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GROWTH AND CHANGES OF STREPTOMYCETES IN NUTRITIOUS MEDIA WITH TOLUENE

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Introduction

Toluene is one of many aromatic pollutants, which comes into waters with the organic waste from petrochemical factories. In its structure, it is methylbenzene, a colourless, easily flammable liquid with the boiling point at 110.8 °C (Noller 1961). It is practically insoluble in water. It was first obtained by heating tolu-balsam, a plant-resin. It is produced by dry destillation of coal or by fractionation of tar. In great quantities, it can be obtained by reforming-operations, i. e. by catalytic conversion of methylyclolexane.

Like all aromatic hydrocarbons, toluene is toxic for organisms when applied in higher concentrations. The limit of toxicity amounts to 10 mg/l for fish, and 440 mg/l for sludge-bacteria (Liebman 1960). Subletal doses of toluene block chemoreceptors for organic materials of some motile marine bacteria (Chet et Mitchell 1976). Low concentrations of that aromatic compound are used in microbiological experiments where breaking of the membrane barrier is desirable, for it makes bacterial membranes permeable (Rezvzn et Chaikovskaya 1972; Rebrov et al. 1974). But as all compounds of natural origin can be biodegraded, there are also microorganisms which can decompose toluene, owing to oxygenase enzymes which break aromatic nuclei and decompose the side-chains, using molecular oxygen (Hayaishi 1964). The most extensively investigated of those microorganisms are *Pseudomonas* (Van Ravenswaay et Van der Linden 1971; Kobal et al. 1973; Jigami et al. 1974; Worsey et Williams 1975) and *Nocardia* (Aristarkhova 1972).

In our work we examined the growth of 11 strains of streptomycetes izolated from river-water and seawater, in media with the addition of various concentrations of toluene. Changes in the pigmentation of the streptomycetes, as well as their abilities to decompose cellulose and gelatine, after treatment with toluene were also investigated. Streptomycetes are primary soil-microorganisms, but can be found also in waters and water-sediments, where they take part in the processes of self-purification of waters (Silevy 1962). We tried to cultivate them also in a mineral solution with toluene as the sole carbon-source, to find out if they were able to decompose that aromatic hydrocarbon.

Almost nothing is known about the interactions of aromatic hydrocarbons and streptomycetes. *Nocardia* is the only genus of *Actinomycetales,* whose abilities to decompose aromatics have been investigated (Aristarkhova 1972; Hartmann et al. 1973; Haider et al. 1974; Skryabin et al. 1974). Grbić (1975) investigated influences of toluene and some other aromatic hydrocarbons upon streptomycetes.

Methods and Materials

Streptomycetes were isolated from the water of the river Sava, in the vicinity of the main collector of Zagreb (5 strains), and from waters of the South Adriatic in the region of Boka Kotorska Bay (6). The medium for isolation and for cultivation of pure strains was Gauze's agar: KNO_3, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; soluble starch, 20,0 g; agar, 20.0 g; distilled water, 1000 ml; pH was adjusted to 7.2—7.5. The strains were clasified into sections and series according to the ISP (International *Streptomyces* Project, Shirling et Gottlieb 1964), considering the shape of their sporophores and pigments of their aerial mycelia. Their ability to decompose cellulose was determined in a mineral solution ($NaNO_3$, 2.5 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; NaCl, 0.1 g; $CaCl_2 \cdot 6H_2O$, 0.1 g; distilled water, 1000 ml; pH adjusted to 7.2—7.5), with a filter-paper — band as a source of cellulose. Liquefaction of gelatine was examined in the medium: glucose, 20.0 g; nutritious broth ("Torlak"), 23.3 g; gelatine, 150.0 g; distilled water, 1000 ml; pH adjusted to 7.2—7.5.

In the experiments with toluene, the aromatic compound was added to a starvation medium, mineral agar (KNO_3, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 20.0 g; disitlled water, 1000 ml; pH adjusted to 7.2—7.5), after sterilization. The concentrations of toluene used were 2, 3, 5, 7 or 10% (v/v). For purposes of control, all the strains of streptomycetes were also inoculated onto pure mineral agar without toluene. Their pigmentation was examined on the mineral agar with without toluene, as well as on Gauze's agar, and their ablities to decompose cellulose and liquefy gelatine were determined before and after growth on the mineral agar with toluene.

The incubation in media with cellulose and gelatine lasted 30 days, at 28 °C, and in all the other media 8 days, at 28 °C.

