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Co-metabolism of 2,4-dichlorophenol and 4-Cl-*m*-cresol in the presence of glucose as an easily assimilated carbon source by *Staphylococcus xylosus*

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ABSTRACT

Comparison of the ability of *Staphylococcus xylosus* to degrade 2,4-dichlorophenol and 4-Cl-*m*-cresol in separate cultures is reported. Bacterial adaptation and the continuous presence of glucose, as a conventional carbon source, were found to stimulate the degrading efficiency of *S. xylosus*. 4-Cl-*m*-cresol exhibited higher substrate-induced toxicity with K_{ig} value at 0.25 mM, comparing to 2,4-dichlorophenol (K_{ig} value at 0.90 mM) at initial concentration ranging from 0.1 to 0.5 mM. Degradation rate of 4-Cl-*m*-cresol was found to decrease only, revealing lower value of inhibition degradation constant (K_i at 0.019 mM) comparing to that of 2,4-dichlorophenol (K_i at 0.41 mM). Both glucose and each one of the chloro-aromatic compounds tested were simultaneously consumed and an increase of chloride ions in the medium appeared, during the exponential phase of growth. The chloride ions increase was nearly stoichiometric in the presence of 2,4-dichlorophenol and one of its several intermediate products identified was 2-Cl-maleylacetic acid. In the case of 4-Cl-*m*-cresol, only one metabolic product was found and identified as 3-methyl-4-oxo-pentanoic acid.

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1. Introduction

Phenolic effluents are produced by several industries including petroleum refineries, pharmaceutical and plastic industries, constituting thus important representatives of hazardous wastes [1]. Among them, 2,4-dichlorophenol (2,4-DCP) and 4-Cl-*m*-cresol are mainly used as disinfectants and intermediates in organic synthesis [2,3]. Their extensive use pose a serious threat for the environment and the human health, since 2,4-dichlorophenol and 4-Cl-*m*-cresol are included in the list of 129 priority pollutants according to US Environmental Protection Agency [4].

In most of the bioremediation processes Gram-negative bacteria are used. Pseudomonads have been extensively studied and constitute the main carrier of biodegradation studies due to their nutrient and metabolic diversity [5]. Gram-positive bacteria, i.e. *Rhodococcus* spp., have been proved recently very effective in bioremediation techniques [6]. Remarkable differences between Gram-positive and Gram-negative bacteria have not been observed, regarding the degradation mechanism of some aromatic compounds, since dioxygenases are mainly responsible for the ring cleavage [7]. However molecular toxicity resulting from enzyme inhibition, protein denaturation and membrane modification is not so apparent in Gram-negative species due to the activation of a broad range of defense mechanisms [8].

Staphylococcus sp. has been used in single cases of phenol and catechol degradation while it was found also able to utilize waste drilling fluid as a substrate [9,10]. Nielsen et al. [11] have isolated recently Gram-positive cocci, *Staphylococcus haemolyticus*, which showed an extreme solvent tolerance. In general, the reports regarding the degrading ability of *Staphylococcus* sp. are limited. Therefore, the previous report [11] may turn the attention towards a new direction.

Most of the studies involve biodegradation of toxic pollutants as sole carbon and energy source [12]. However, the tolerance of microorganisms towards the exhibited toxicity of these compounds is not unlimited. High concentrations of these compounds may change the activity of one or more enzymes, affect enzyme synthesis by interaction with genome or transcription process, alter cell's permeability and finally influence the total functional activity of the cell [13]. It is evident that many of these systems are not effective at high concentrations of pollutants, since the main result is low growth and degradation rates [14].

In order to prevent the unfavorable impacts on cells metabolism, many researchers have recently studied the effect of an easily assimilated carbon source on the biodegradation of a xenobiotic compound [15]. According to these studies, microorganisms have to perform different modes of action. In the wide majority of cases, conventional carbon sources at low concentrations stimulate

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microbial growth and thus more enzymes are available in order to attack the toxic compound [16]. However, in high concentrations, carbon catabolite repression may happen, suspending thus the microbial degradation [17].

Another issue, that has to be pointed out here, is the importance of culture history on the effective biodegradation of priority pollutants, since it affects strongly the physiological state of the cells, meaning their macromolecular composition, which is the synthesis and reaction of enzyme systems [18]. It has been also reported that bacteria continuously exposed in xenobiotic compounds have improved their degradation abilities [19,20]. In microbial communities, a natural selection between microbial species takes place due to pollution. This imminent change is referred as microevolution, according to Medina et al. [21]. If adaptation is too slow under experimental conditions, the addition of a variety of microbial strains carrying some desired genes might speed up evolution (directed evolution) [22].

Many mechanisms are responsible for this result: induction, mutation and horizontal gene transfer. The later is reported in bacteria of mixed and not single populations [23,24].

