

IV. MICROORGANISMS AND ENVIRONMENT

RESISTANCE OF YEAST AND ACTINOMYCETE POPULATIONS AT CULTIVATION IN A MEDIUM WITH INSECTICIDES

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Abstract: Soil microflora, as a component of biogeocenoses is experiencing different impacts by pesticides. Acting on the individual microbial species they affect and modify the ecosystems as a whole. On the other hand, many microorganisms can perform transformation and mineralization of pesticides. The aim of the study is to establish the nature of the interaction and sustainability of the model yeast and actinomycete populations with insecticide λ -cyhalothrin in laboratory conditions, as well as their ability to mineralize it. It has been found that a concentration of 0, 02% insecticide enhances the efficiency of utilization of the carbon substrate by the yeast population and inhibited actinomycetes at all concentrations tested. The addition of an insecticide in the medium decreases the activity of amylolytic enzymes and increases that of superoxide dismutase. The yeast strain *Hansenula anomala* var. Schneggii can use λ -cyhalothrin as a sole carbon source.

Keywords: pesticides, λ - cyhalothrin, yeast, actinomycete, growth, enzyme activity

INTRODUCTION

Global pollution of the biosphere with different chemicals is one of the problems giving rise to legitimate concerns about disruption of the ecological balance in ecosystems. Particular hazards are the synthetic compounds entering the environment as a result of the industrial production and human activity. Important role among them play the chemicals for plants and animals protection - pesticides [2, 15, 19].

The problem of the toxicity of pesticides on living organisms stands very strongly to contemporary science. In their application as means of protection, they have a biocidal effect on both the plants useful for life and on plant and soil microflora. The main danger lies in the fact that they may be included in food chains and reach higher animals and man, concentrating in each unit of these chains [6, 15]. Contamination of hydrosphere is carried out by groundwater, and hence it goes to drinking water.

Soil microflora, as a component of biogeocenoses is experiencing different impacts by pesticides. Certain reactions that inhibit or stimulate the growth of the communities [8, 12, 14] or some enzymatic reactions performed by them are registered [4, 14]. Acting on the individual microbial species, pesticides manipulate biodiversity in ecosystems, modifying them as a whole. Often such modifications lead to disturbance in the ecological balance. Many microorganisms can perform transformation and mineralization of pesticides [9, 16, 13, 18]. This capability is allowing their use for the detoxification of these xenobiotics.

In natural conditions between microbial populations, various types of interactions, mostly

related to the trophic levels of existence are created. They consist of a variety of competitive relationships and are executed through separation or consumption of various metabolites in the medium or from it. [1,7]. Positive or negative impact of these metabolites or competition, leads to a different end result - a full shift or domination of one population or coexistence of both. These outcomes, in the presence of multi- component microbial communities, give an idea of the wide range of options in the dynamics of associations and the complexity of their study.

The aim of the present study is to establish the nature of the interaction and sustainability of the model of yeast and actinomycete populations and insecticide λ -cyhalothrin in laboratory conditions, as well as their ability to mineralize it.

MATERIALS AND METHODS

Microorganisms The investigations were conducted with the actinomycete strain of the species *Proactinomyces niveus* 01 isolated and identified in our laboratory and yeast strain *Hansenula anomala* var. Schneggii from the collection of the Institute of Microbiology at BAS. They were chosen as they are both involved in the first step of degradation of the plant remains in the soil, i.e. utilize starch as a carbon source. Microorganisms were maintained on conventional media for the yeasts - on beer agar and for the actinomycetes - on starch-ammonia medium (compositions are shown below). The actinomycete strain was isolated from black soil taken from the region of Pleven. The main vegetation of the area is planted

orchard for which data exist that has been treated with pesticides.

