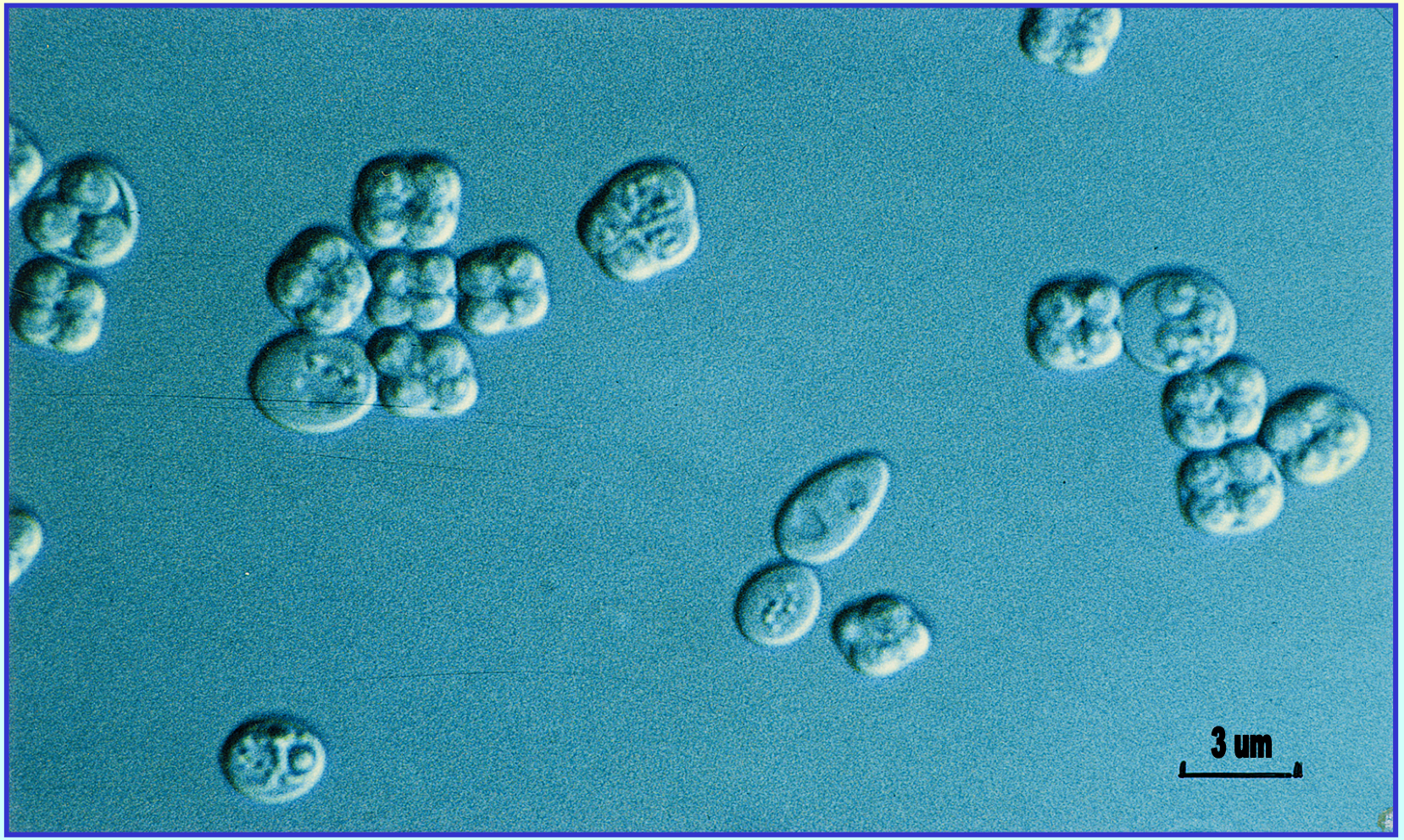
III. KILLER PROTEIN BIOCHEMISTRY

- Characteristic of killer proteins
- Selection of biochemical methods for purification of killer protein
- Biological activity of killer proteins
- The best killer toxins - a lethal effect for pathogenic microorganisms

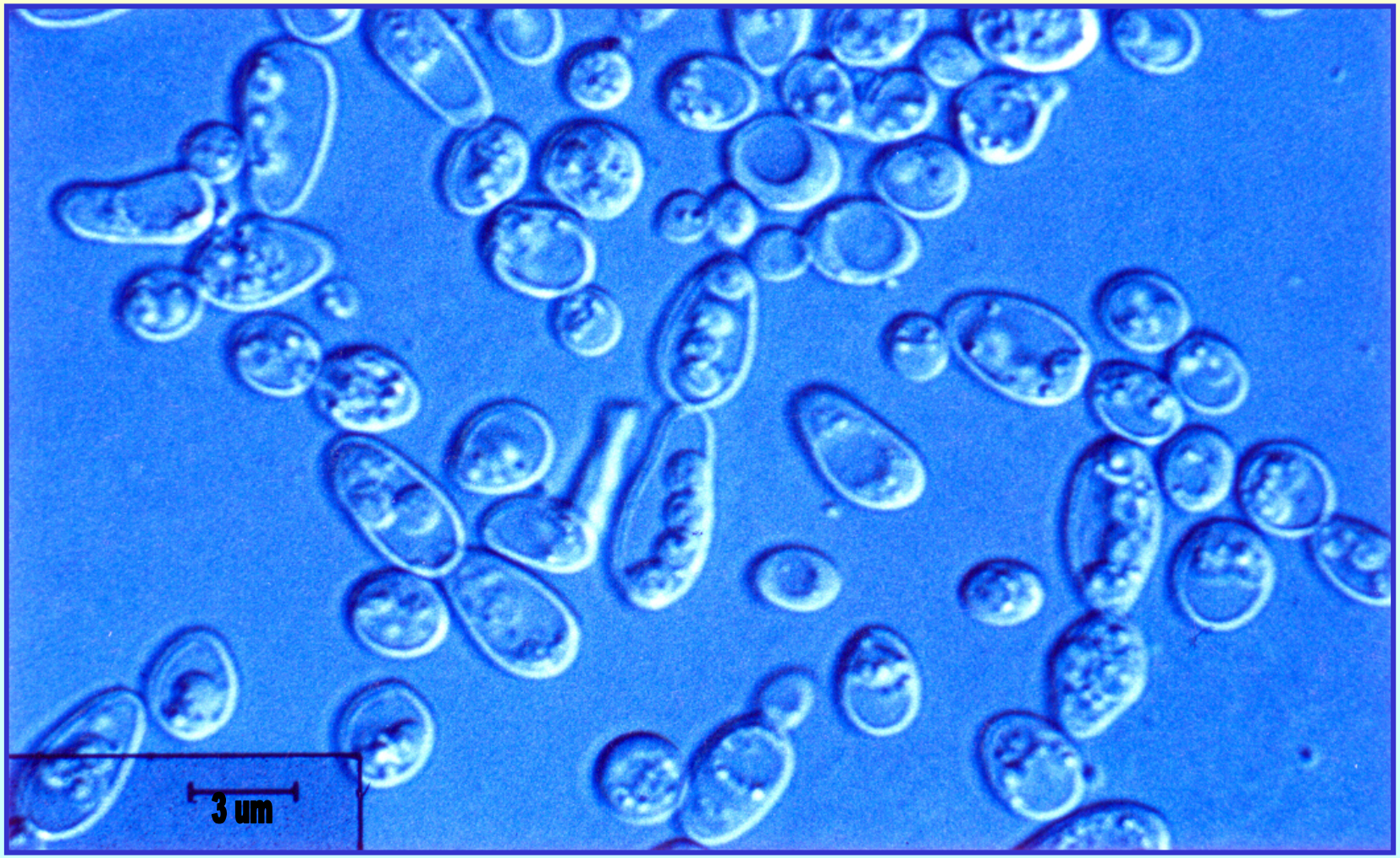
IV. INFLUENCE OF YEAST KILLER TOXINS ON THE CITOTOXICITY OF SHIGA - LIKE TOXINS

- Effect of killer toxin on mammalian cells
- Killer protein binding to Shiga-Like-Toxin receptors

Yeast *Saccharomyces cerevisiae and Hanseniaspora valbyensis cells*



Yeast *Williopsis mrakii cells*



I. BIOTHERAPEUTIC EFFECT OF KILLER TOXINS

It has been observed that an increasing number of pathogens are becoming resistant to antibiotics in current use. The need for novel, broad-spectrum antimicrobial agents is increasingly important in today´s medical field.

Therefore, biotechnology is turning to the natural product to find new biotherapeutic agents, active against pathogenic bacteria or yeast.

Recently, a prophylactic and therapeutic antimicrobial strategy, based on a specific physiological target, has become effective due to the use of killer yeast directed against their natural competition.

On several occasions, differential susceptibility to the toxic effect of yeast killer protein has been proposed as a potentially useful biotherapeutic agent for improvement of the human or animals health as well as for environmental control.

II. KILLER PHENOMENON

The killer phenomenon has been reported for strains of the genera *Saccharomyces, Kluyveromyces, Hansenula* (or *Pichia*), *Hanseniaspora, Williopsis, Candida, Torulopsis, Debaromyces, Cryptococcus* and *Ustilago*. The above-mentioned yeasts produce toxins which act against sensitive strains of the same or closely related species as well as against unrelated microorganisms, including pathogenic yeasts.

The mechanisms of toxicity are various: 1). a pore-forming channels in the cell wall (i.e. enter the cytosol and attack essential constituents), 2). an inhibition of protein synthesis, or 3). an arrest of the G_1 phase of the cell cycle. Many of yeast killer toxins are glycoproteins.

III. KILLER PROTEIN BIOCHEMISTRY

A phylogenetic study on killer yeasts of genus *Hansenula* showed that yeast species *Williopsis mrakii* or *Pichia anomala* with saturn-shaped ascospores had a strong killer activity to ward various yeast species and will be useful for breeding in wine making and will have a wide application as „antibiotics“.

Despite, our knowledge about above genera, i.e. toxins from some killer strains, including those *Williopsis* (or *Hansenula*) sp., have undefinited genetic origins. For instance, *Williopsis mrakii* LKB 169 secreted two toxins (a protein and a single polypeptide with molecular weight 10.7 and 8.9 kD, respectively) which showed identical killer activities (disruption of the impermeability of the cell membrane leading to ATP leakage) and killed yeast which belong to various genera. Strain of *Williopsis mrakii* NCYC 500 secreted only one active acidic polypeptide with molecular weight 1.8-5.0 kD.

A study performed by Ashida and Yamamoto showed that *Williopsis mrakii* LKB 169 secrete into culture media two toxins (K-I and K-II). Toxin K-I is composed of 88 amino acids residues with a molecular size of 10.7 kD. The K-II toxin is a single polypeptide with molecular weight of about 8.9 kD. Both toxins were very stable against heat (boiling for 3 min at pH 4) and in the pH range of 4-11 at 25^{o} C. They showed identical killer actions (disrupts the permeability of the cell membrane and ATP leakage) and killed yeasts which belong to various genera (e.g. *Hansenula, Pichia, Candida, Saccharomyces, Kluyveromyces, etc.*).

The therapeutic effect of killer protein from well genetically-characterized laboratory yeast strain of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* is presently unknown, because their proteins are weak killer to variety pathogenic microorganisms. *Kluyveromyces lactis* strains, harboring pGKL1 and pGKL2 plasmids, secrete a killer toxin (glycoprotein), consisting of three subunits, α (97 kD), β(31 kD) and γ (28 kD). This toxin has a very broad killer spectrum against yeasts of different genera and species.

In the light of above-mentioned study it was performed biochemical characteristic of other yeast proteins. Established tools in life-sciences research, such various chromatography and electrophoresis techniques, are now being more useful in protein biochemistry. Therefore, in the case of purification of killer proteins some of their methods were used.

Table 1. A content of total proteins and specific killer activity.