In the second series of the experiments, the streptomycetes were inoculated into mineral solution (KNO_3, 2.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CaCl_2 \cdot 6H_2O$, 0.1 g;

distilleed water, 1000 ml; pH adjusted to 7.2—7.5) with addition of 2, 3, 5, 7 or 10% (v/v) of toluene as the sole carbon-surce. The mineral solution proved to be a good medium, because the streptomycetes all grew in it when glucose was added as the only carbon-cource. For purposes of control, all the strains were also inoculated into pure mineral solution with no carbon-source.

The toluene used in the experiments was the product of Organic-Chemical Industry OKI in Zagreb. It was 98% pure; the rest (2%) were benzene, ethylbenzene and cyclohexane. As toluene is very volatile, its concentrations in the media were reduced during incubation, for control, the final concentrations in sterile media were measured by help of IR-spectrometry, on a Perkin-Elmer apparatus.

Results

An the mineral agar with addition of various concentrations of toluene, the growth of streptomycetes was reduced in relation to Gauze's agar, the more the concentration of toluene was higher, but they all germinated and produced hyphae. The shape of their sporophores did not change. Pigmentation of their aerial mycelia changed in some instances, and their vegetative mycelia were all totally depigmented, except with the strain R_3 *F*-red. On pure mineral agar, where the streptomycetes could grow utilizing only agar and where their mycelia were weaker than on the plates with the addition of toluene, a slight decrease of pigmentation of vegetative mycelia as noticed, but not total depigmentation. Soluble pigments were all lost in the mineral agar with toluene.

In Table 1. shows changes of the aerial mycelia of streptomycetes on the mineral agar with toluene. Names of the series after ISP (Shirling et Gottlieb 1964) show the colour of aerial mycelia.

It is worth mentioning that all the normal characteristics of vegetative and aerial mycelia, as well as of soluble pigments, reversed after reinoculation onto Gauze's agar.

Table 2. gives changes in the ability of streptomycetes treated with toluene to decompose cellulose, and table 3. changes in the ability to liquefy gelatine.

In the experiments with pure mineral solution, no strain grew in the medium without a carbon-source, but the majority of them grew in the solution with the addition of various concentrations of toluene. The results are presented in Table 4.

The concentrations of toluene in the mineral solution weree considerably reduced during incubation (10 days) at someewhat raised temperature (29 °C), owing to its greaet volatility, as can be seen from Table 5; but, in any case, the initial concentrations were very high.

Table 1. Changes of the aerial mycelia of the streptomycetes on the mineral agar with toluene

Tabela 1. Promjene zračnog micelija streptomiceta na mineralnom agaru s dodatkom toluena.

Sign of the strain (Oznaka soja)	Section and series after ISP (Sekcija i serija po ISP)	Concentrations of toluene (Koncentracije toluena) (% v/v):				
		2	3	5	7	10
R_1	*S*-blue	norm.	norm.	norm.	norm.	grey
R_2	*RF*-grey	M	M	M	M	M
R_3	*F*-red	norm.	norm.	norm.	norm.	norm.
R_4	*R*-yellow	norm.	norm.	norm.	norm.	norm.
R_5	*R*-yellow	norm.	norm.	norm.	norm.	norm.
M_1	*F*-yellow	norm.	norm.	norm.	norm.	norm.
M_2	*S*-blue	grey	grey	grey	grey	grey
M_3	*RF*-yellow	norm.	norm.	norm.	norm.	norm.
M_4	*F*-yellow	grey	grey	grey	grey	grey
M_5	*RF*-yellow	grey	grey	grey	grey	grey
M_6	*R*-grey	M	M	—	—	—

Legend to the table 1.

R strains isolated from river-water
M „ „ „ seawater
Section after ISP (shapes of sporophores):
R — *Rectus* (sporophores straight)
F — *Flexibilis* (sporophores twisting)
RF — *Rectus-Flexibilis*
S — *Spira* (sporophores spiral)
norm. — mycelium unchanged
M — mycelium only microscopically visible
grey — colour of the mycelium changed to grey
— — aerial mycelium has not developed

Legenda uz tabelu 1.