Culture media. For cultivation of the microorganism - basic medium "Ryder" was used with the following composition (g.dm^{-3}): $(\text{NH}_4)_2\text{SO}_4 - 3,0$; $\text{MgSO}_4 - 0,7$; $\text{KH}_2\text{PO}_4 - 1,0$; $\text{NaCl} - 0,5$; $\text{CaCl}_2 - 0,2$; yeast extract (Difco) - 2, 0; starch - 10, 0. Salts and sugars are dissolved in distilled water in a sequence to exclude the formation of a precipitate. Acidity of the medium was 5, $5 \pm 0, 1$. To establish the number of cells able to form colonies "Ryder" medium was used for the yeast and starch-ammonia medium for actinomycetes, with the following composition (g.dm^{-3}): $(\text{NH}_4)_2\text{SO}_4 - 1, 0$; $\text{MgSO}_4 - 1, 0$; $\text{KH}_2\text{PO}_4 - 1, 0$; $\text{NaCl} - 0,5$; $\text{CaCO}_3 - 3, 0$; starch - 10, 0. To suppress the growth of yeasts in the medium, antifungal antibiotic nystatin in an amount of 100,000 units of 15 cm^3 of liquid was added. For the purpose of the study a pyrethroid insecticide λ -cyhalothrin was used, which is a chlorofluoric derivative of chrysanthemum acid. As a chemical compound it is an alpha-cyano-3-phenoxybensil 3-(2-chloro-3, 3, 3-trifluoropropnyl) -2, 2- dimetil-cyclopanecarboxylate. In the experiments the commercial preparation of the company "Zeneca" was used in concentration of active substance 25 g.dm^{-3} . Concentrated solutions were prepared from all components, which were sterilized at 105°C for 15 min. Prior to experiments implementation, the components were mixed in a sequence that did not permit the formation of sludge. The insecticide is added after sterilization.

Cultivation. To prepare the inoculum, cells of the yeast or actinomycete cultures were prepared on slant agar medium and were washed with sterile distilled water. With this suspension $0, 5 \text{ dm}^3$ Erlenmeyer flasks, containing 100 sm^3 "Ryder" medium were inoculated. The microorganisms were cultivated on a rotary shaker at 220 rpm / min at 30°C - yeasts for 48 hours and actinomycetes for 72 hours. The biomass was removed by centrifugation aseptically, and a suspension in a "Ryder" medium was prepared which was used to inoculate the bioreactor. Batch cultivation was used for accumulation of biomass in a bioreactor with a working volume of $2, 5 \text{ dm}^3$, equipped with devices for adjusting and maintaining the temperature (30°C), pH and dissolved oxygen. The active pH is controlled and maintained at 5, $5 \pm 0, 1$ by addition of $0, 1 \text{ N NaOH}$. Dissolved oxygen saturation reached 40-50% and it was stabilized at this level by regulating the speed of the stirrer ($400\text{-}600 \text{ rpm / min}$) and aeration air expense ($0, 8\text{-}1, 0 \text{ dm}^3.\text{dm}^{-3}$

min^{-1}). To establish the effect of different concentrations of the insecticide on the synthesis of biomass and the enzyme activity the method of "acute experiments" was performed. Monocultures were separately cultivated until reaching the exponential growth phase in the bioreactor. The biomass was collected aseptically by centrifugation at 3500 rpm min for 5 min and was suspended in a medium "Ryder" containing 7 g.dm^{-3} starch. The cell suspension of 80 cm^3 was dispensed into $0, 5 \text{ dm}^3$ Erlenmeyer flasks and the insecticide in the following concentrations (%): 0,002, 0,005, 0,010, 0,015, 0,020, 0,050, 0,100 and 0,150 was added. Thus, the experimental variants contain active λ -cyhalothrin substance in quantities, as follows (mg.dm^{-3}): 0.50, 1.25, 2.50, 3.75, 5.00, 12.50, 25.00 and 3.75 respectively. Cultivation continued on a shaker for 6 h. At the end of the process the changes in biomass, residual sugars and enzyme activity were analyzed.