Specific activity Supernatant of killer strain cells/ml cult.]	Medium	Protein [μg/ml]	Activity [U/ml culture]	Specific activity [U/mg protein] x 10^5	[dead x 10^6
Williopsis mrakii AS/15ρ$^-$	YNBglu2	0.8	255	3.2	1.96
	YPG	1.2	455	3.8	3.51
Pichia anomala USCS 25F	YNBglu2	0.9	410	4.6	3.16
	YPG	1.1	600	5.5	4.63
Saccharomyces globosus BKM y-438	YNBYEglu2	0.6	190	3.2	1.47
	YPG	1.3	330	2.5	2.54
Pichia subpelli-culosa NCYC 16	YNBglu2	0.5	130	2.6	1.00
	YPG	0.9	220	2.4	1.70
Hansenula anomala NCYC 435	YNBglu2	0.4	105	2.6	0.81
	YPG	0.8	160	2.0	1.23
Hanseniaspora valbyensis 13cs/6ρ$^-$	B_m	0.3	80	2.7	0.62
	MRS	1.2	160	1.3	1.23

Table 2. Contents and activity of separated killer proteins.

Nr. fraction	Absorbtion (at 280 nm)	Protein (μg/ml)	Killer activity (Units/ml)
Elution:			
5	0.223		0
6	0.159		0
7	0.140		0
8	0.292		±
9	**0.722**	**235**	**52**
10	**0.629**	**206**	**69**
11	0.481		52
12	0.421		±
13	0.330		±
14	**0.330**	**102**	**21**

Fig. 2a. Molecular weight of killer proteins, purified by IEX chromatography

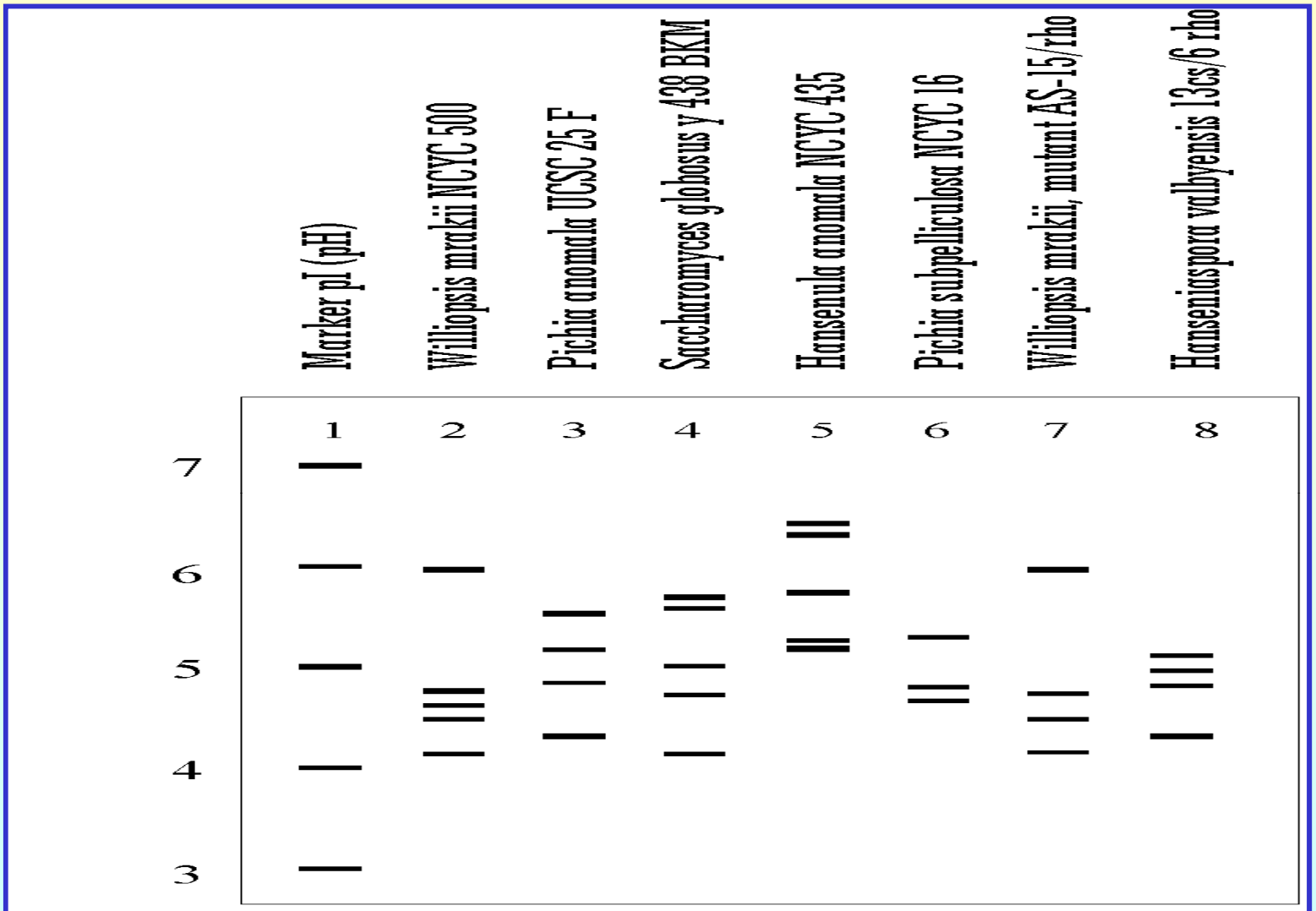
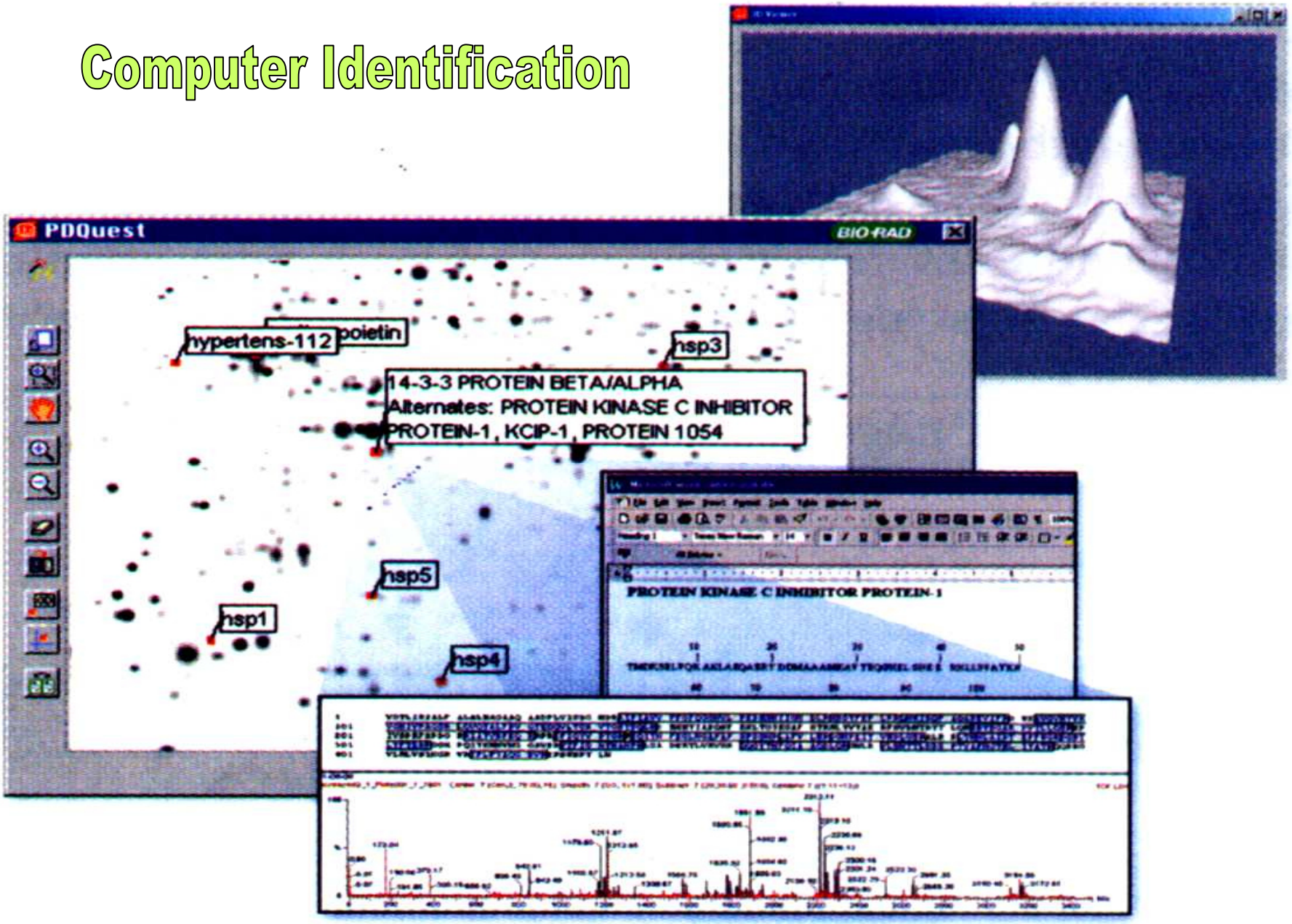
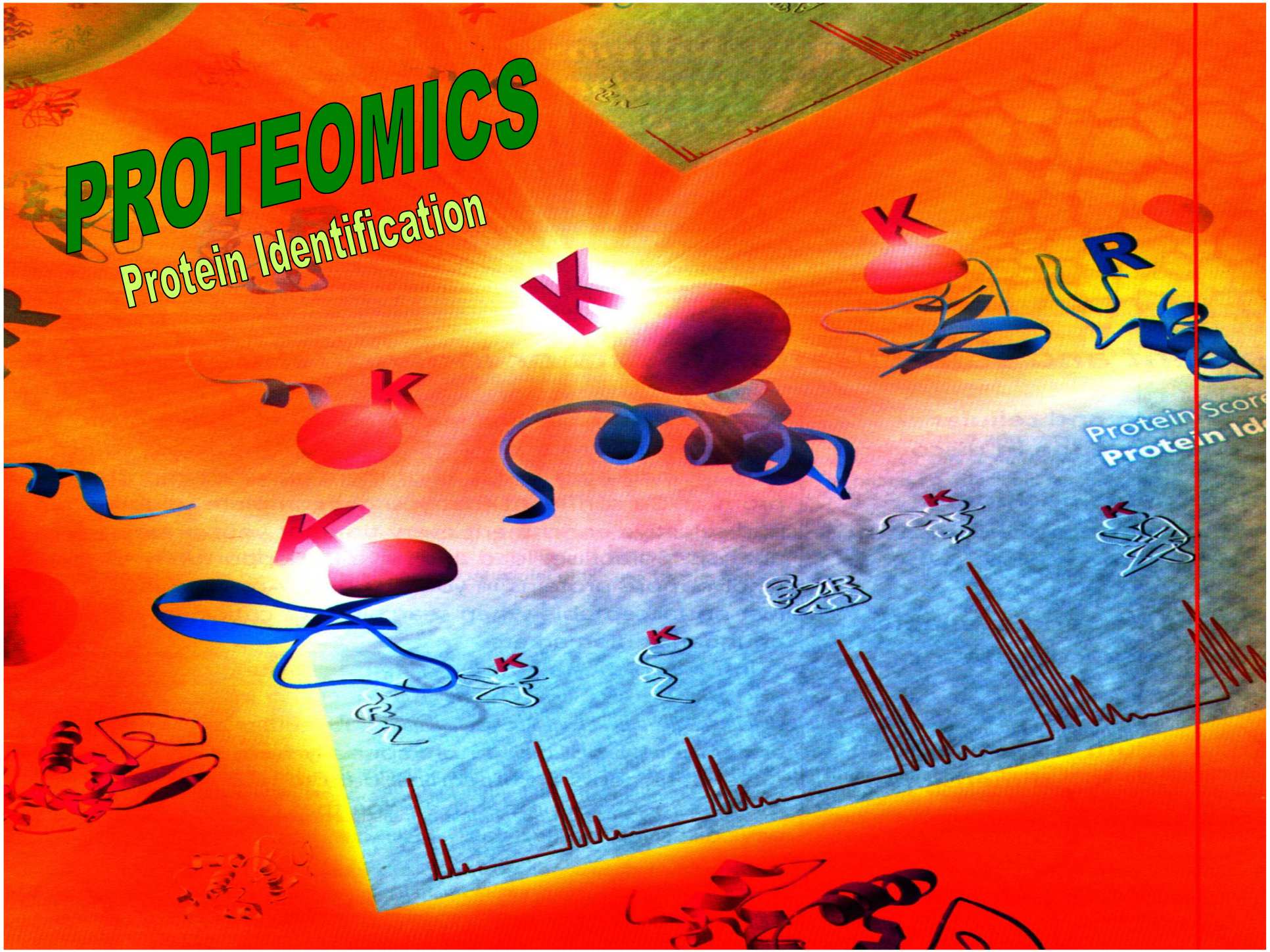


Fig. 2b. pI (isoelectric point) of killer proteins separated by gel filtration chromatography (GF)

2D-Gel

Computer Identification




PROTEOMICS
Protein Identification
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Summary

Many yeasts secrete proteins which are toxic for pathogenic and non-pathogenic microorganisms. These toxins, mostly glycoproteins, consist of membrane-binding subunits which interact with carbohydrate (e.g. 1,6-β-D-glucan or α-mannan) on the cell wall of sensitive strains. The killing effect is presented by membrane permeation, cell lysis or inhibition of the cell cycle.

