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Influence of Humic Acids on the Growth of the Microorganisms Utilizing Toxic Compounds (Comparison between Yeast and Bacteria)

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Abstract: Humic acids, a heterogenic group of natural organic macromolecules with a complex polymeric structure, form a significant part of the soil and water environment. They have a high surface activity, thus they are able to interact with other components in the environment (mineral and microbial cell surfaces, organic and inorganic compounds). This work is focused on humic acid effects on the growth of microorganisms utilizing phenolic compounds as the sole carbon and energy source. For this purpose a bacterial population *Rhodococcus erythropolis* and a yeast strain *Candida maltosa* were used. Both microorganisms had been adapted to the degradation of phenol and phenolic compounds for many years. However, a toxic effect of these compounds still occurs. The addition of humic acids into the cultivation medium extended toleration of the bacteria to higher concentrations of phenolic compounds and increased the growth of this microbial population as well. In the case of the yeast, humic acids inhibited the growth. Humic acids easily form an additive layer on the surface of the investigated microorganisms. The generated humic acid layer can probably serve as a transport barrier and thus influence the growth of microbial populations.

Keywords: *Candida maltosa* · Humic acid · Microbial growth · Phenolic compounds · *Rhodococcus erythropolis*

1. Introduction

Humic acids (HA) form an inseparable part of the soil and water environment. Sixty to eighty percent of the total organic carbon in soil occurs in the form of humic substances (humic acids, fulvic acids, and humin). Fifty to seventy-five percent of dissolved organic matter in water consists of fulvic and humic acids. As described by Pitter [1], HA originate from plant, animal and microorganism residues and are formed by coupling reactions and polymerizations among intermediates of the organisms' decomposition, either spontaneously or supported by natural microbial activities.

It is generally known that HA are macromolecular compounds with a complex polymeric structure. Aromatic rings and carboxylic and phenolic functional groups are characteristic. Stegmann *et al.* [2] have described how the heterogeneity of HA make it difficult to define the individual molecules. As shown by Janoš [3], the structure and the composition of the functional groups depend on their origin as well as on the applied isolation procedure.

Esparza-Soto and Westerhoff [4] and Murphy and Zachara [5] studied the sorption of HA onto a microbial surface and onto a mineral surface. Pitter [1], among others, mentions binding of various inorganic ions with HA and Karthikeyan and Chorover [6] and Jeffé [7] dealt with interactions between HA and organic compounds, especially with hydrophobic ones. HA can interact with almost every component of their surrounding area and thus affect the distribution of microorganisms, pollutants, and themselves in the environment. On the basis of these interactions, humic substances possess a potential ability to influence the activity of degrading microorganisms.

Furthermore, HA can serve as an electron donor for Fe(III)-reducing microorganisms, as shown by Lovley *et al.* [8] or by Chen *et al.* [9], or for the reduction of Cr(VI) and U(VI), as shown by Gu and Chen [10]. The stimulation of activity and growth of nitrifying bacteria as well as the negative effects were proved, for instance by Vallini *et al.* [11] and Visser [12], respectively. However, there is still a lack of studies about the potential effect of HA on microbial activity.

In this work the effect of HA on microbial growth inhibition in the presence of phenolic compounds (phenol and catechol) was investigated. The studied microorganisms can utilize a wide range of phenolic compounds as a sole carbon and energy source, but some of these substances can be toxic at higher concentrations as well. The effect of HA on the growth of a yeast and a bacterial strain is discussed.

2. Material and Methods

2.1. *Microorganisms*

A yeast strain, *Candida maltosa*, was isolated by modified aerobic enrichment

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of activated sludge and consequent phenol-utilizing single species isolations as described by Masák *et al.* [13]. The yeast population was grown aerobically in YNB medium (Difco Laboratories, USA).

Bacterium *Rhodococcus erythropolis* CCM 2595 was obtained from the Czech culture collection (Masaryk University, Brno, Czech Republic) and was grown aerobically in BSM as reported by Cejkova *et al.* [14].

2.2. *Growth Media*

A basic mineral medium for yeasts (YNB), used for cultivation of *Candida maltosa*, contains Bacto-Yeast Nitrogen Base (1.7 $g \cdot l^{-1}$), $(NH_4)_2SO_4$ (5.0 $g \cdot l^{-1}$), L-histidine (10 $mg \cdot l^{-1}$), D,L-methionine (20 $mg \cdot l^{-1}$) and D,L-tryptophan (20 $mg \cdot l^{-1}$). The pH value of the medium is approximately 5.0.