Insecticide biodegradability determination. The medium was dispensed in $24, 5 \text{ cm}^3$ sterile Erlenmeyer flasks and inoculated with $0, 5 \text{ cm}^3$ microbial suspension. Control variants contain all chemical components as the tested samples with the exception of the microbial suspension, which is replaced by the corresponding quantity of distilled water. Cultivation was carried out on a shaker at 30°C for 21 days. At the end of the process biomass and λ -cyhalothrin concentrations were defined.

Methods of analysis. The amount of dry biomass in the culture fluid was determined after drying of a specific volume at 105°C . The concentration of residual sugars was measured after hydrolysis with $0, 1 \text{ N HCl}$ at 100°C for 30 min. with an iodometric method [10]. The residual concentration of the insecticide was determined in samples of the culture liquid after separation from the biomass. The supernatant was filtered through a blue filter Sartorius, Minisart SM 16 534, pore size $0, 2 \mu\text{l}$ for assay. The concentration of the active substance λ -cyhalothrin insecticide was measured by automated HPLC system of the company La Chrom, Hitachi, Germany. A chromatographic column "Intersil" of the company Penomenex was used. Solution of λ -cyhalothrin in acetonitrile at a concentration of 10 mg.sm^{-3} was taken as a standard. The results were received automatically by a graphic recorder.

Total amylolytic activity was assayed by a fast colorimetric method [11]. One unit of enzyme activity (U/cm^3) is assumed as the enzyme quantity, catalyzing the release of $1 \mu\text{mol}$ glucose from 1% starch solution at 60°C and pH 4.3 for 1 min.

Superoxide dismutase activity was determined in cell free extracts according to the method of Beaushamp, Fridovich [21]. Cell extracts were obtained by grinding the frozen with liquid nitrogen biomass in a mortar with glass beads. For measuring the superoxide dismutase the released after centrifugation cell extract was used. The concentration of proteins in it was estimated by the method of Lowry.

RESULTS AND DISCUSSION

Growth of microbial populations, depending on the concentration of the insecticide. As stated above, insecticides also affect soil microflora.

Acting on the individual species, they affect the ecosystem by modifying it. On the other hand, the very xenobiotics undergo destruction by many soil microorganisms. All this prompted us to investigate the effect of different concentrations of insecticide on the viability of the two microbial species by "acute experiments".

The graphs in Figure 1. show that in a medium without insecticide (zero concentration), the actinomycete population synthesized 17% more biomass compared to yeast one. The addition of λ -cyhalothrin reliably reduced the number of populations for both species.

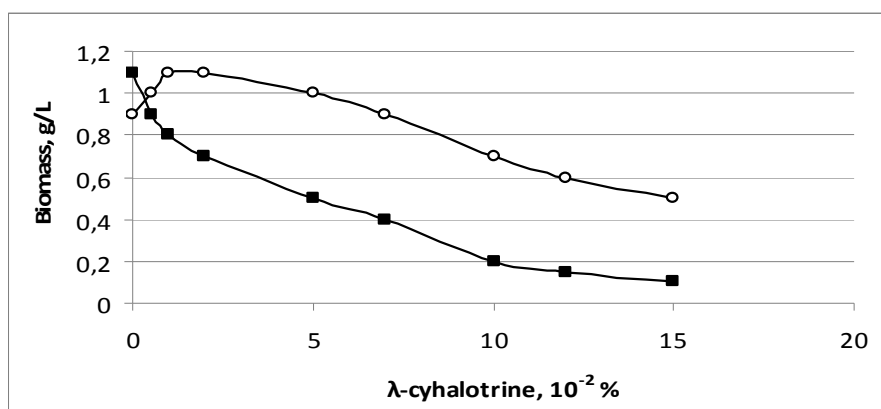


Fig. 1. Biomass synthesis by yeasts (○) and actinomycetes (■) in a medium with insecticide.

For the yeasts, however, concentrations of insecticide up to $2 \cdot 10^{-2}$ % caused a pronounced stimulatory effect. With the actinomycetes, the quantity of biomass at the designated concentration dropped sharply, after which a steady reduction was established, as with the yeasts. Consequently, actinomycete population is more sensitive than the

yeast one in the presence of low doses of insecticide.

