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THE CELLULAR BIODEGRADATION OF DI- AND TRIHYDROXYBENZENES

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ABSTRACT. The enzymatic transformation of di- and trihydroxybenzenes with water-suspended mixed *Pelobacter acidigallici* and *Pseudomonas putida* cells was investigated.

Key words: *Pelobacter acidigallici, Pseudomonas putida, enzymatic hydroxylation, cellular biodegradation, dihydroxy benzene*

INTRODUCTION

Phenols widely exists in many industry wastewaters and effluents. Their derivatives like chlorophenol, especially 4-chlorophenol and di- and trihydroxy benzenes are been detected in several industries (wood preservation, agriculture by using pesticides, fungicides and herbicides, byproducts formed during bleaching of pulp with chlorine or chlorination of drinking water [1-3]. Subsequently they have widely contaminated soil and groundwater, and their toxicity seriously affects living organisms. Even low concentration of these phenols could seriously damage the enviroment.

The efficient removal of these compounds from industrial aqueous effluents is of great practical significance for environmental protection. Many environment protection problems caused by phenols can be solved by employing the ability of microorganisms to degrade these compounds [4-11].

Pelobacter acidigallici have the ability to metabolise gallic acid into acetate via trihydroxybenzenes. As reported by us this bacterial strain can be used succesfully for the biodegradation of several kind of trihydroxibenzenes. In the present work we describe the interaction of mixed *Pelobacter acidigallici* and *Pseudomonas putida* cells with dihydroxybenzenes.

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RESULTS AND DISCUSSION

First we investigated the interaction of the dihydroxybenzenes (cathecol, resorcine, hydroquinone) with *Pseudomonas putida* cells. It was shown that this microorganism has the ability to introduce in *ortho* position a hydoxylic group when the substrate is a substituted or not substituted phenol. Using HPLC-MS (UV) first the chromatographic separation of the comercially available dihydroxybenzenes and their possible reaction products, pirogallol for the hydroxylation of catechol and rezoricine and 1,2,4-trihydroxy benzene for the transformation of hydrochinone was set up. As we aspected all the three dihydroxy benzenes were ben transformed into the corresponding products as it is shown in Figure 1.

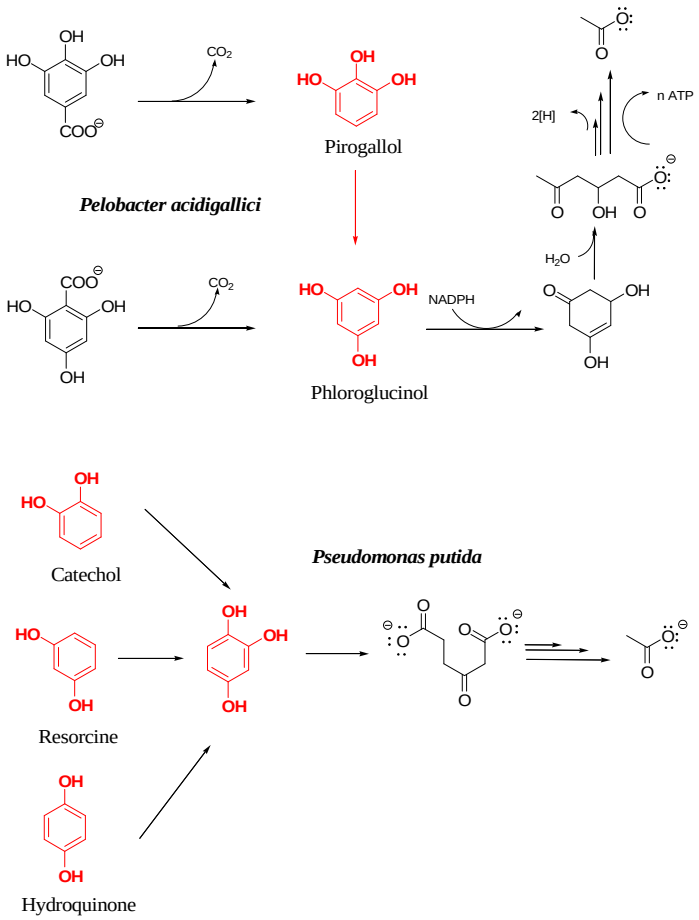

Figure 1. The enzymatic transformation of the dihydroxybenzenes followed by the metabolism of the formed trihydroxybenzenes

For all of the cases kinetic data shown an approximative 25μmol/min/mg enzyme activity.