It is also suggested that these killer glycoproteins, similar in structure to lectins, can mediate self-adhesion of the pathogenic microorganisms, so stimulating their excretion from the intestines of infected mammals. It is supposed that above interactions could be important for therapeutic application, especially to enteric diseases.

IV. INFLUENCE OF YEAST KILLER TOXINS ON THE CYTOTOXICITY OF SHIGA-LIKE TOXINS

1. Effect of killer toxin on mammalian cells

As we told before, biotechnology is turning to the natural product to find new biotherapeutic agents, active against pathogenic bacteria or yeasts.

A prophylactic and therapeutic antimicrobial strategy, based on a specific physiological target, has become effective (seems to be) due to the use of killer yeasts directed against their natural competition.

The killer yeasts produce toxins, which are mostly glycoproteins and similar in structure to lectins. They act against sensitive strains of the same or closely related species as well as against unrelated microorganisms, including pathogenic yeasts or bacteria such enterohemorrhagic *Escherichia coli* (EHEC), producing Shiga-Like-Toxins (SLT).

The activity of yeast killer toxins are lethal due to the presence of specific cell wall receptors. Therefore, they have been proposed as a potentially useful biotherapeutic agents for improvement of the human or animals health as well as for environmental control.

The toxic effect of killer protein can not be comprehensively utilised without a very specific study. Such studies aim at characterising:

- **an influence of yeast killer proteins on standard mammalian cells (examination of cell viability and cytotoxicity),**
- **receptors (ligands) from pathogens or which bind the killer glycoprotein.**

It was found that killer toxins from the yeasts *Williopsis mrakii* NCYC 500, *Pichia anomala* UCSC 25F, *Pichia subpelliculosa* NCYC 16 and *Hanseniaspora valbyensis* 13cs/ρ^- do not have inhibitory effects on mammalian cells (Fig.1-10).

However, some emphatic inhibition of mammalian cells activity (except human liver cells, Hep-2 and monkey kidney cells, Vero-79) has been observed when 100-times concentrated killer toxin from *Saccharomyces globosus* BKM y 438 was used.

Fig. 1. Toxicity of different killer toxin for EBL´ cells.

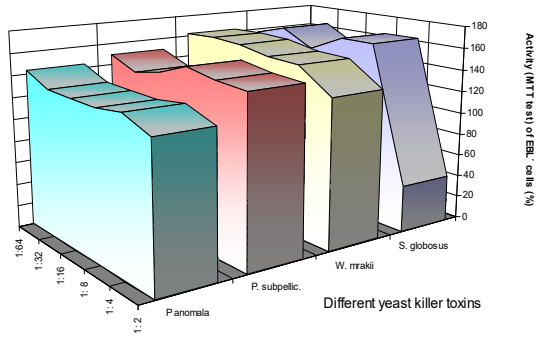


Fig. 2. Toxicity of different killer toxins for HeLa cells.

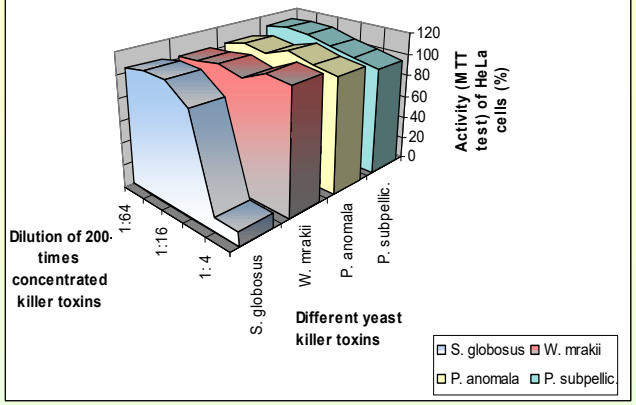


Fig. 3. Toxicity of different killer toxins for PK-15 cells.

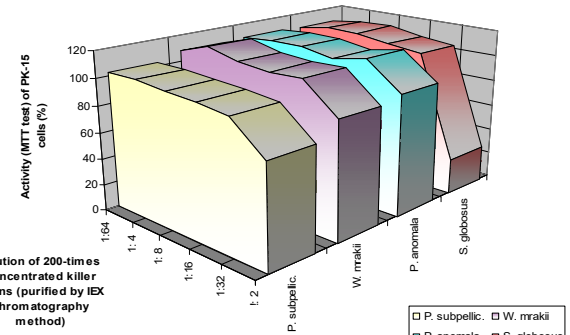


Fig. 4. Toxicity of different killer toxins for HEP-2 cells.

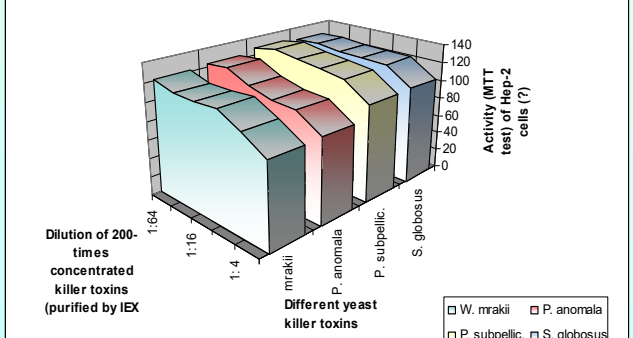


Fig. 5. Toxicity of different killer toxins for V-79 cells.

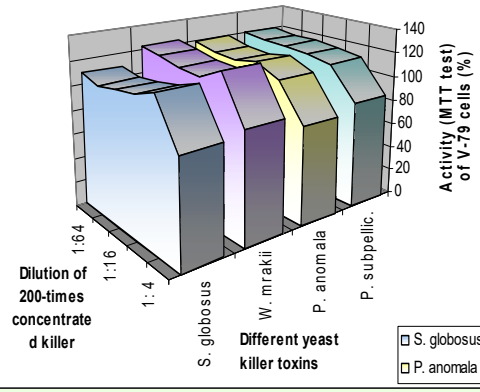


Fig. 6. Toxicity of different killer toxins for VERO cells.

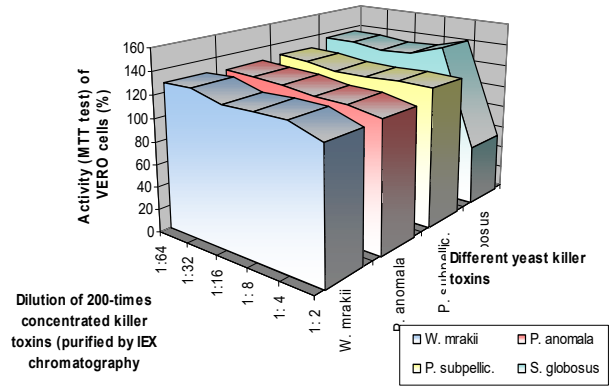


Fig. 7. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for HEP-2 cells.

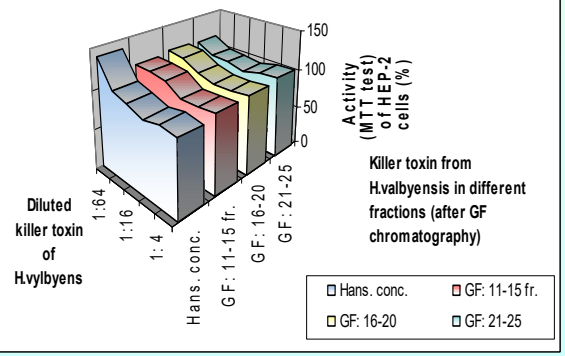


Fig. 8. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for VERO cells.

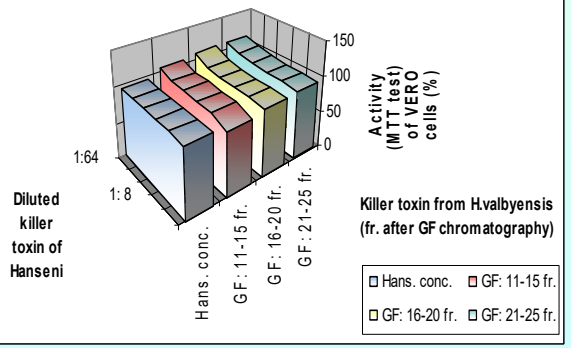


Fig. 9. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for PK-15 cells.

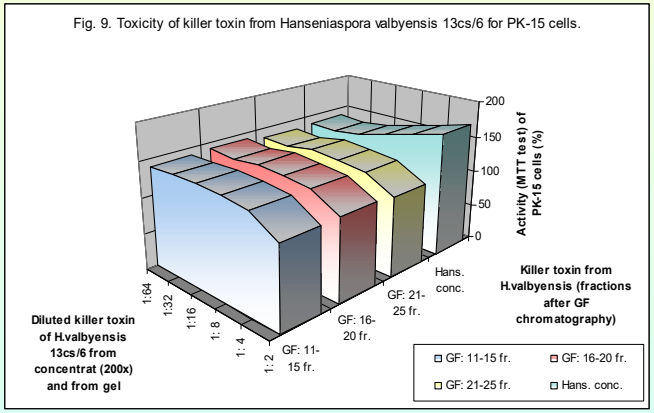
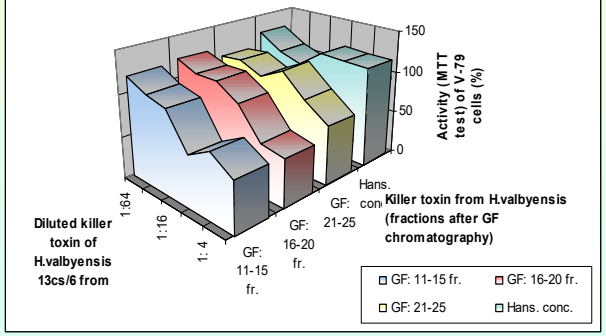


Fig. 10. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for V-79 cells.



Finally, we concluded, that killer toxins, which are used for examinations, display a lethal effect to a wide spectrum of pathogenic microorganisms. From preliminary experiments is clear that the results with 5 different yeast killer proteins, presented on Fig. 1 - 10, did not show emphatic cytotoxicity (especially toxin from *Williopsis mrakii*) or any adverse effect in any mammalian and embryo-cells.

In other words they are likely to be harmless to animals and humans tissues cells. Therefore, they could be used for explain pretherapeutic effect on animal cells in the case of EHEC infections.

2. Killer protein binding to Shiga-Like-Toxin receptors

The allocation of mammalian cell receptors, specific for different drug-proteins and hormones as well as other transmitters, into groups or types, is sufficient known (but not for killer toxins). The need to know how killer toxins work, both in physiology and in pathology, has driven the process of investigating receptors through which they act.

This is not only academic interest but also has practical consequences.

Since pharmacology has been for a long time, and still is, concerned with description of drug effects on living systems (usually in relationship to physiology or pathophysiological consequences), our interest, important for therapeutic reasons, was to know effects of eventual competitions between two different toxins, SLT and killer toxin, about binding of receptors, for instance, on standard mammalian cells, HeLa, or in some organs, i.e. kidney (e.g. Vero-cells, e.g. PK-15- and V-79), liver (Hep 2) as well as lung (EBL – embryo cells).

As a primary receptors blocker (antagonist) was used yeast killer toxin from *Williopsis mrakii* AS/15ρ^-, which is glycoprotein (like lectin with oligosaccharide sequences).

Shiga-Like-Toxins from *E. coli* DSM 2403 and *E. coli* DSM 2430 were tested for eventual secondary binding of cell receptors.