A mineral medium for bacteria (BSM), used for cultivation of *Rhodococcus erythropolis*, consists of KH_2PO_4 (0.17 $g \cdot l^{-1}$), K_2HPO_4 (0.13 $g \cdot l^{-1}$), $(NH_4)_2SO_4$ (0.71 $g \cdot l^{-1}$), $MgCl_2 \cdot 6H_2O$ (0.34 $g \cdot l^{-1}$) and trace elements ($MnCl_2 \cdot 4H_2O$ at 1.0 $mg \cdot l^{-1}$, $CaCl_2 \cdot 2H_2O$ at 0.26 $mg \cdot l^{-1}$, $FeSO_4 \cdot 7H_2O$ at 0.6 $mg \cdot l^{-1}$ and $Na_2MoO_4 \cdot 2H_2O$ at 2.0 $mg \cdot l^{-1}$). The pH value of the medium is approximately 6.5.

2.3. *Humic Acids*

Humic acids (PAB 32, PAB 54 and S5) were isolated from oxihumolite, which originated from brown coal fields in the north of the Czech Republic (Bilina and Sokolov). They were provided by the Institute of Inorganic Chemistry (VUAnCh), Usti nad Labem, Czech Republic as 20 $g \cdot l^{-1}$ potassium humate solutions. For each experiment a concentration of 0.05 $g \cdot l^{-1}$ of HA was used.

2.4. *Experimental Set-up*

The inhibition of the microbial growth was monitored using a Bioscreen C Analyser (Labsystem, Finland). The cultivations were carried out in a volume of 350 µl, at a temperature of 25 °C, with a high intensity shaking. Phenol or catechol was used as the sole carbon and energy source.

2.5. *Electron Microscopy*

An electron microscope (Hitachi S 4700, Canada) was used for the investigation of the HA–cell surface interaction. The isolated biomass was spread onto cover slides and dried by infrared light. After a gold and palladium covering of the slides, the obtained samples were studied. The electron microscopy was performed by Martin Maryska from the Department of Glass and Ceramics of the ICT Prague.

3. Results

3.1. *Effect of HA on Microbial Growth Inhibition*

The experiments showed that none of the investigated HA preparations can be used as a carbon and energy source in the concentration range 0.01 $g \cdot l^{-1}$–0.2 $g \cdot l^{-1}$ (not shown).

At the optimal concentration of phenol (1.0 $g \cdot l^{-1}$), the *C. maltosa* growth was inhibited by all investigated HA preparations (Fig. 1). A shorter lag-phase and a higher biomass yield occurred when the cell population was not exposed to HA. The strongest growth inhibition was shown using PAB 54.

Fig. 2 shows, at the optimal concentration of phenol (0.3 $g \cdot l^{-1}$), the greatest inhibition of *R. erythropolis* growth when it was cultivated in the absence of HA. When HA were applied, the bacteria started to grow earlier and slightly higher biomass yields were reached.

The situation of the microorganisms which were grown at phenol concentrations higher than optimal (1.5 $g \cdot l^{-1}$ for *C. maltosa* and 0.7 $g \cdot l^{-1}$ for *R. erythropolis*), is shown in Fig. 3 and 4. In the presence of HA, *C. maltosa* started to grow after a nearly 90 h lag-phase, whereas in the absence of HA the lag-phase was about 35 h. On the other hand, the biomass yield of the population which was exposed to HA reached a higher value compared to the population cultivated in the absence of HA.

When the *R. erythropolis* population was exposed to a higher concentration of phenol (0.7 $g \cdot l^{-1}$) and HA was not added, microbial growth was inhibited (Fig. 4). A significantly longer lag-phase occurred during the cultivation without HA (90 h lag-phase in the absence of HA and about 20 h lag-phase in the presence of HA). However, in the absence of HA, the bacteria reached the highest biomass yields. The smallest inhibition of the *R. erythropolis* growth was shown when the population was exposed to HA S5.

Catechol is less toxic towards both investigated microorganisms than phenol.

The inhibition of the *C. maltosa* growth in the presence of catechol as the sole carbon and energy source is shown in Fig. 5. HA influenced neither the lag-phases nor the growth rates, but they significantly decreased the biomass yields. The strongest inhibition occurred in the presence of PAB 54.