R sojevi izolirani iz riječne vode
M „ „ „ morske „
Sekcije po ISP-u (oblici sporofora):
R — *Rectus* (ravni sporofori)
F — *Flexibilis* (vijugavi sporofori)
RF — *Rectus-Flexibilis*
S — *Spira* (spiralni sporofori)
norm. — micelij nepromijenjen
M — „ vidljiv samo mikroskopski
grey — boja micelija promijenjena u sivu
— — zračni micelij se nije razvio

Table 2. Changes of the ability to decompose cellulose with the streptomycetes treated with toluene

Tabela 2. Promjene sposobnosti razgradnje celuloze kod streptomiceta tretiranih toluenom

Sign of the strain (Oznaka soja)	Before treatment (Prije tretmana)	After treatment with toluene in concentrations (Nakon tretmana toluenom u koncentracijama) (%v/v): 2	3	5	7	10
R_1	+++	++	++	+++	++	1–
R_2	+	–	–	–	–	–
R_3	+++++	++++	++++	+++	++++	+++++
R_4	+++++	+++++	+++++	++++	++++	+
R_5	++++	+++	+++	+++	+++	+
M_1	++++	++	++	++	+	++
M_2	–	–	–	–	–	–
M_3	++++	++	+	+++++	+++++	+++++
M_4	+	–	–	–	–	–
M_5	–	–	–	–	–	–
M_6	–	–	–	–	–	–

Legend to the table 2.:

R strains isolated from river-water
M „ „ „ seawater
– no decomposition of cellulose
+, ++, +++, ++++, +++++ various intensities of cellulosedecomposition

Legenda uz tabelu 2.:

R sojevi izolirani iz riječne vode
M „ „ „ morske „
– nema razgradnje celuloze
+, ++, +++, ++++, +++++ različiti stupnjevi razgradnje celuloze

Table 3. Changes of the ability to liquefy gelatine with the streptomycetes treated with toluene

Tabela 3. Promjene sposobnosti likvefakcije želatine kod streptomiceta tretiranih toluenom

Sign of the strain (Oznaka soja)	Before treatment (Prije tretmana)	After treatment with toluene in concentrations (% v/v): (Nakon tretmana toluenom u koncentracijama)				
		2	3	5	7	10
R_1	+	+	+	+	+	+
R_2	–	–	–	+	–	–
R_3	+	+	+	+	+	+
R_4	+	+	+	+	+	–
R_5	+	+	+	+	+	–
M_1	+	+	+	+	+	+
M_2	+	–	–	–	–	–
M_3	+	+	+	+	+	+
M_4	+	+	+	+	+	+
M_5	+	+	+	+	±	–
M_6	–	–	–	–	–	–

Legend to the table 3.:

R strains isolated from river-water
M „ „ „ seawater
– no liquefaction of gelatine
± liquefaction of gelatine doubtful
+ „ „ „ positive

Legenda uz tabelu 3.:

R sojevi izolirani iz riječne vode
M „ „ „ morske „
– nema likvefakcije želatine
± likvefakcija želatine dvojbena
+ „ „ pozitivna

Table 4. Growth of the streptomycetes in the mineral sclution with toluene as the sole carbon-source

Tabela 4. Rast streptomiceta u mineralnoj otopini s toluenom kao jedinim izvorom ugljika

Sign of the strain (Oznaka soja)	Concentrations of toluene in the medium (Koncentracije toluena u mediju) (% v/v):				
	2	3	5	7	10
R_1	++++	++++	+++	++++	++++
R_2	++++	—	—	—	—
R_3	—	—	±	+	—
R_4	—	++++	++++	—	—
R_5	—	++++	+++++	++++	+++
M_1	—	+	+	+	+
M_2	+++	++	+++	—	++++
M_3	+++	++	+++	+++	—
M_4	++	++	+	++	++
M_5	++	+++	++	+++	++
M_6	—	—	—	—	—

Legend to the table 4.:

R strains isolated from river-water
M „ „ „ seawater
— no growth
± growth doubtful
+, ++, +++, ++++, +++++ increasing number of developed colonies

Legenda uz tabelu 4.:

R sojevi izolirani iz riječne vode
M „ „ „ morske „
— nema rasta
± rast dvojben
+, ++, +++, ++++, +++++ rastući broj razvijenih kolonija

Table 5. Final concentrations of toluene in the sterile mineral solution after 10 days of incubation at 28°C

Tabela 5. Konačne koncentracije toluena u sterilnoj mineralnoj otopini nakon 10 dana inkubacije na 28°C

Initial concentrations (Početne koncentracije) (% v/v)	Concentrations after 10 days of incubation (Koncentracije nakon 10 dana inkubacije) (% v/v)
2	0,00113
3	0,00065
5	0,00109
7	0,08670
10	0,77100

Discussion

Streptomycetes, bacteria able to utilize various compounds for their nutrition, appeared to be greatly resistant to inhibitory effects of the aromatic. They all grew on the starvation medium, i. e. in unfavourable conditions, with the addition of very high initial concentrations of toluene. The most pronounced changese, due to the toluene-influence, appeared in their pigmentation; a loss of soluble pigments and of pigments in vegetative mycelia, and changes of colours to grey of the aerial mycelia in some strains. As all the pigments were regained after reinoculation onto Gauze's agar, it is obvious that the changes have no permanent character, but that they — probably — represent extractions of pigments, caused by the toluene-damage of the membrane-systems.