Fig. 11 shows that obtained Shiga-Like-Toxins in this case were very active and toxic for HeLa cells. All results are presented in Fig. 12, 13, 14 and 15.

In these figures we can see a distinct effect of yeast killer toxin from *Williopsis mrakii* and *Saccharomyces glubosus*, which appears to be protection of mammalian cells against the cytotoxicity of Shiga-Like-Toxins.

A some dilution of yeast killer toxin from *Williopsis mrakii* reduces slowly protective effect for HeLa cells (Fig. 12), however, at lower concentration of killer protein (17 μg/ml, i.e. 5 Units killer activity) that effect is still observed.

Fig. 11. Toxicity of **Shiga-Like-Toxins** from different strains of *Escherichia coli* and yeast killer toxin (fr. 1-10/GF) from *Williopsis mrakii* AS/15.

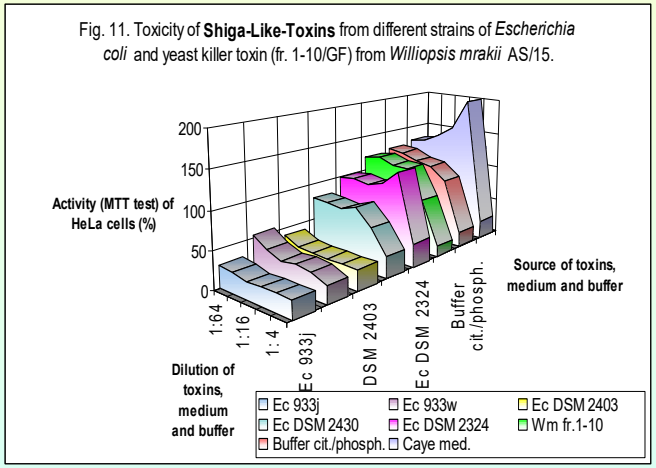
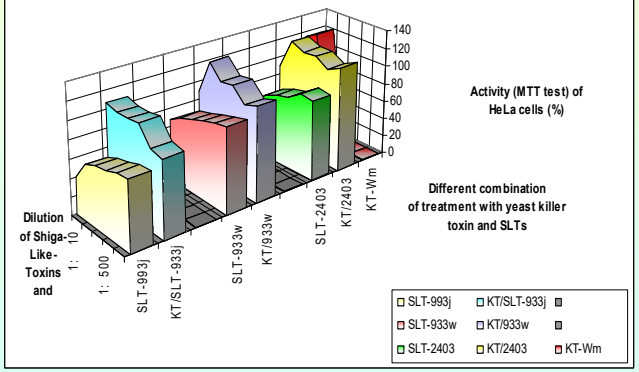


Fig. 12. Receptors binding effect. Pretreatment of **HeLa** cells by yeast killer toxin from *Williopsis mrakii*.



A some dilution of yeast killer toxin from *Williopsis mrakii* reduces slowly protective effect for HeLa cells (Fig. 12), however, at lower concentration of killer protein (17 μg/ml, i.e. 5 Units killer activity) that effect is still observed.

Fig. 13. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Williopsis mrakii* .

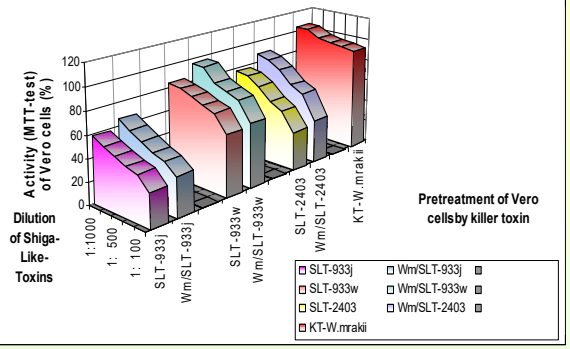


Fig. 14. Receptors binding effect. Pretreatment of **HeLa** cells by killer toxin from *Saccharomyces globosus* .

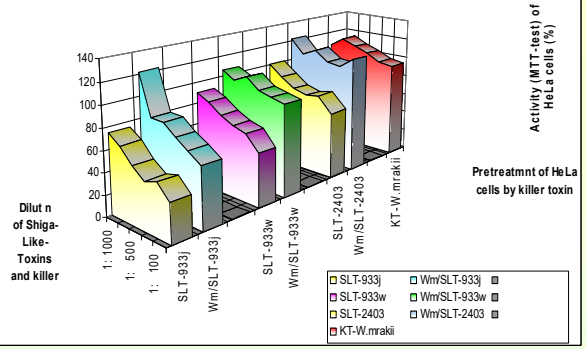
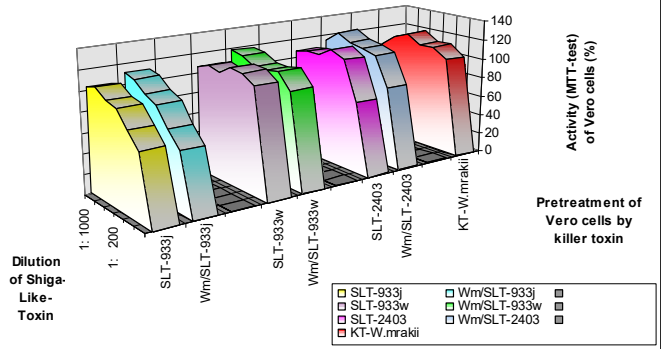


Fig. 15. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Saccharomyces globosus* .



Yeast killer proteins form *Williopsis mrakii* and *Saccharomyces glubosus* play evident protective role for mammalian cells, especially for HeLa cells, which are most sensitive in the case of intoxication by Shiga-Like-Toxins (Fig. 12 and 14). Vero cells are more resistant, therefore, the protective effect is not so big (Fig. 13 and 15).

Therefore, we concluded, it was found that yeast killer toxin from *Williopsis mrakii* can protect mammalian cells such HeLa and Vero cells against challenge by Shiga-Like-Toxins (derived from cultures of pathogenic strains of *Escherichia coli*, called EHEC), probably by occupation of intestinal receptors during competitions with infected microorganisms.

The final activities of tested mammalian cells are better when they are pre-treated by killer protein, i.e. before challenge with Shiga-Like-Toxins.

It appears that this prophylactic effect could be very interesting for veterinary what has been proof, by orally treatment a big population (about 2000) of healthy and ill (with diarrhoea) pigs (manuscript – confidential data).

Generally, we can concluded that yeast killer strains are probiotic, i.e. could eliminate fecal shedding of EHEC strains in pigs when animals prior are treated with developed yeast toxins.

Treatment with yeast killer toxin in pigs following challenge of EHEC can reduce the fecal shedding of SLT when compared with the control animals.

Summary for Part IV

It is evident that the results of preliminary experiments with 5 different yeast killer proteins did not show emphatic cytotoxicity or any adverse effect in any mammalian and embryo-cells. Moreover, they are likely to be harmless to animals and humans tissues cells. Therefore, could be used for explain pre-therapeutic effect on mammalian cells (mostly animals) in the case of infections by strains *Escherichia coli*, called EHEC.

It was found that yeast killer toxin from *Williopsis mrakii* can protect mammalian cells such HeLa and Vero cells against challenge by Shiga-Like-Toxins (derived from cultures of pathogenic strains of *Escherichia coli).* The final activities of tested mammalian cells are better when they are pre-treated by killer protein, i.e. before challenge with Shiga-Like-Toxins. It appears that this prophylactic effect could be very interesting for veterinary what has been proved on a big population (about 2000) of healthy and ill (with diarrhoea, i.e. haemorrhagic colitis) pigs (manuscript – confidential data).

Fig. 1. Toxicity of different killer toxin for EBL´ cells.

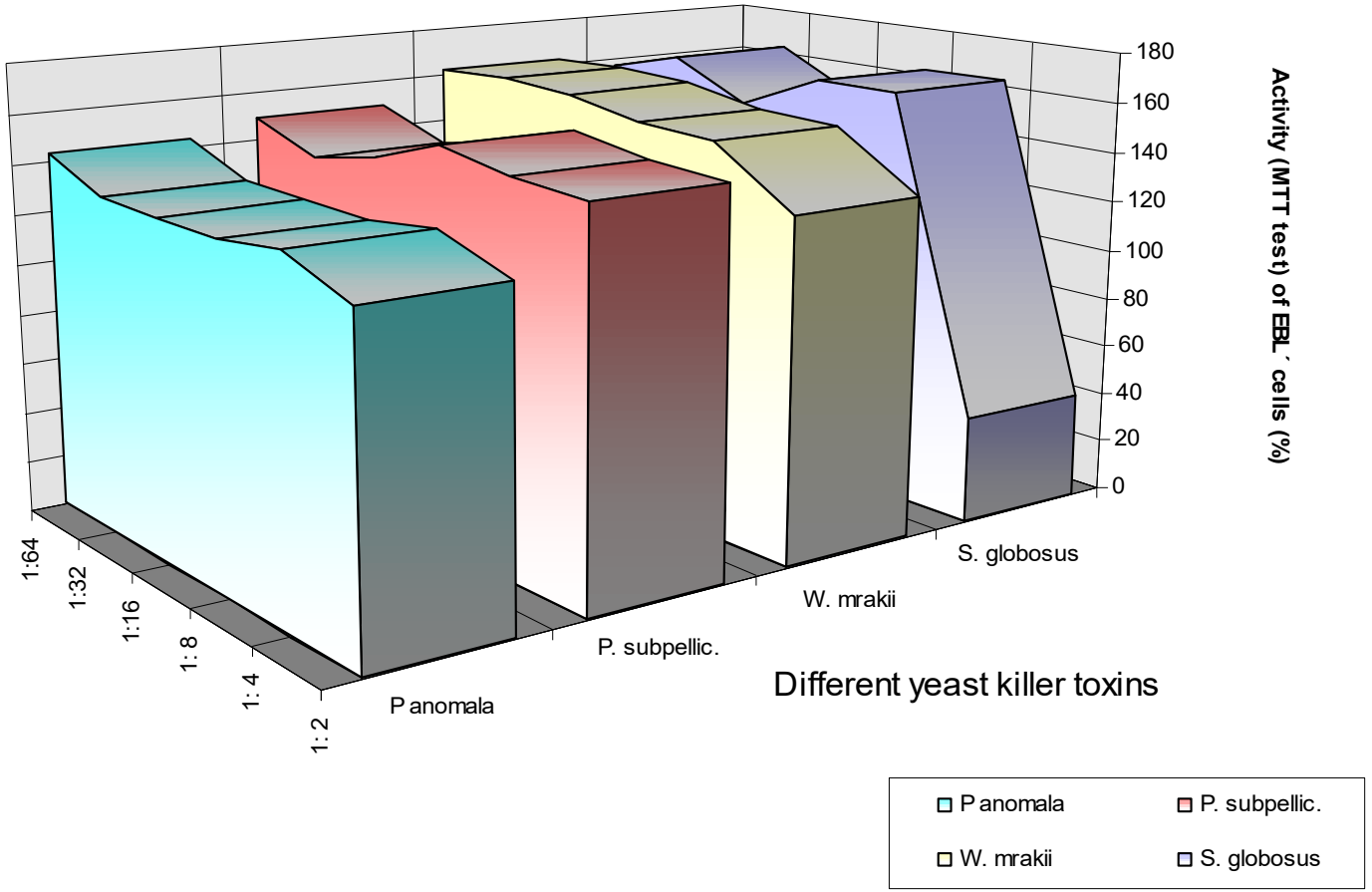


Fig. 2. Toxicity of different killer toxins for HeLa cells.