When the *R. erythropolis* cultivation on catechol was carried out without HA, a considerably faster growth of the bacteria occurred in the initial hours (Fig. 6). However, the biomass yields of the cell populations

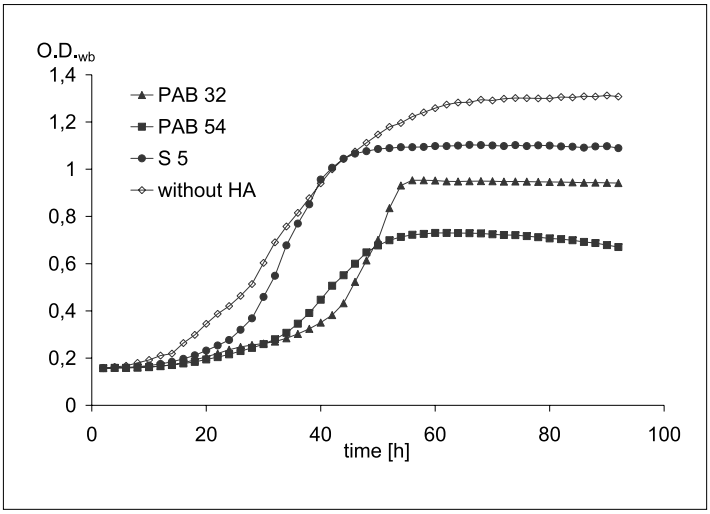

Fig. 1. *Candida maltosa* growth curves at the optimal concentration of phenol (1 $g \cdot l^{-1}$) in the presence of HA (PAB 32, PAB 54 and S5, concentration 0.05 $g \cdot l^{-1}$)

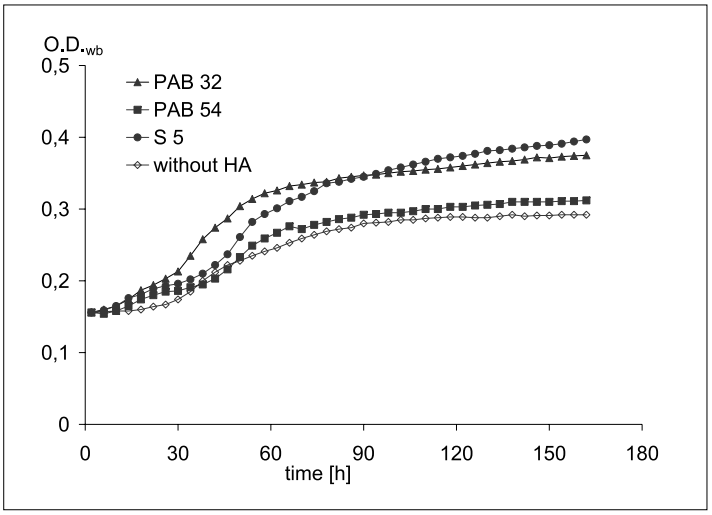

Fig. 2. *Rhodococcus erythropolis* growth curves at the optimal concentration of phenol (0.3 $g \cdot l^{-1}$) in the presence of HA (PAB 32, PAB 54 and S5, concentration 0.05 $g \cdot l^{-1}$)

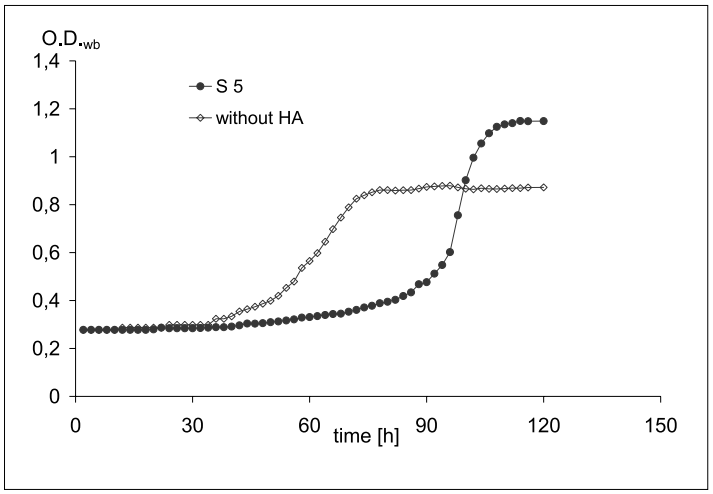

Fig. 3. *Candida maltosa* growth curves at higher concentration of phenol (1.5 g·l^{-1}) in the presence of HA (S5, concentration 0.05 g·l^{-1})