As it was demonstrated by us using *Pelobacter acidigallici* cells pirogallol can be converted into phloroglucinol. The lattes compound will be further transformed by the cells into acetic acid .

In contrast to the metabolism of pirogallol, 1,2,4 trihydroxibenzene will be enzymatically converted into succinyl acetate, which will be further transformed in the Krebs cycle into acetyl-CoA.

In this way we demonstrated that enzymes presents in *Pelobacter acidigallici* are able to transforme di- and trihydroxybenzenes. Further we investigated the interaction of the above mentioned compounds with *Pelobacter acidigallici* harvested cells.

As reference we used the kinetic data for the biodegradation of gallic acid, a natural substrate of *Pelobacter acidigallici*. Using solution with variable concentrations of the natural substrate first the specific activity of the cells was determined. For this, after the incubation of the gallic acid with *Pelobacter acidigallici* cells periodically samples were taken which were centrigugated and subjected to HPLC-UV analysis.

Further all the three dihydroxybenzenes were incubated in the same manner with the *Pseudomonas putida* cells. In Table 1 it is shown the values for the specific activity of the cells for the transformation of gallic acid in *Pelobacter acidigallici* compared with the degradation of resorcine in *Pseudomonas putida*.

Table 1. Specific activity for the cellular degradation of gallic acid and resorcine.

Entry	$c_{substrate}$ [mM]	Specific activity [U/g cell] Gallic acid	Specific activity [U/g cell] resorcine
1.	0.05	3.61	-
2.	0.1	3.53	0.59
3.	0.25	7.23	0.88
4.	0.5	7.44	1.37
5.	0.75	7.53	1.76
6.	1	7.78	1.84
7.	1.5	8.39	2.13
8.	2	8.29	2.27
9.	2.5	8.39	2.45
10.	3	8.61	-

Using the Lineweaver-Burke liniarization method it was demonstrated that the global rate of the transformation of the polihydroxybenzenes is guverned by enzymes. Further it was calculated the k_M and the v_{max} for the biodegradation of all the mentioned compounds.

Table 2. The Michaelis constant and the relative rate of the cellular transformation of polihydroxybenzenes.

Substrate	K_M [μM]	$V_{max}/V_{max\ gallic\ acid}$
Gallic acid	0.032	1
catechol	0.076	0.34
pirogallol	0.134	1.00
phloroglucinol	0.048	1.00
resorcine	0.032	0.29
hydrochinone	0.048	0.49

After that we studied the kinetc parameters for the transformation of the natural substrate of the cells in presence of di- and trihydroxybenzenes. For this we suspended the *Pellobacter acidigallici* and *Pseudomonas putida* cells in solution of gallic acid and we add different amount of polihydroxy benzenes into the suspension. The working-up and analytical procedures were the same as used for activity measurement. It was shown that all of the used phenols are weak competitive inhibitors for the biodegradation of the gallic acid as shown in Table 3.