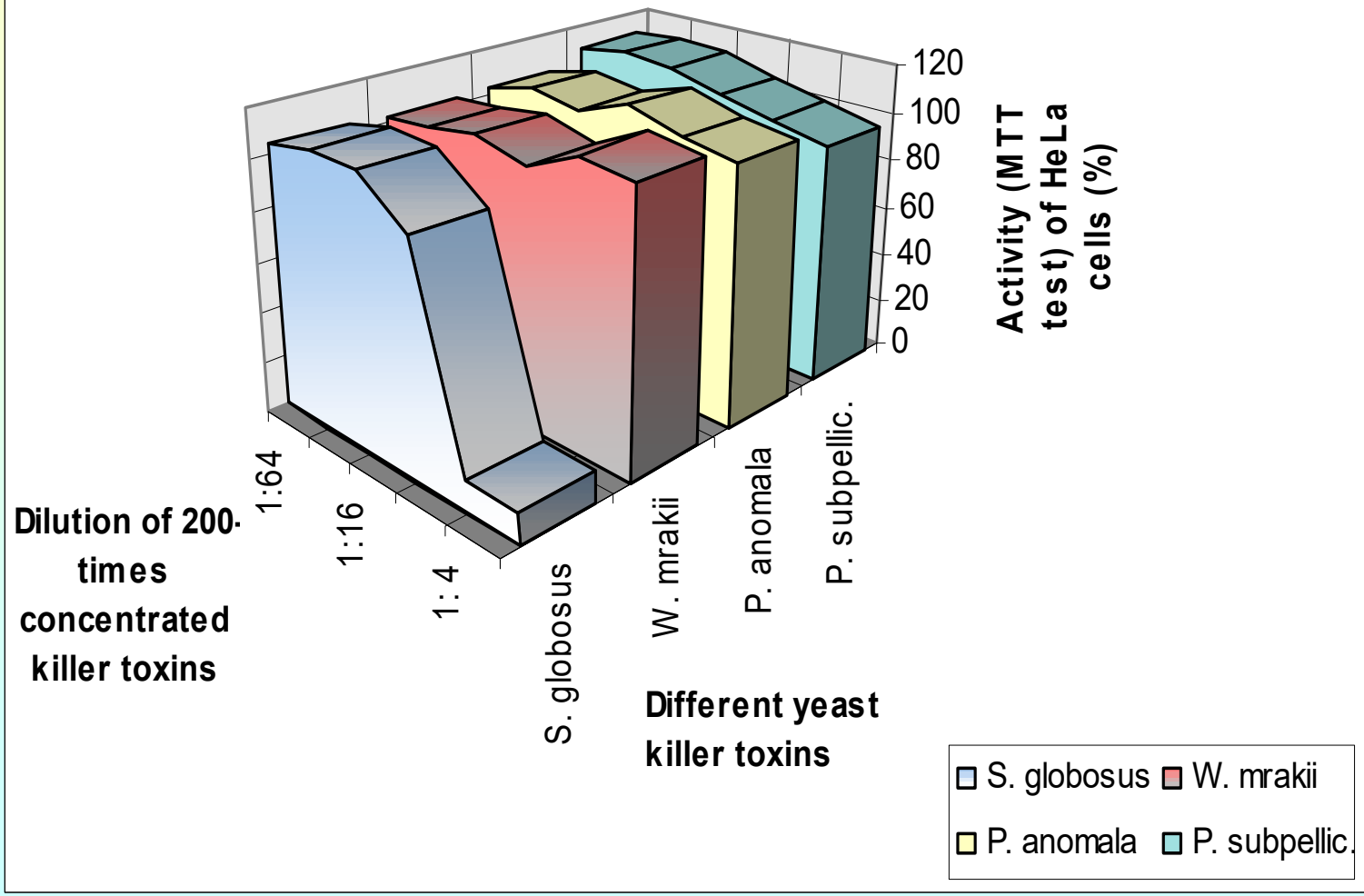


Fig. 3. Toxicity of different killer toxins for PK-15 cells.

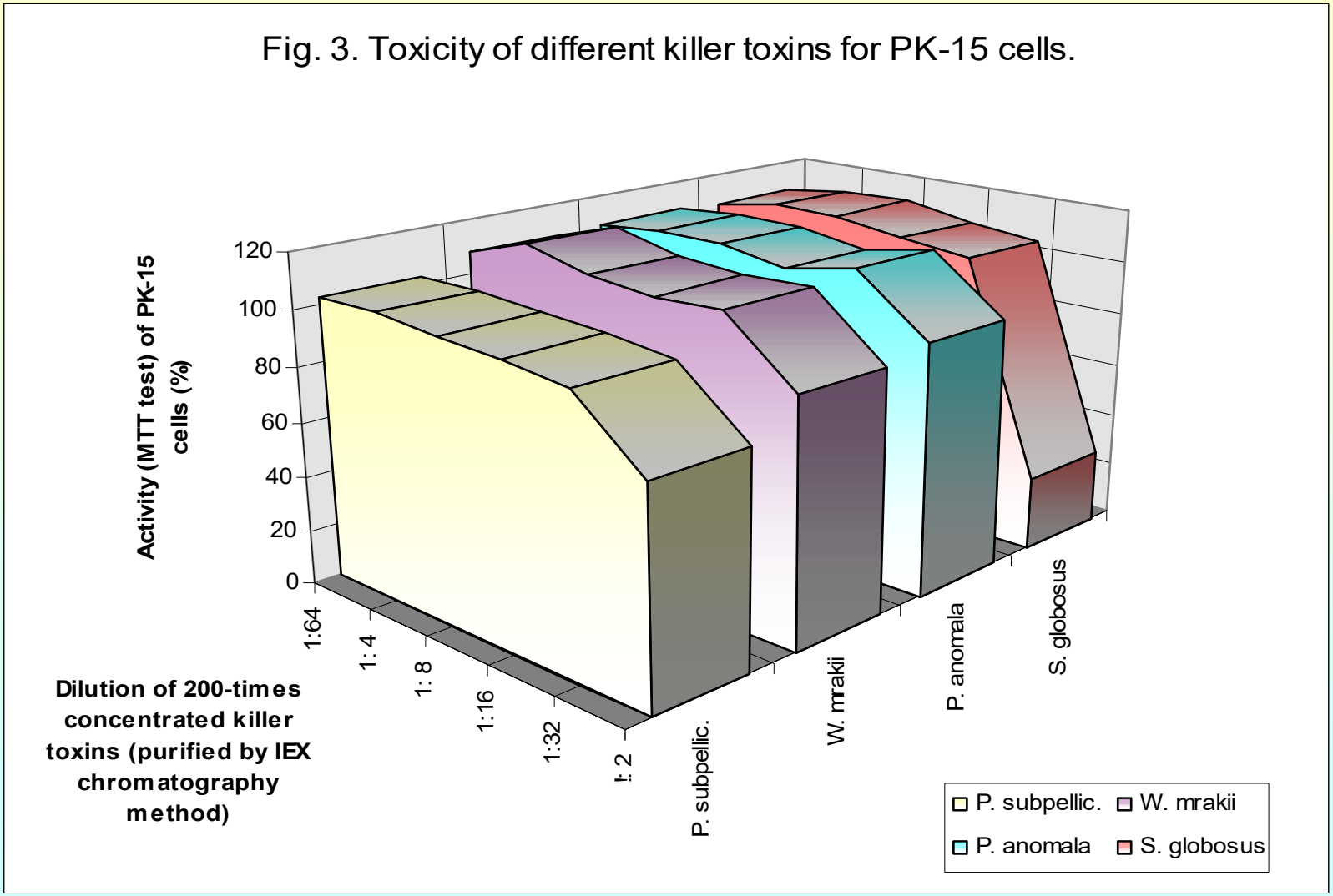


Fig. 4. Toxicity of different killer toxins for HEP-2 cells.

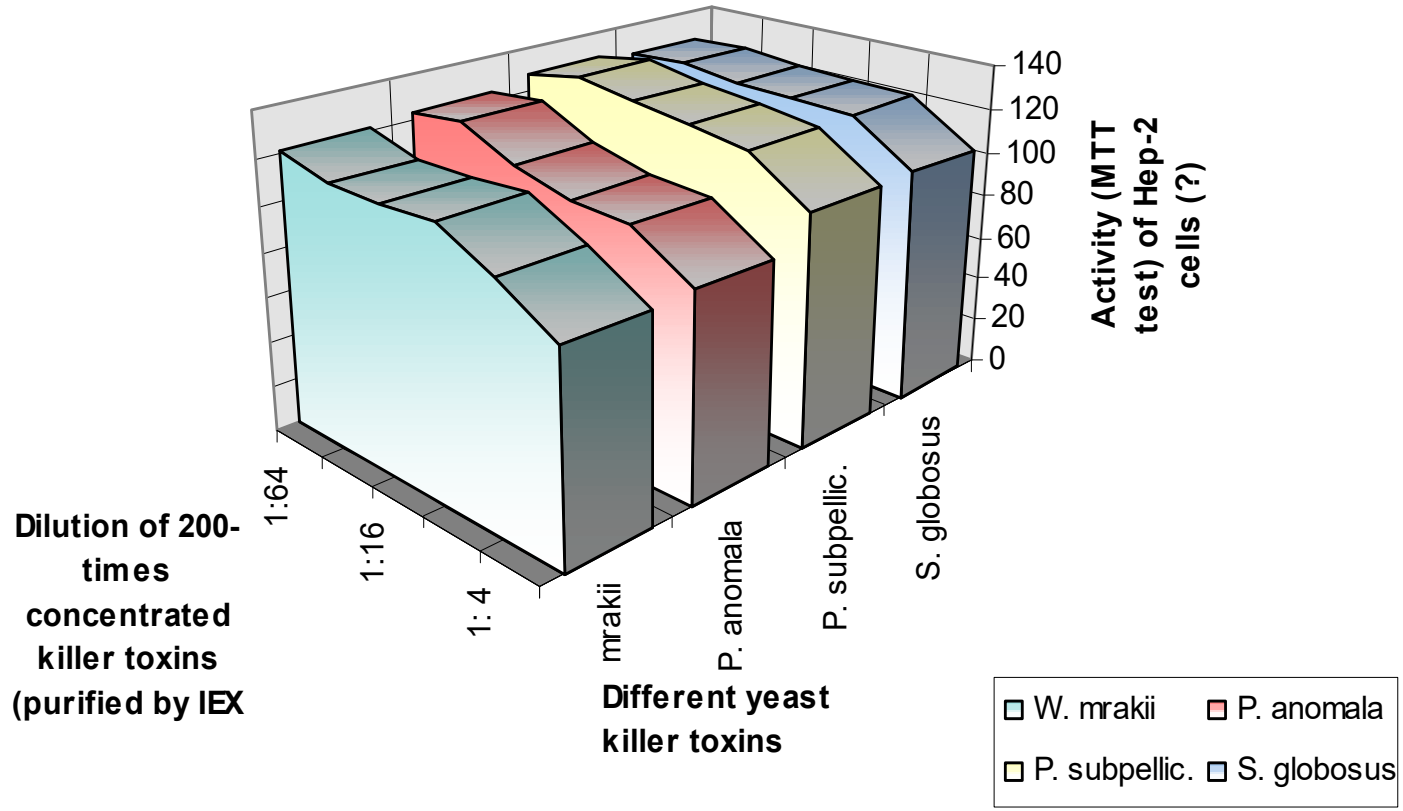


Fig. 5. Toxicity of different killer toxins for V-79 cells.