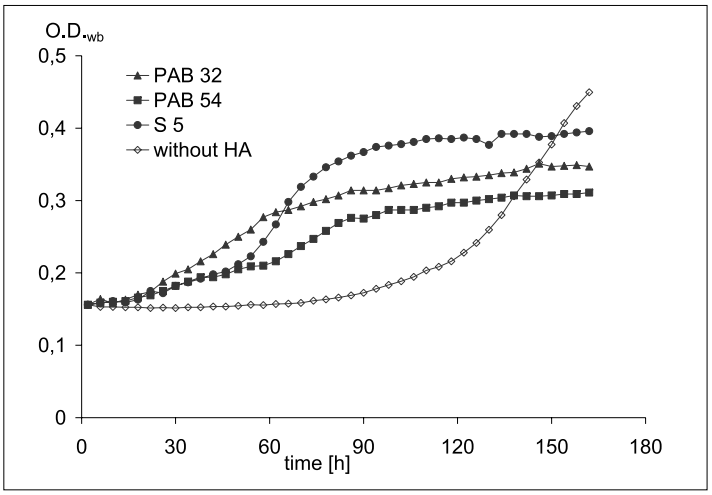

Fig. 4. *Rhodococcus erythropolis* growth curves at higher concentration of phenol (0.7 g·l^{-1}) in the presence of HA (PAB 32, PAB 54 and S5, concentration 0.05 g·l^{-1})

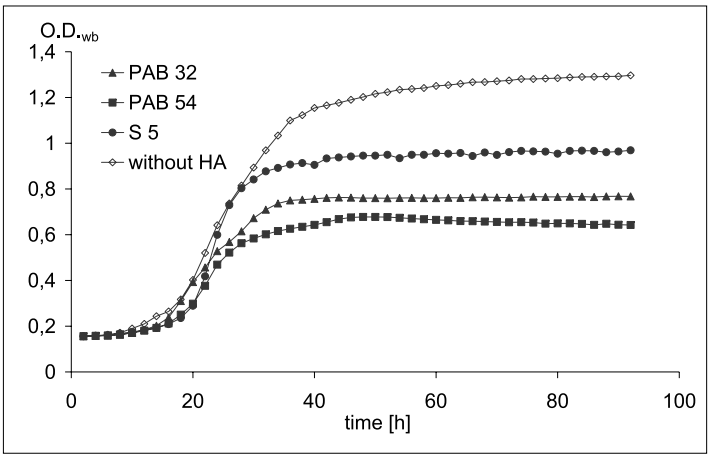

Fig. 5. *Candida maltosa* growth curves on catechol (0.5 g·l^{-1}) in the presence of HA (PAB 32, PAB 54 and S5, concentration 0.05 g·l^{-1})

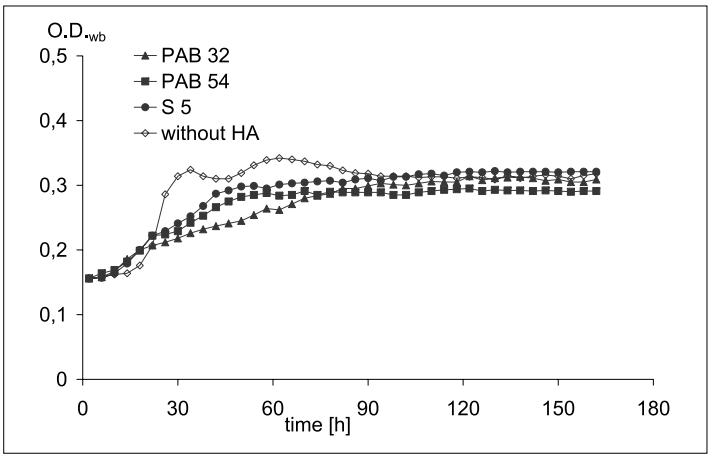

Fig. 6. *Rhodococcus erythropolis* growth curves on catechol (0.1 g·l^{-1}) in the presence of HA (PAB 32, PAB 54 and S5, concentration 0.05 g·l^{-1})

exposed and not exposed to HA did not differ significantly.

3.2. *Study of the Microbial Surface*

The electron microscopy investigation of the cells showed that HA easily form an additive layer on microbial surfaces. A marked covering of the cell surfaces by HA was visible after a 12 h contact of HA with the yeast sample and after a 48 h contact with the bacterial sample (Fig. 7–10).

4. Discussion

We have confirmed that the influence of HA on the microbial growth inhibition by phenolic compounds differs according to their origin. As reported by Janoš [3], the structure and the composition of the HA molecules are highly dependent on their origin and the isolation process used. This probably causes the different effects on the microbial growth of the single HA samples. However, certain tendencies are similar.

None of the studied HA can be used as a satisfactory carbon and energy source. HA seem to influence the microbial growth indirectly, by forming a layer on the microbial surface *via* adsorption. This layer probably serves as a transport barrier, which causes a decrease in concentration of phenolic compounds in the inner cell space.