The amount of residual starch (measured as glucose) correlated with the changes in the biomass (Figure 2). The increase of its concentration in parallel with the increase of the insecticide in the medium suggests a change in the mechanism of its utilization.

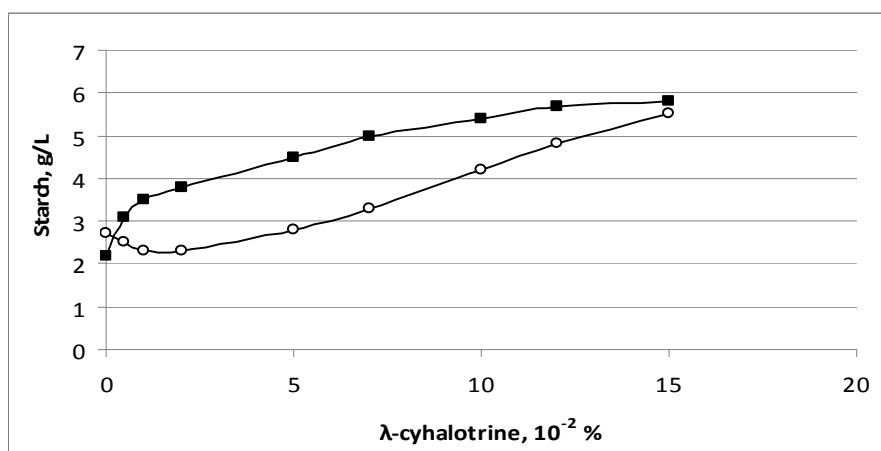


Fig. 2. Residual sugars at yeast (○) and actinomycetes (■) cultivation in a medium with insecticide.

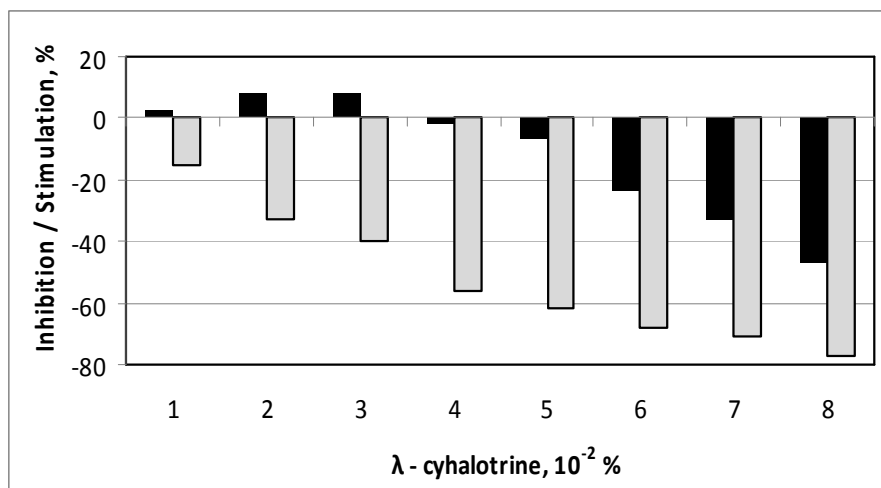


Fig. 3. Degree of inhibition or stimulation at yeast (■) and actinomycetes (□) cultivation in a medium with insecticide.

Quantitative inhibitory effect of the insecticide on the two populations is shown in Figure 3. In the yeast population the inhibitory effect grew in proportion after adding 0.05% of inhibitor, reaching the highest value of 45% at the highest xenobiotic concentrations. At low concentrations of the insecticide in the medium (to 0.02 %) the growth of the yeasts was stimulated by about 7.5 %. In

actinomycete populations the degree of inhibition increased sharply even with the addition of minimal doses of insecticide. The maximum value of this effect reached nearly 77 %. Characteristic for this effect is that it is achieved not linearly proportional to the concentrations of the insecticide in the medium but is described by a hyperbolic curve, showing a saturation zone.