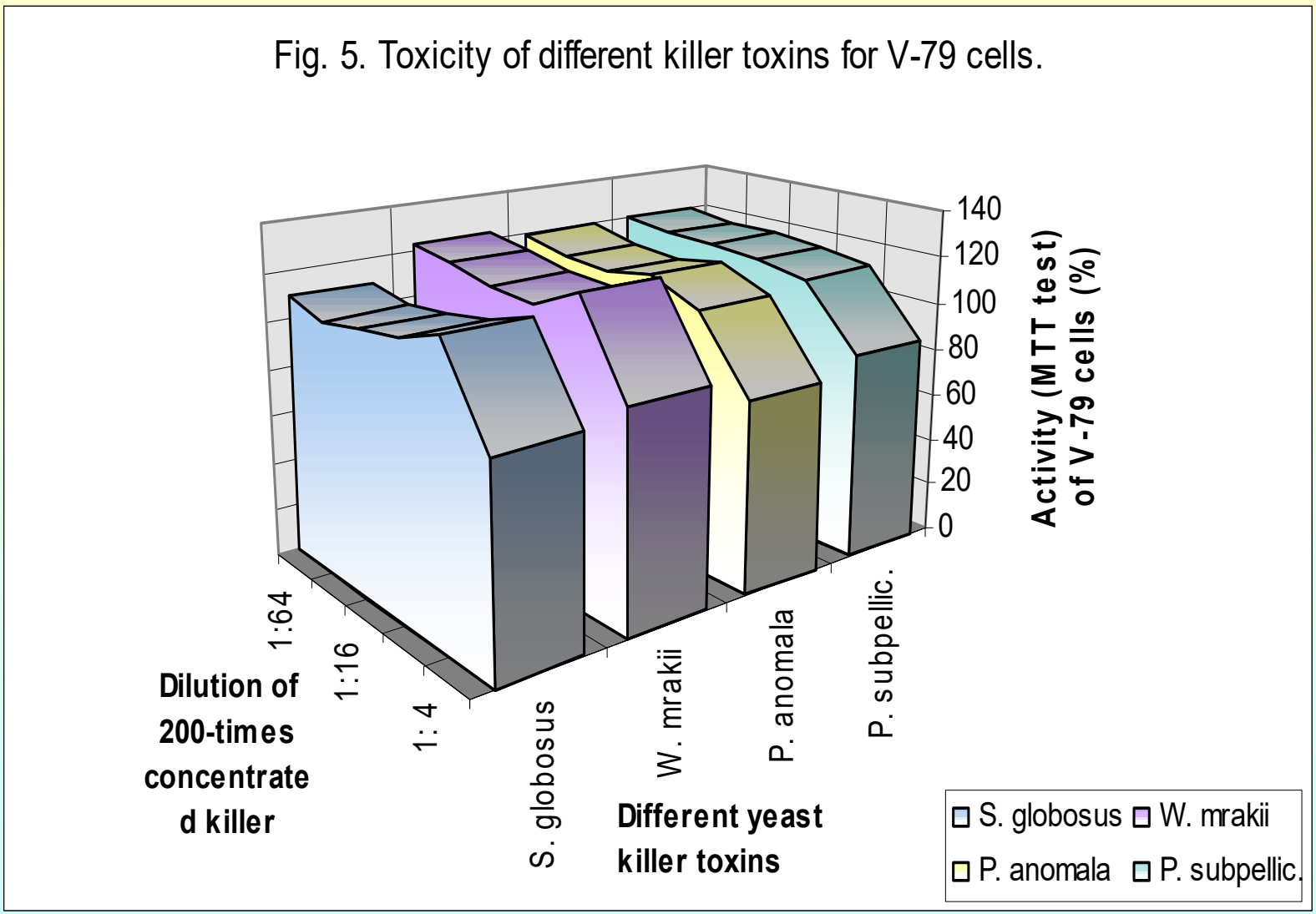


Fig. 6. Toxicity of different killer toxins for VERO cells.

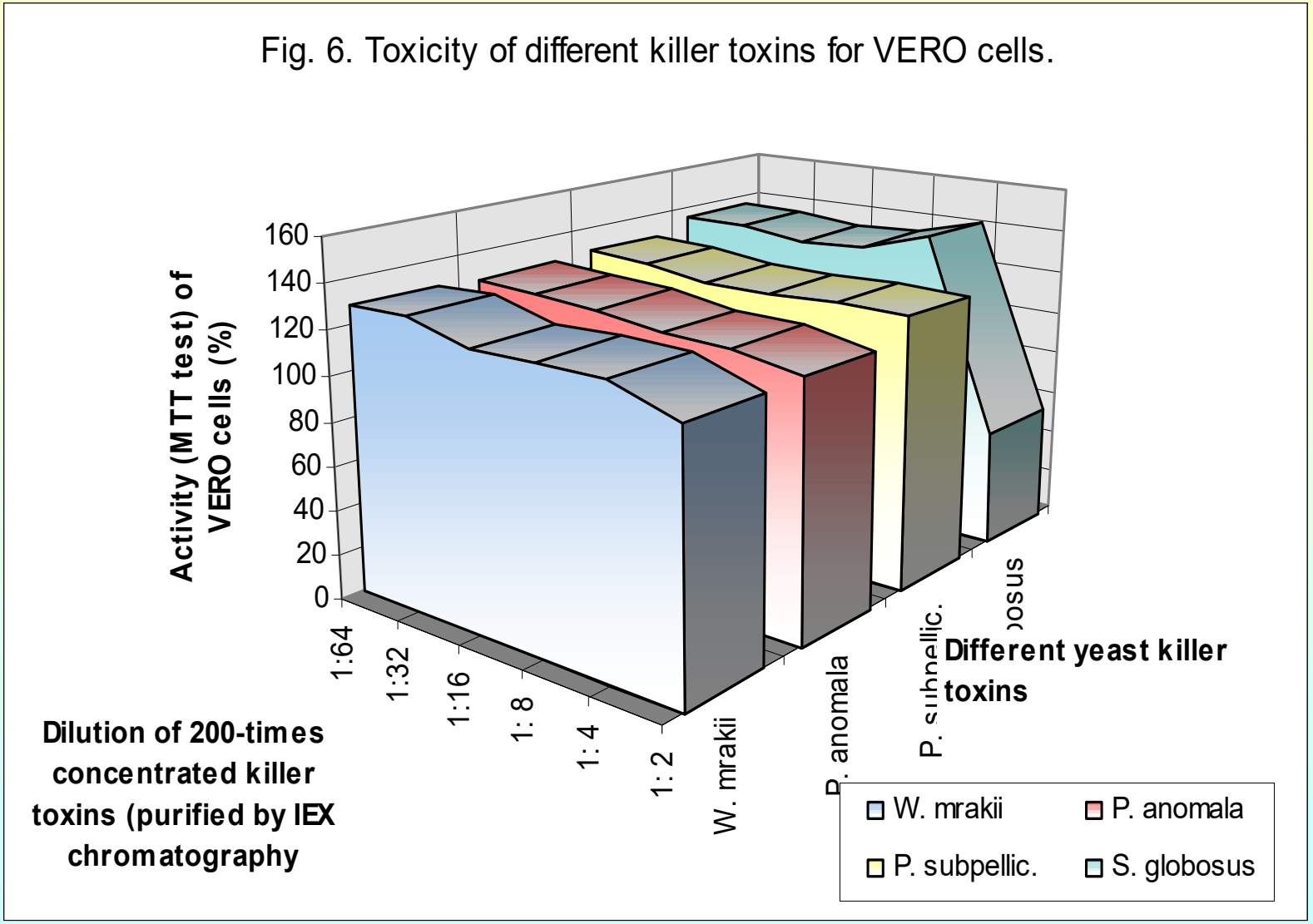


Fig. 7. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for HEP-2 cells.

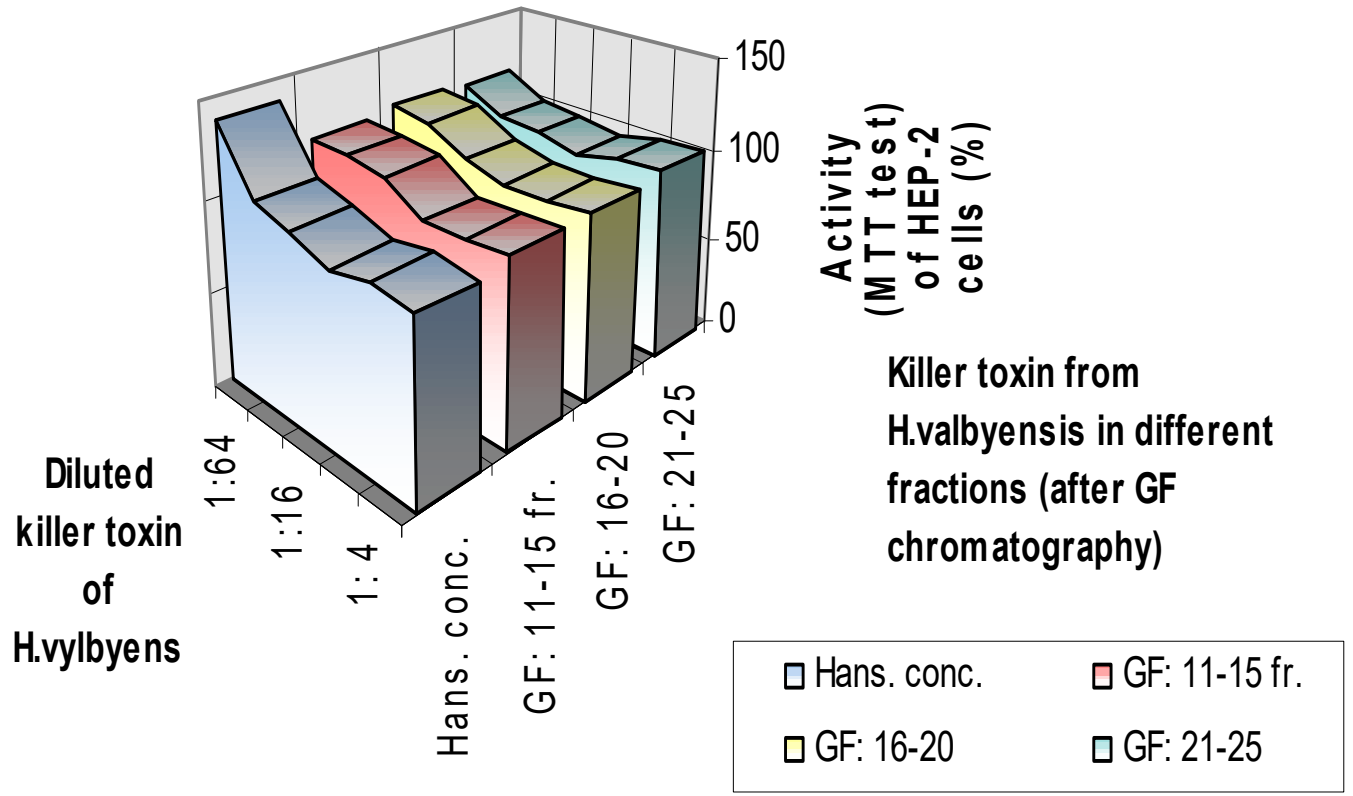


Fig. 8. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for VERO cells.

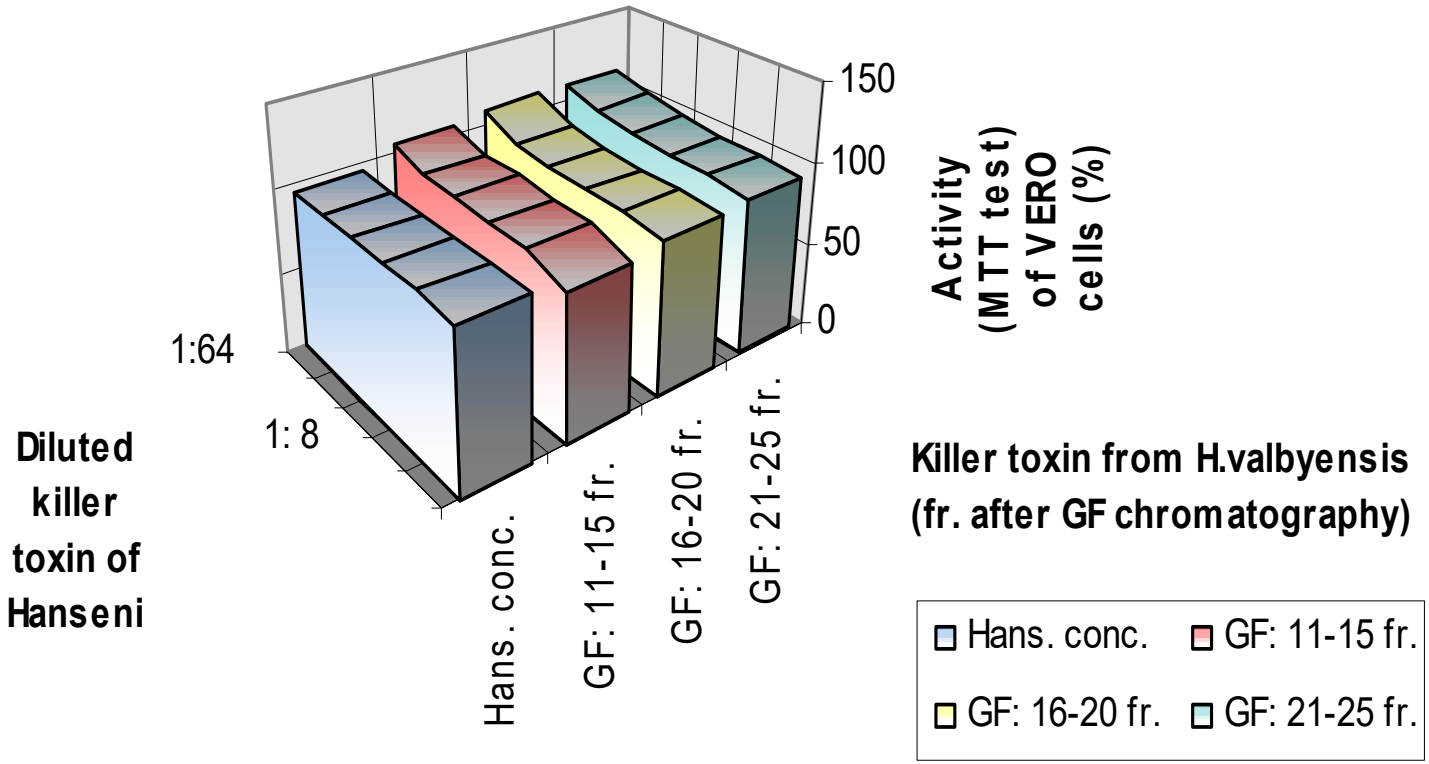


Fig. 9. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for PK-15 cells.