This deficit seems to be positive in the case of the bacteria. The grow rates and the biomass yields were higher when the microorganism was grown in the presence of HA. Even at low and optimal concentrations of phenolic compounds, some toxic effects were indicated. Nevertheless, the HA layer can reduce the penetration of phenolic compounds into the cells, and thus protect the microorganism.

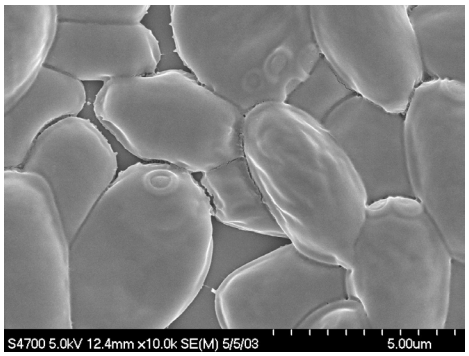

Fig. 7. Photo of *Candida maltosa* cells after 12 h cultivation in phenol; electron microscope (Hitachi, Canada)

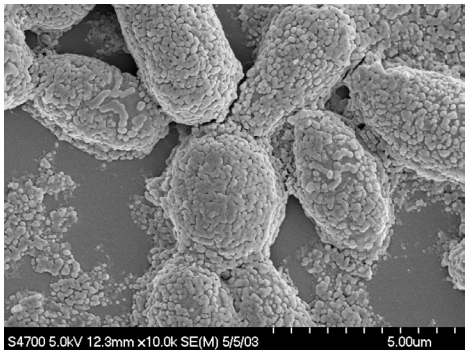

Fig. 8. Photo of *Candida maltosa* cells after 12 h cultivation in phenol and in the presence of 0.05 g·l^{-1} HA S5; electron microscope (Hitachi, Canada)

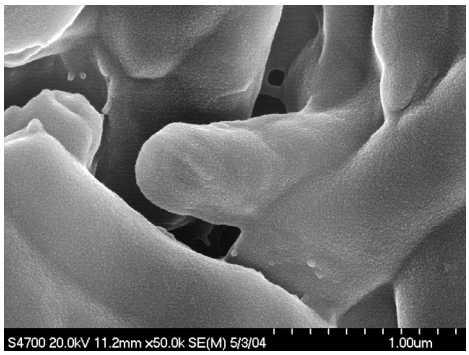

Fig. 9. Photo of *Rhodococcus erythropolis* cells after 48 h cultivation in phenol; electron microscope (Hitachi, Canada)

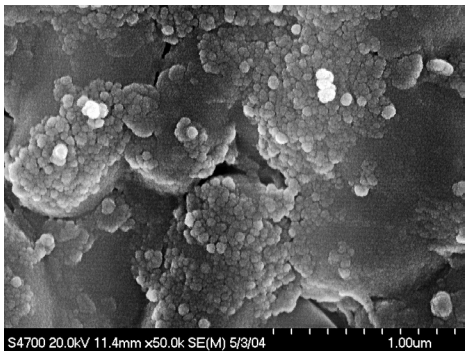

Fig. 10. Photo of *Rhodococcus erythropolis* cells after 48 h cultivation in phenol and in the presence of 0.05 $g \cdot l^{-1}$ HA S5; electron microscope (Hitachi, Canada)

On the other hand, the yeast population grew faster and the biomass yields were higher when the cultivation was carried out without HA. As mentioned above, the HA layer possibly reduces the penetration of phenolic compounds into the cells. However, the phenolic compounds used did not show any marked toxic effect towards the yeast cells. Even at relatively high concentrations of phenol the population grew very well. The HA layer on the yeast cell surface causes a lack of carbon and energy source in the inner cell space, and therefore the growth of microbial populations is inhibited.

5. Conclusions

The different humic acids under investigation vary in the intensity of their effects on the studied microorganism growth. However, the tendencies are similar for all of them. None of the humic acid preparations can be used as a satisfactory carbon and energy source for the yeast *Candida maltosa* or the bacteria *Rhodococcus erythropolis*. Humic acids are easily adsorbed onto the cell surface of the investigated microorganisms, and thus they form a layer. The humic acid layer probably serves as a transport barrier, which decreases the penetration of molecules into the inner cell space. In the case of the bacteria, the humic acid layer protects the cells against the toxic effect of phenolic compounds (even at optimal concentration). In the case of the yeast, the humic acid layer causes a lack of a carbon and energy source in the inner cell space (even at higher concentrations), and therefore inhibits the growth.

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