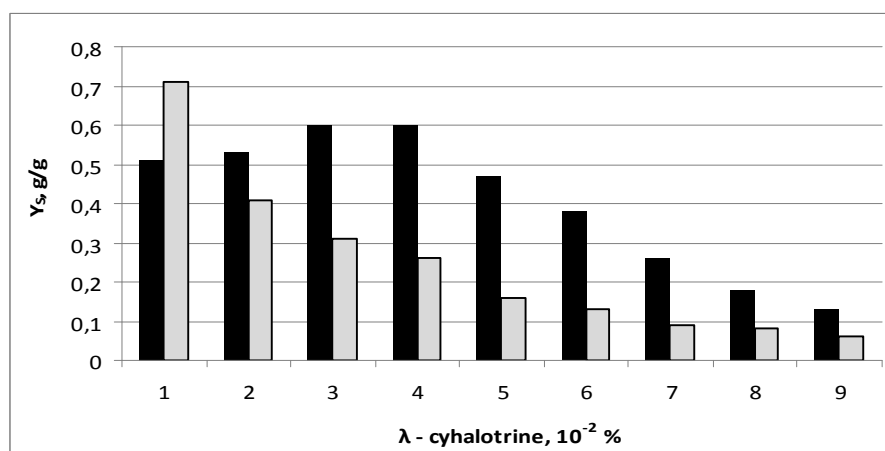


Fig. 4. Changes of the economic coefficient (Y_s) in relation to the concentration of the insecticide at cultivation of yeasts (■) and actinomycetes (□).

The established experimental changes in biomass and utilized sugars can be explained by the calculated values for the economic coefficient (Y_s). These show the synthesis effectiveness at the given conditions (Figure 4). In the absence of the insecticide Y_s of actinomycetes is higher than that of the yeasts, i.e. the already known fact for this type of microorganisms that they utilize starch more effectively was confirmed. This is reflected in the

biomass synthesis. Addition of insecticide logically and quickly lowers the effective conversion of substrate into biomass.

The yeast population effectively utilized the substrate up to concentrations of 0.02 % of insecticide and then Y_s decreased, but remains twice as high compared to this of actinomycetes.

Influence of the insecticide on the enzyme activity of the populations. The main carbon

substrate in our experiments is starch. Its degradation involves various enzymes which are synthesized by the microbial cells. We chose amylolytic enzymes, since they participate in the first step of the hydrolysis of this polysaccharide. As a result of their action glucose is obtained, which is further included in one or other metabolic cycle.

Figure 5 shows that under the conditions of the experiment, the yeast population had a lower amylolytic activity than the actinomycete one. According to it is the

amount of degraded starch. When adding the insecticide in the medium, the activity of these enzymes decreased for both species. The differences are in the concentration of the inhibitor at which this effect occurs. For the yeasts it was 0, 02% and for actinomycetes - 0.05%. In quantities greater than those, the amylolytic activity remained constant, but higher for actinomycetes. Therefore, we can assume that the mechanism of influence of the insecticide is the same for both species- the curves are almost parallel.