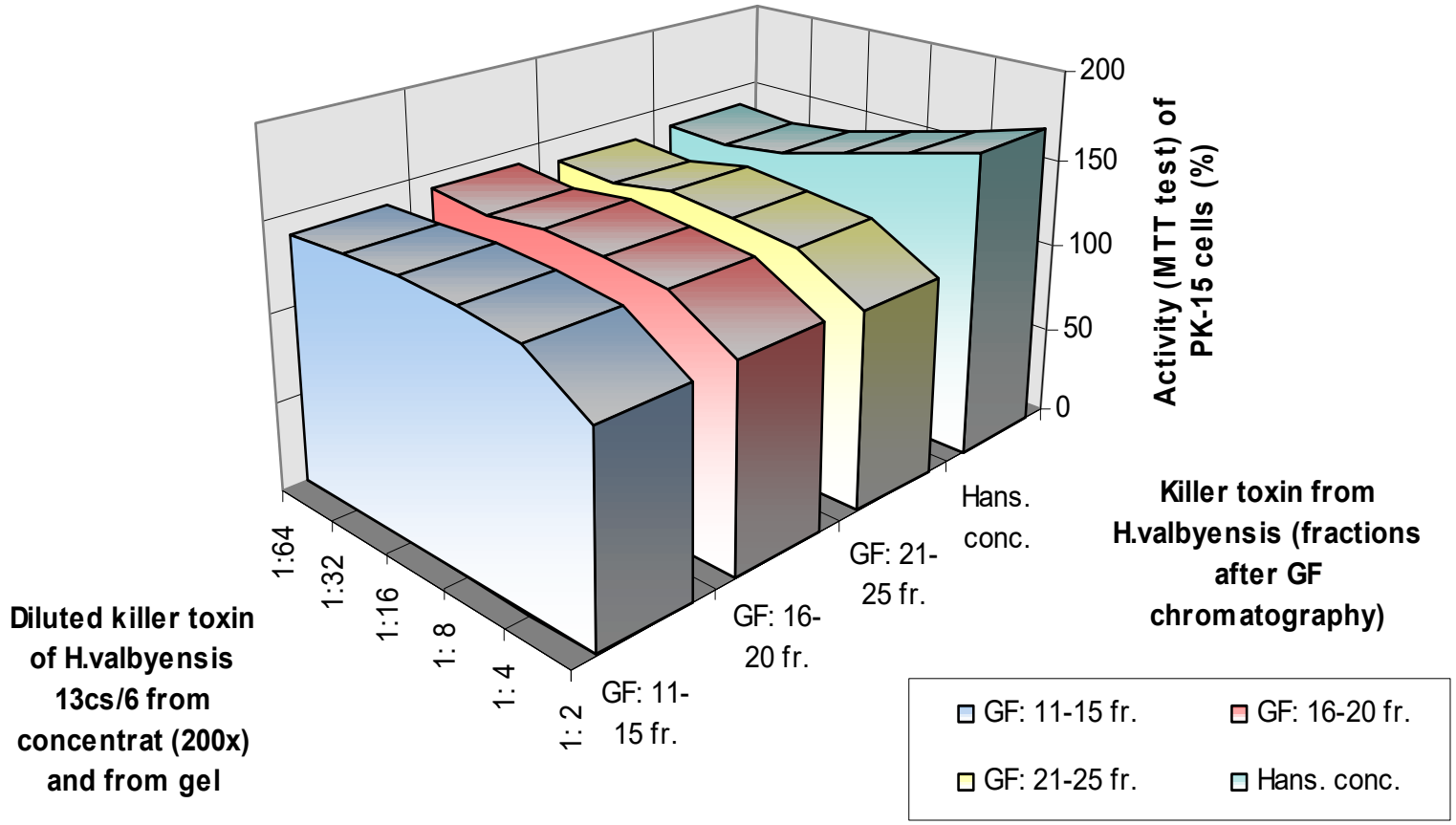


Fig. 10. Toxicity of killer toxin from Hanseniaspora valbyensis 13cs/6 for V-79 cells.

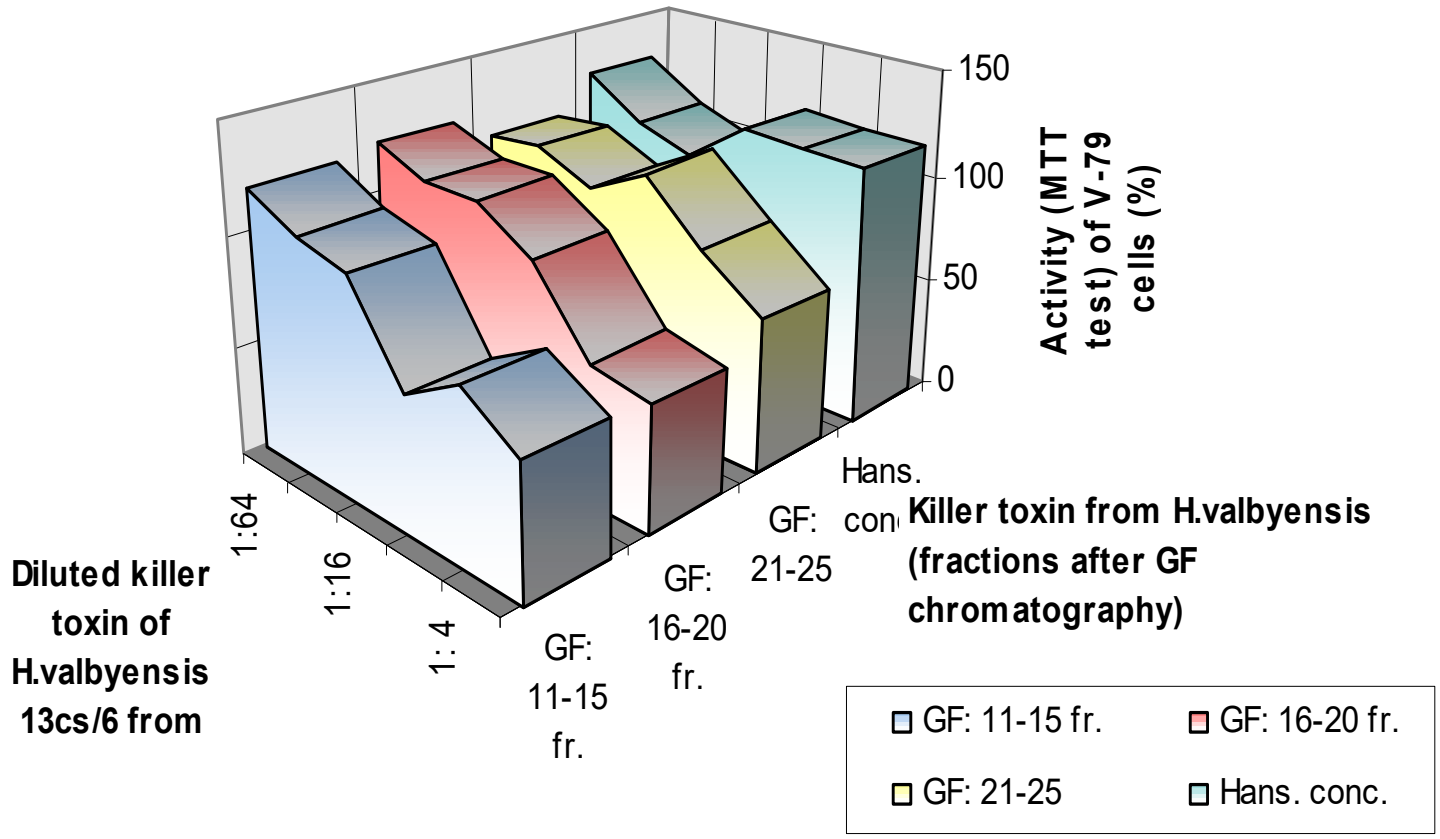


Fig. 11. Toxicity of **Shiga-Like-Toxins** from different strains of *Escherichia coli* and yeast killer toxin (fr. 1-10/GF) from *Williopsis mrakii* AS/15.

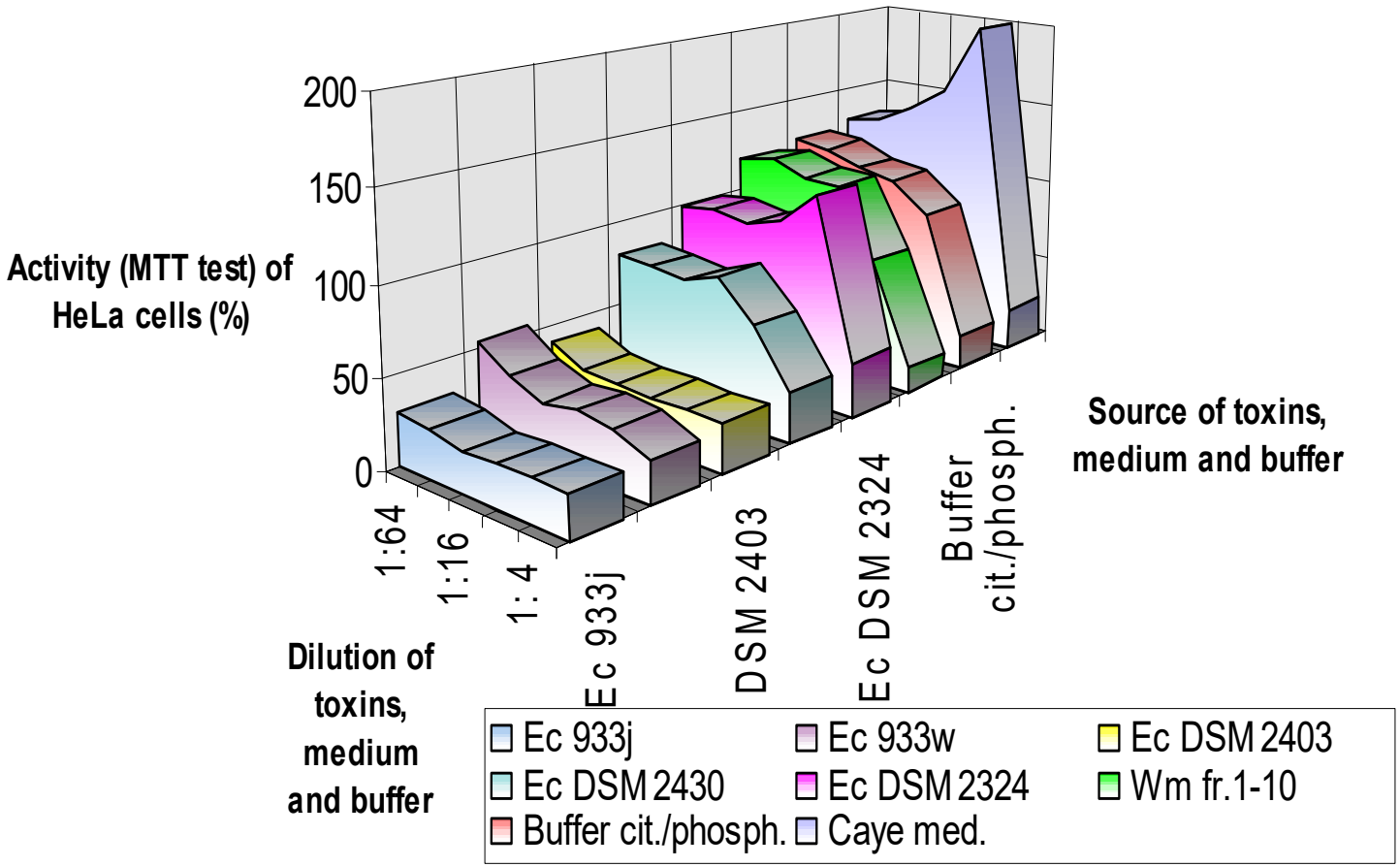


Fig. 12. Receptors binding effect. Pretreatment of **HeLa** cells by yeast killer toxin from *Williopsis mrakii.*