The most sensitive to toluene were the strains R_2 *RF*-grey from river-water, and M_3 *R*-grey from seawater. The aerial mycelium of the former was reduced to microscopical size on all the concentrations of toluene applied, and the latter did not develop its aerial hyphae at all at 5, 7 and 10% (v/v) of the aromatic (see Table 1).

The abilities of the streptomycetes to decompose cellulose and liquefy gelatine were not considerably damaged after the treatment with toluene. As can be seen from Tables 2. and 3., the majority of the strains was not affected at all. Only the strains R_2 *RF*-grey from river-water and M_4 *F*-yellow from seawater stopped decomposing cellulose already after the treatment with 2% (v/v) of toluene; R_4 *S*-blue from river-water was affected only at 10% (v/v). The loss of the ability to liquify gelatine was noticed only with 2 strains from river-water and 2 strains from seawater, and that only at 10% (v/v) of toluene, except with the strain M_2 *S*-blue from seawater which lost that ability already at 2% (v/v).

All the examined strains of streptomycetes, except M_3 *R*-grey from seawater, grew in the mineral solution with toluene as the sole carbon-source (se Table 4); as none of them germinated in pure mineral solution, it is obvious that they are not autotrophic and that they decomposed toluene, using it as the source of carbon and energy. It is interesting that two strains from river-water and one from seawater started growth at 3% (v/v) of toluene, which means that 2% (v/v) was too low a concentration for them. As many as six of the strains grew well at 10% (v/v) of toluene, the highest concentration applied.

The great resistance of the streptomycetes to influences of toluene, as well as their capability to decompose it, could be explained by the characteristics of the habitat they were isolated from. The water of the river Sava at the main city-collector of Zagreb is considerably polluted, and it also receives wastewaters from the petrochemical industry OKI. Small quantities of oil could also be found in seawater, because ships let their oil-waste flow into the sea. Possibly owing to the mechanisms of adaptation to their habitat, the streptomycetes are able to resist higher concentrations of aromatic compounds and even to biodegrade them.

Owing to the great volatility of toluene, its concentrations were reduced in the medium during incubation (see Table 5). But the spores of the streptomycetes which grew in the mineral solution with toluene had to survive the high initial concentrations and then germinate, producing the hyphae and finally mycelia of the colonies.

Summary

By adding various quantities of toluene to media for streptomycetes, it was established that the streptomycetes isolated from polluted river-water and from seawater were highly resistant to effects of toluene; all of them grew even at the highest concentration of the compound — 10% (v/v); their abilities to decompose cellulose and liquefy gelatine were not changed much, but on the medium with toluene they were highly depigmented, possibily owing to the toluene-effects upon the cell-membrane. The depigmentation had no permanent charcter. As many as 10 out of 11 strains grew in the mineral solution with toluene as the sole carbon-source, which means that they could decompose the aromatic hydrocarbon, probably due to adaptations to the habitant they were isolated from

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SADRŽAJ

RAST I PROMJENE STREPTOMICETA U HRANJIVIM PODLOGAMA S TOLUENOM

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U radu je ispitan utjecaj toluena na 11 sojeva streptomiceta iz riječne i morske vode. Streptomiceti su pokazali znatnu rezistenciju na djelovanje toluena, vjerojatno zbog mehanizama adaptacije na staništa odakle su izolirani. Svi su razvili micelij na siromašnoj podlozi (mineralni agar) sa čak 10% (v/v) toluena. Njihove sposobnosti da razgrađuju celulozu i želatinu nisu se znatno izmijenile. Na podlozi s toluenom micelij streptomiceta pretrpio je znatnu depigmentaciju, a topljivi pigment potpuno je nestao. Međutim, te promjene nisu imale trajni karakter. Deset od 11 sojeva streptomiceta raslo je u mineralnoj otopini s toluenom kao jedinim izvorom ugljika, što upućuje na mogućnosti biorazgradnje tog spoja.

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