Here, the adaptation of *Staphylococcus xylosus* in the continuous presence of 2,4-dichlorophenol and 4-Cl-*m*-cresol separately, in combination with the addition of an easily assimilated substrate (glucose) is used for the stimulation of the degrading ability of this microbial strain. According to this, we report on the kinetics of biodegradation of 2,4-dichlorophenol and 4-Cl-*m*-cresol separately by *S. xylosus* in the presence of glucose as the carbon source. Mathematical models were adopted also in order to describe the relation between the degradation rates, the specific growth rates and the initial chloro-compound concentration. Measurements of total chloride ions liberated from each chlorinated compound were carried out, as well as a first estimation was made involving the molecular structures and identity of some metabolites, which are reported here.

2. Materials and methods

2.1. Bacterial strains

S. xylosus cells were obtained from the collection of the laboratory of Food Microbiology and Hygiene in the School of Agriculture, A.U.Th, Greece (Prof. E. Litopoulou-Tzannetaki). The strain was isolated from a mining industry near Stratoni, Chalkidiki, Greece and was identified according to the criteria described in Bergey's Manual of Systematic Bacteriology [25].

2.2. Adaptation experiments

Adaptation experiments of *S. xylosus* were performed by successive cultivations in the mineral medium described by Dorn et al. [26] in the presence of 0.1% (5.5 mM) glucose and 0.1 mM 2,4-DCP. Acclimation period was completed, when there was no further change in the residual concentration of 2,4-DCP in the medium. Cells were collected at the end of their exponential phase and spread onto minimal agar plates containing 2,4-DCP and glucose as carbon source. Plates were incubated at 30 °C for 2 days. Similar adaptation experiments were performed in the presence of 0.1 mM 4-Cl-*m*-cresol and 0.1% glucose.

2.3. Culture conditions

S. xylosus cells were grown in the mineral medium described above in the presence of 0.1% glucose and 2,4-DCP at 0.1–1.0 mM concentrations. The increment in the final concentration of 2,4-DCP was made subsequently, starting from 0.1 mM, according to bacteria's growth characteristics and degradation ability. The addition of toxic substrate in each culture was done at the early beginning of the exponential phase in two doses (OD_{600} approx. 0.1) 5 min apart from each other. Batch cultivations were carried out aerobically at 30 °C in Erlenmeyer flasks in a water bath at 150 rpm, with an air to liquid ratio of 5:1 (v/v). Liquid stock cultures were maintained in the refrigerator at −70 °C, in the presence of 10% glycerol. The same culture procedure was followed in the case of 4-Cl-*m*-cresol, as the other toxic substrate, at 0.1–0.5 mM concentrations.

2.4. Measurement of cell growth

Growth of the cultures was monitored, by measuring optical density at 600 nm, with a Shimadzu UV–Vis spectrophotometer. The specific growth rates were estimated by the slope of the exponential phase of each growth curve at all concentrations of 2,4-DCP and 4-Cl-*m*-cresol investigated in this work.

2.5. Extraction of 2,4-DCP and 4-Cl-*m*-cresol

Samples of 5 ml from culture medium were taken hourly mainly during the exponential phase, and at the end of the stationary phase (24 h). They were centrifuged at $2000 \times g$ for 20 min and the supernatant was extracted three times with two-fold volumes of ethyl acetate. The organic layers were collected and processed as previously reported [27]. The average recovery for 2,4-DCP and 4-Cl-*m*-cresol, respectively was 95 ± 3%.

2.6. Analytical methods

2,4-DCP and 4-Cl-*m*-cresol were determined by reverse phase high-pressure liquid chromatography (HPLC) using a C18 column (250 mm length and 4.6 mm internal diameter) and UV detection at 270 nm. The mobile phase consisted of acetonitrile/H_2O/CH_3COOH in the ratio 70/30/0.5 at a flow rate of 0.75 ml min^{-1}. The detection limit of both compounds was 0.35 mg l^{-1}. Retention times of reference 2,4-DCP and 4-Cl-*m*-cresol were estimated at 3.5 and 3.8 min, respectively.

Calibration curves were obtained from their reference solutions by extracting 2,4-DCP and 4-Cl-*m*-cresol, respectively with ethyl acetate, following exactly the above-mentioned procedure.

Metabolites were determined by GC–MS analysis, using a GCQ Plus MS system (EI, ThermoQuest Finnigan, USA), connected with a Trace GC 2000 (ThermoQuest CE Instruments, USA) gas chromatography system.

Glucose was determined photometrically, by measuring the absorbance at 575 nm, using dinitrosalicylic acid method [28].

2.7. Chloride ions release

Chloride ions in the culture medium were monitored by anion chromatography, using Dionex 4500i model with a Dionex Ion Pak column (AS9-HC, 4 mm).

2.8. Chemicals

All the solvents and reagents used in the present study were purchased from Merck Chemicals industry and were HPLC grade.

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