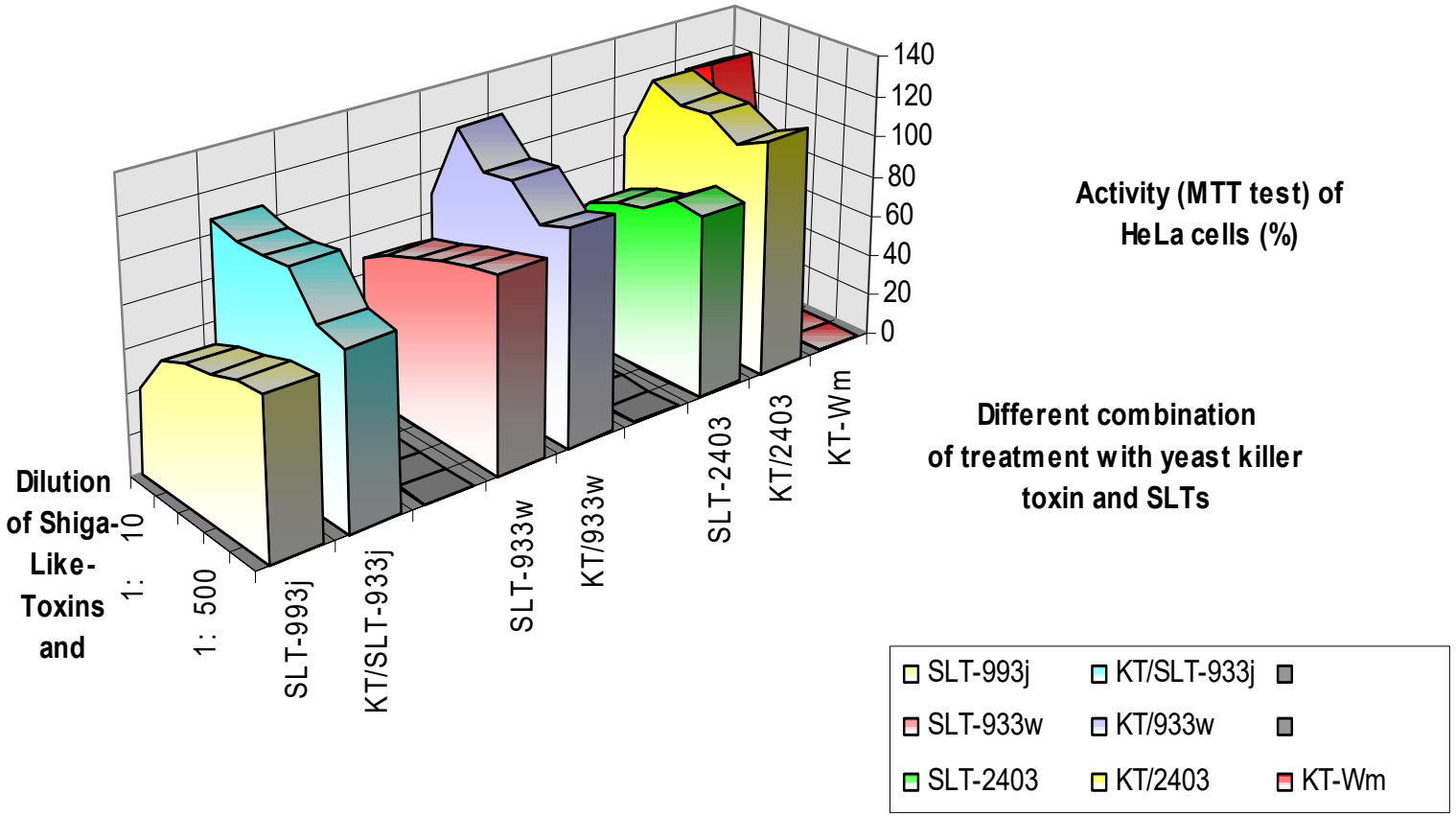


Fig. 13. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Williopsis mrakii* .

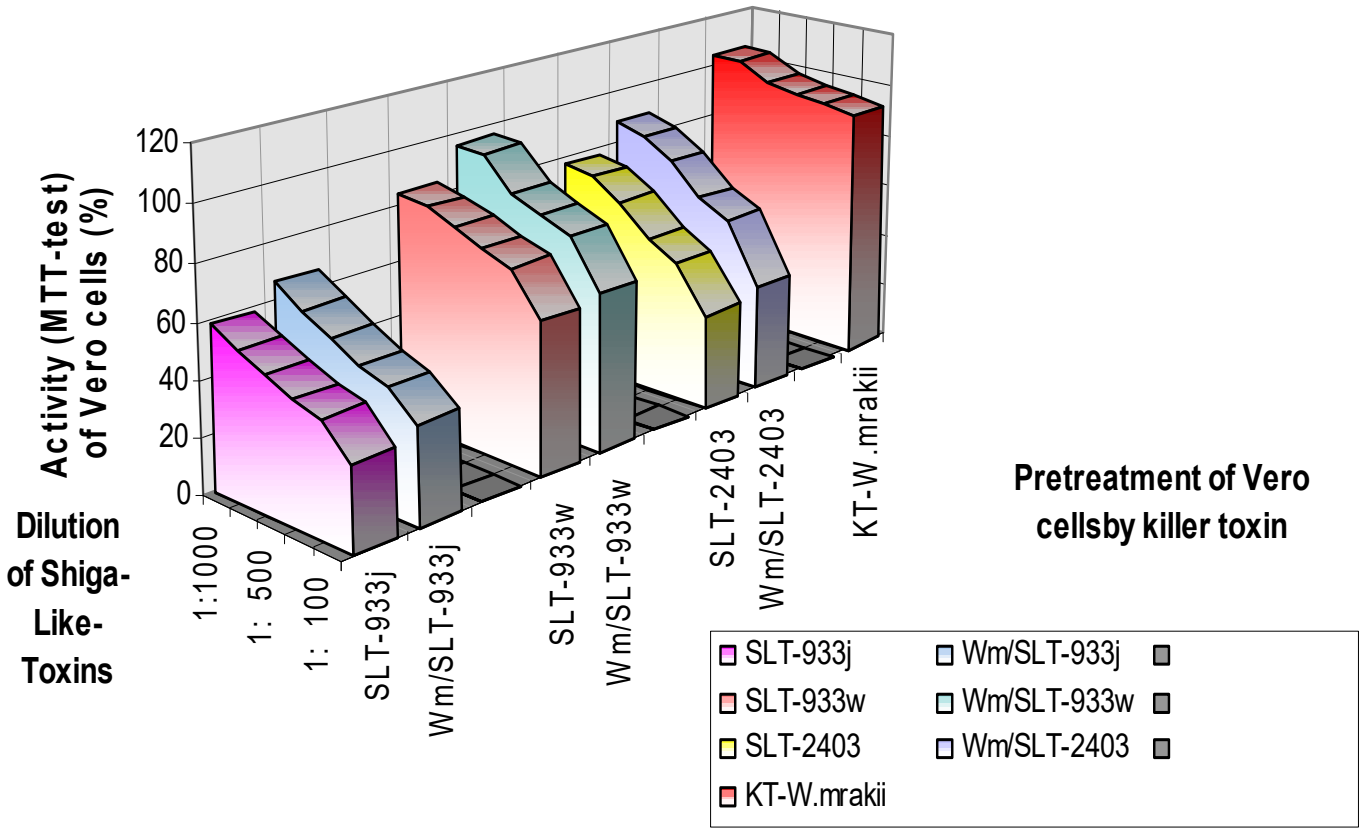


Fig. 14. Receptors binding effect. Pretreatment of **HeLa** cells by killer toxin from *Saccharomyces globosus* .

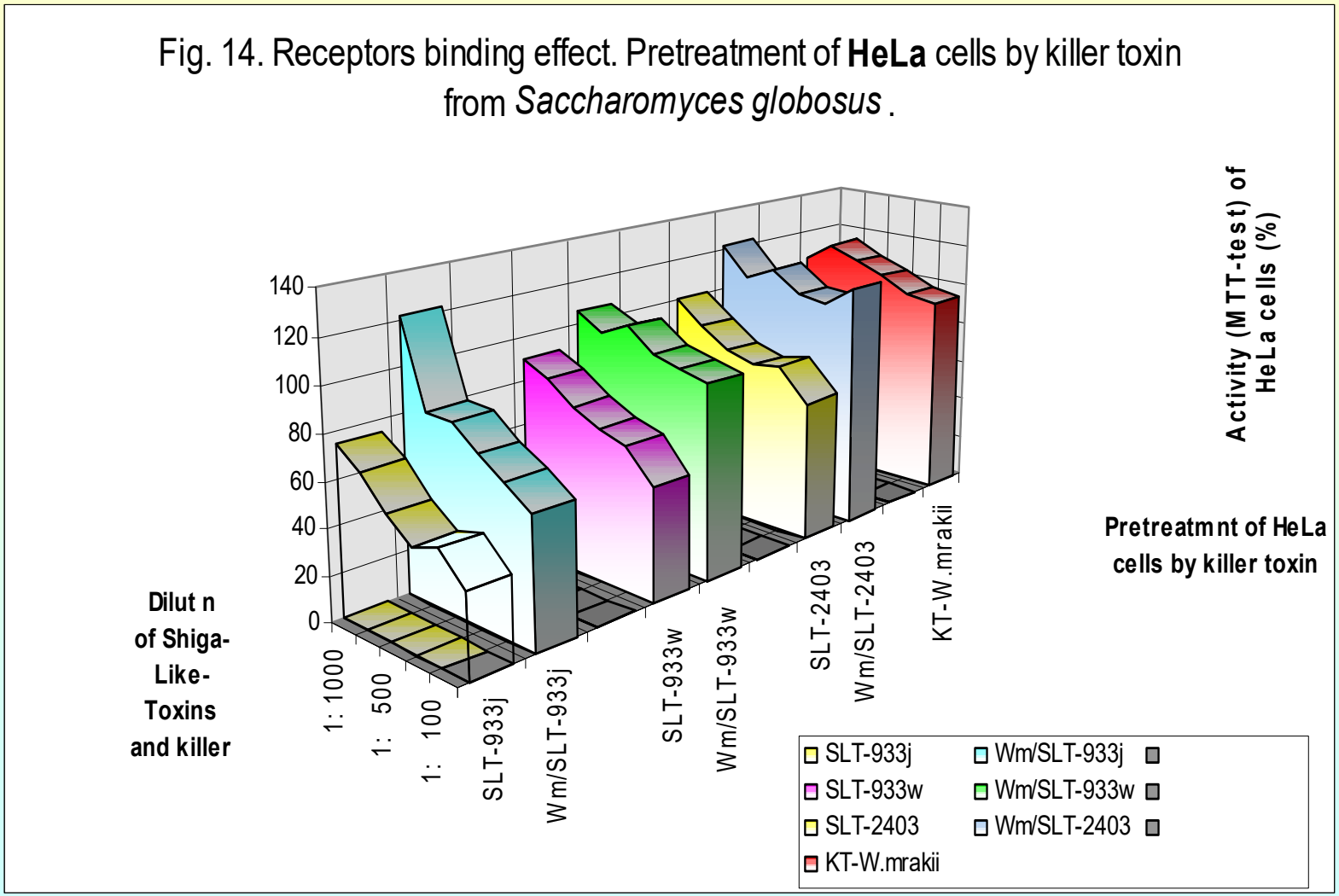


Fig. 15. Receptors binding effect. Pretreatment of **Vero** cells by killer toxin from *Saccharomyces globosus* .