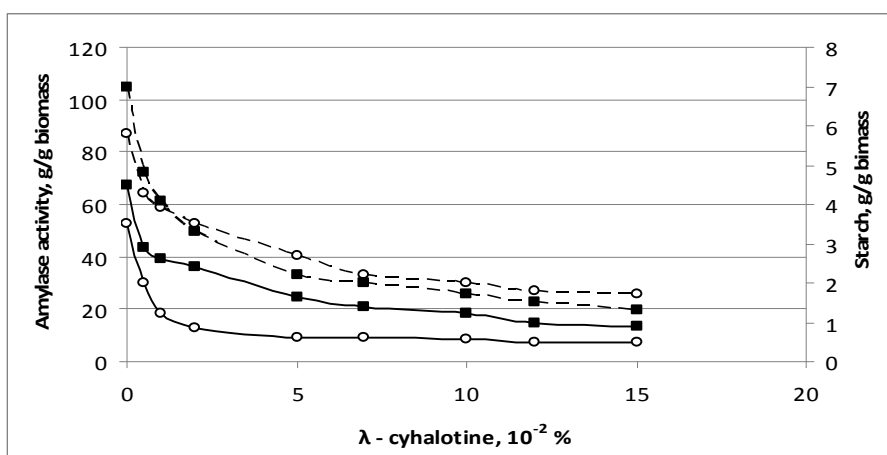


Fig. 5. Amylolytic activity (----) and degraded starch (—) by the yeasts (○) and actinomycetes (■) in a medium with insecticide.

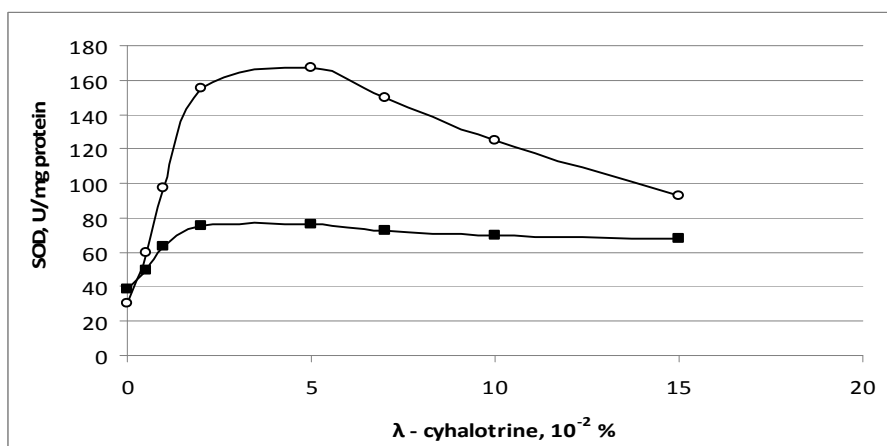


Fig. 6. Superoxide dismutase activity at cultivation of yeasts (○) and actinomycetes (■) in a medium with insecticide.

In recent years, particular attention has been paid by a number of authors to the so-called stress enzymes. One of them is the superoxide dismutase (SOD) [3, 17]. This enzyme is intracellular and is considered a natural defense against the reactive and highly toxic forms of oxygen. Our results on the activity of SOD in relation to the concentration of the insecticide are shown in Figure 6. For the yeasts, with increasing the amount of the insecticide in the

medium to 0, 02- 0. 04 %. the activity of the enzyme increased. Higher concentrations of the inhibitor did not cause an answer reaction in the enzyme activity - the activity remained at the same level or slightly decreased, but remained higher in comparison to this in the medium without insecticide.

With the actinomycetes, the enzyme activity increased slightly up to a concentration of 0.02 % of

the insecticide and then remained at the same level regardless of the amount of the inhibitor.

Comparing these results with the data for the synthesis of biomass (Figure 1), we can conclude that the enzyme superoxide dismutase is involved in the defense mechanisms of the two microbial species when there is an insecticide in the medium. In a defensive plan the yeast population is more flexible and responded to the inhibitor. This also affected the biomass synthesis.

Insecticide biodegradation. The chemical structure of λ -cyhalothrin, together with the highly toxic chlorine and fluorine atoms, contains sufficient functional groups that are easily digestible $-\text{CH}_3$, $\text{C}=\text{C}$, CO . Therefore, the logical question is, if it is possible and how this insecticide could serve as a

carbon source for growth of yeasts and actinomycetes. To get an answer, experiments were performed in which the same medium was used, but where the starch lacked. The obtained results are presented in Table 1 and Table 2.