Tabele 3. V_{max} K_M and competitive inhibition constants for the biodegradation of gallic acid in presence of various phenols

Compound	K_M [mM]	V_{max} [U/mg]	K_i [μM]
catechol		25.76884	18±2
pirogalhol		23.16081	33±5
phloroglucinol	15.72	24.14445	41±10
resorcine		23.20162	25±5
hydrochinone		22.76602	39±2

Finally we investigated the biodegradation of the mixture of all of the studied compounds using an initial concentration of 10mM for each compound. Due to their weak inhibitory effect the global biodegradation rate of each compound was pourly affected, the individually decreassing of the reaction rate for each compound was not higher than 10%. After 24 hours no phenolic compond was detected using HPLC-UV analysis. It is known that the detection limit for these compounds is arround 1μM. To detect lower concentration of untransformed phenols, the cellular suspension was centrifuged and the supernatant was filtered on a small size C18 cartridge. The cartridge was eluted with a mixture of acetonitrile-water (50:50, v/v). from the obtained concentrated solution was determined a rezidual untransformed mixture of phenols with 0.1-0.15 μM concentration calculated for the volume of the initial cellular suspension.

CONCLUSIONS

It can be concluded that the mixture of *Pelobacter acidigallici* and *Pseudomonas putida* cells in water suspension can be succesfully used for the biodegradation of polihydroxy phenols. The biochemical path of these biotransformation was demonstrated and kinetic measurement were performed for each individual compound. Activity measurements using a two component mixture of the natural substrate and a poliphenol demonstrated that the global rate for the transformation of gallic acid was weakly afected. Moreover the biodegradation of the complex mixture of phenols undergoes with good rate, the untransformed phenol concentration was not higher than 0.15 μM.

EXPERIMENTAL SECTION

Pelobacter acidigallici and *Pseudomonas putida* cells were grown and isolated as described in standard methods. The enzyme assay was carried out at 30 ^{o}C in a discontinuous fashion by HPLC analysis of the products.

Their concentrations in the assay mixture were as follows: 100 mM potassium phosphate buffer, (pH 7.2), 10 mmol dihydroxybenzene, and 1 U of enzyme in 1 mL. Samples (20 μL) were withdrawn with a unimetric-pipet and the reaction was terminated by adding 0.1 M H_3PO_4 (5 μL) and diluted 10 times with double distilled water followed by the centrifugation before injection. The withdrawals occurred soon after start of the reaction and then at regular intervals. The conditions for the HPLC analyses were: on a C18 ec column using as eluent 20 mM HCl in water: acetonitrile (95: 5, v/v) in an isocratic manner at a 1 mL$\times$min^{-1} flow rate.

For the cellular biodegradation a concentration of 1g harvested cell in 1 mL of buffer was used. The substrate concentration was varied in 10-50 mM range. Before injection cells were centrifugated and the supernatant was filtered. The HPLC analysis was performed as previously described.

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REFERENCES

1. A.R. Bielefeldt, H. D. Stensel. *Biodegradation,* **1999**, 10:1.
2. V. Kavitha, K. Palanivelu. *Chemosphere* **2004**, 55:1235.
3. S.H. Yuan, X. H. Lu. *Journal of Hazardous Materials*, **2005**, 118:85.
4. V.V. Vel'kov, *Biotekhnologiya*, **1995**, 3−4:20.

5. F.M. Khabibulina, A.A. Shubakov, I.B, Archegova, G.G. Romanov. *Biotekhnologiya*, **2002**, 6:57.
6. T.O. Anokhina, V.V. Kochetkov, N.F. Zelenkova, V.V. Balakshina, A.M. Boronin. *Prikladnaia Biokhimia Mikrobiolgiya*, **2004**, 40:654.
7. F. Bux, B. Akkinson, K. Kasan. *Water ScienceTechnology*, **1999**, 39:127.
8. A. Zumriye, A. Derya, R. Elif. *Process Biochemistry*, **1999**, 35:301.
9. A. Fialova´, E. Boschke, T. Bley. *Biodegradation*, **2007**, 18:719.
10. Z. Ai, P. Yang, X. H. Lu. *Journal of Hazardous Materials*, **2005**, 124:147.
11. Z. Aleksieva, D. Ivanova, T. Godjevargova. *Process Biochemistry*, **2007**, 37:1215.