It is obvious that the introduction of insecticide into the medium without microorganisms and after micro aeration at 220 rpm/min and a temperature of 30°C , the active substance of the preparation decreased. Therefore, in these conditions degradation was 35.0 %. This disruption depends on the time of treatment. When microbial species was introduced in the medium, the residual amount of λ -cyhalothrin, after 21 days of cultivation of the yeasts was $11,0 \text{ mg.dm}^{-3}$, representing 22,0 % of its starting concentration.

Table 1. Residual concentrations of λ -cyhalothrin at cultivation of yeast and actinomycete strains with 0,2 % insecticide.

Cultivation, days	Control, without microorganisms		With yeasts		With actinomycetes	
	Insecticide mg.dm^{-3}	%	Insecticide mg.dm^{-3}	%	Insecticide mg.dm^{-3}	%
0	50,0	100	50,0	100	50,0	100
3	49,5	99	44,0	89,0	48,2	96,3
5	47,5	95	39,7	79,4	44,4	88,8
7	45,2	90,4	26,4	52,8	41,5	83,0
9	42,3	84,6	23,0	46,0	36,6	73,2
11	40,4	80,8	18,5	37,0	34,2	68,4
14	37,5	75,0	14,5	29,0	32,7	65,4
18	34,4	68,8	12,9	25,8	31,6	63,2
21	32,5	65,0	11,0	22,0	30,8	61,7

Table 2. Biomass synthesis (g.dm^{-3}) at cultivation of yeasts and actinomycetes in a medium with insecticide as a sole carbon source

Cultivation days	Yeasts	Actinomycetes
0	2,35	2,12
5	2,41	2,18
14	2,98	2,21
21	3,56,	2,26

In comparison with the variant without microorganisms that is more than a 30 % decrease. For actinomycetes population at the end of the process it reached $30,8 \text{ mg.dm}^{-3}$, which was 61.7 %, i.e. almost as much as in the control variant. It can therefore be stated that the yeast can use λ -

cyhalothrin as a carbon source. The change of biomass, however, in these experiments was negligible (Table 2). This may be due to the extremely low concentration of insecticide or that microorganisms performed only transformation of the active substance of the preparation. Whatever the

mechanism of this effect was, with the concentration of insecticide applied, it could not have a significant impact on the total biomass synthesis. These facts, suggest that among the species of microorganisms some of them can be selected to be used for the controlled degradation of this insecticide. Probably for the intensification of this process a concomitant carbon source is necessary to serve as a co-substrate [11]. It is assumed that for the full degradation of insecticides makes sense not pure (mono) cultures, but a mixture of populations sequentially metabolizing the intermediates to be used [20]. Such method of combining co-metabolism with comensalism, i. e. the coordinated interaction of cellular and population levels open great opportunities for intensification of the treatment processes in biosphere as well as under controlled conditions.

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УСТОЙЧИВОСТ НА ДРОЖДЕВА И АКТИНОМИЦЕТНА ПОПУЛАЦИИ ПРИ КУЛТИВИРАНЕ В СРЕДА С ИНСЕКТИЦИД

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Резюме. Почвената микрофлора, като компонент на биогеоценозите, изпитва различно въздействие от страна на пестицидите. Действайки на отделните микробни видове, те влияят и модифицират екосистемите като цяло. От друга страна, много микроорганизми могат да осъществят трансформация и минерализация на пестицидите.

Целта на разработката е да установи, в лабораторни условия, характера на взаимодействие и устойчивостта на моделна дрождева и актиномицетна популация на инсектицида λ -цихалотрин, както и способността да го минерализират. Установено е, че до концентрация 0,02 % инсектицидът стимулира ефективността на усвояване на въглеродния субстрат от дрождева популация и инхибира актиномицетите при всички изследвани концентрации. Добавянето на инсектицид в средата намалява активността на амилалитичните ензими и повишава тази на супероксиддисмутазата. Дрождевият щам *Hansenula anomala* var. *Schneggii* може да използва λ -цихалотрина като единствен въглероден източник

Ключови думи. пестициди, λ -цихалотрин, дрожди, актиномицети, растеж, ензимна активност.

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