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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.

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 $95 \pm 3\%$.

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.

3. Results and discussion

3.1. Adaptation of the bacterial strain S. xylosus

First, a series of growth experiments were conducted using 2,4-DCP and 4-Cl-*m*-cresol as the only carbon source in separate cultures at concentrations ranging from 0.05 to 0.2 mM, in the presence of vitamins and amino acids as growth factors. Since no real growth was observed under these experimental conditions, attempt was made to use 2,4-DCP and 4-Cl-*m*-cresol in combination with glucose (0.1%), where the biomass increase was evident. The amount of glucose used was the minimum one required, to fortify and not to repress bacterial growth.

In order to investigate the evolutionary ability of *S. xylosus* and improve its degradation ability, successive cultivations of the bacterial stain were carried out in the continuous presence of 0.1 mM 2,4-DCP and 4-Cl-*m*-cresol separately in combination with glucose.

In most cases reported, bacterial adaptation is achieved by gradually increasing the initial xenobiotic concentration. However, in these studies the organic pollutant is used as the main carbon source by the bacterial biomass, and it obviously exhibits wider limits of tolerance [20]. It has to be mentioned that despite the continuous exposal in a persistent compound, still there is a threshold concentration above which gradual decrease in total cell number is not reported [20]. In addition, Spain and Van Veld [29] have reported that the extent of adaptation in *p*-nitrophenol was not always directly proportional to its pre-exposure concentration. According to these and due to limited growing ability of *S. xylosus* higher concentrations of 2,4-DCP were not tested.

Fig. 1a and b gives the total percentage of consumption and the specific growth rate of 2,4-DCP and 4-Cl-*m*-cresol, respectively, at the end of the exponential phase after each culture. As it is shown in this figure, three phases are clearly discriminated for both parameters measured, in relation to the number of cultivations. During the first phase, no change in specific growth or in percentage of consumption is evident. The cells seem to prepare themselves in order to better assimilate each xenobiotic compound. The improved performance of *S. xylosus* becomes apparent from the second-active phase, where an immediate increase in its specific growth rate and the percentage of 2,4-DCP and 4-Cl-*m*-cresol consumption is observed. The end of the active phase, where no further increase in both parameters is observed, indicates the end of the adaptation period.

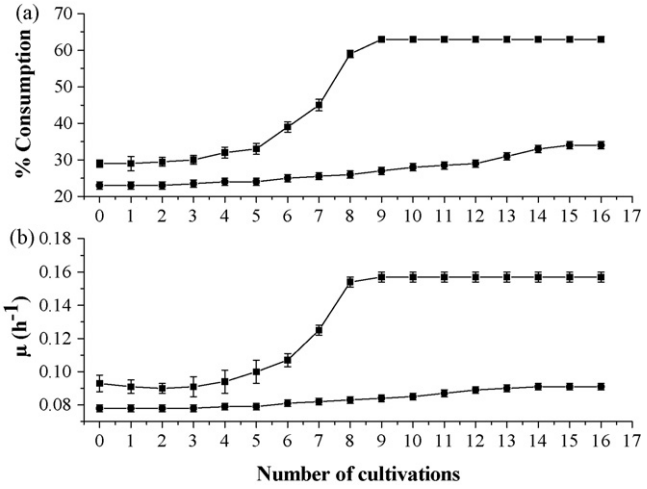

Fig. 1. Percentage of consumption (a) and specific growth rates (b) of *Staphylococcus xylosus* successive cultivations in 0.1 mM 2,4-DCP (■) and 4-Cl-*m*-cresol (●), in the presence of glucose. Error bars indicate the deviation of triplicate experiments.

From Fig. 1 also it can be seen clearly that less cultivations are needed, in order the cells to be adapted in the presence of 2,4-DCP. The percentage of consumption as well as the specific growth rate approaches remarkable higher values (63% and 0.157 h^{-1}, respectively) in the case of 2,4-DCP, contrary to 4-Cl-*m*-cresol where the two parameters have distinctively reached lower values (34% and 0.091 h^{-1}, respectively), showing higher persistence in biodegradation. Similar results were obtained with *Pseudomonas* sp. showing higher resistance of 4-Cl-*m*-cresol to biodegradation [27].

The increase in specific growth rate with successive cultural passages of the same bacterium reflects the important role that exposure to unusual compounds could play in the adaptation of microorganisms to xenobiotics, in order to obtain higher efficiency of degradation [30].

The increment in both parameters observed could be attributed to changes in macromolecular composition, DNA modifications and RNA synthesis, which is known as physiological adaptation [18], and in our case explains better *S. xylosus* performance.

According to the above, the acquisition of degradation ability by the microorganism can be proved, as it has already been reported [31], very useful in bioremediation processes.

3.2. Effect of 2,4-DCP and 4-Cl-m-cresol concentration on bacterial growth

The highest specific growth rate was attained in the presence of glucose only (0.177 h^{-1}), as it was expected, whereas in the presence of 2,4-DCP and 4-Cl-*m*-cresol only, there was an extremely weak growth of *S. xylosus.*

Cell's growth is continuous and there is no delay, despite the addition of 2,4-DCP 2 h approx. after the inoculation of the medium. However, the initial concentration of 2,4-dichlorophenol affects the duration of the exponential phase, since at low initial concentrations (0.1 mM) the growth is completed after 7 h, whereas at higher initial concentrations 10 h are needed. Similar results were obtained in the case of 4-Cl-*m*-cresol (data not shown).

In order to better understand the effect of the initial concentration on cell's growth, specific growth rates were calculated at all concentrations studied. Fig. 2a and b gives the reciprocal value of specific growth rate of *S. xylosus* at various concentrations of 2,4-DCP and 4-Cl-*m*-cresol, respectively. As it is indicated, the specific growth rate is decreased with increasing substrate concentration for both 2,4-DCP and 4-Cl-*m*-cresol, possibly because of substrate-induced toxicity, which is more evident with 4-Cl-*m*-cresol.

Using a non-competitive inhibition model for the growth of *S. xylosus* in the presence of these two xenobiotic compounds, each one separately, the growth inhibition constant and the maximum specific growth rate were calculated from the following linearized Eq. (1), according to Banerji and Bajpai [32].

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{I}{\mu_{max} K_{ig}} \quad (1)$$

where μ is the specific growth rate (h^{-1}), μ_{max} the maximum specific growth rate (h^{-1}), I the 2,4-DCP or 4-Cl-*m*-cresol initial concentration (mM) and K_{ig} is the growth inhibition constant (mM). The calculation of growth inhibition constant is very important since indicates the loss of multiplication ability of microorganisms due to the continuous presence of an organic pollutant [33].

In the case of 2,4-DCP only (Fig. 2a), the yielded group of points were not possible to be linked by one common regression straight line, since at higher concentrations than 0.5 mM there is a steep drop in the values of specific growth rate. Because of this non-linear inhibition, two straight lines, 1 and 2 each for one concentration area between 0 and 0.5 mM and 0.5 and 1.0 mM, respectively were used in order to better characterize the system.

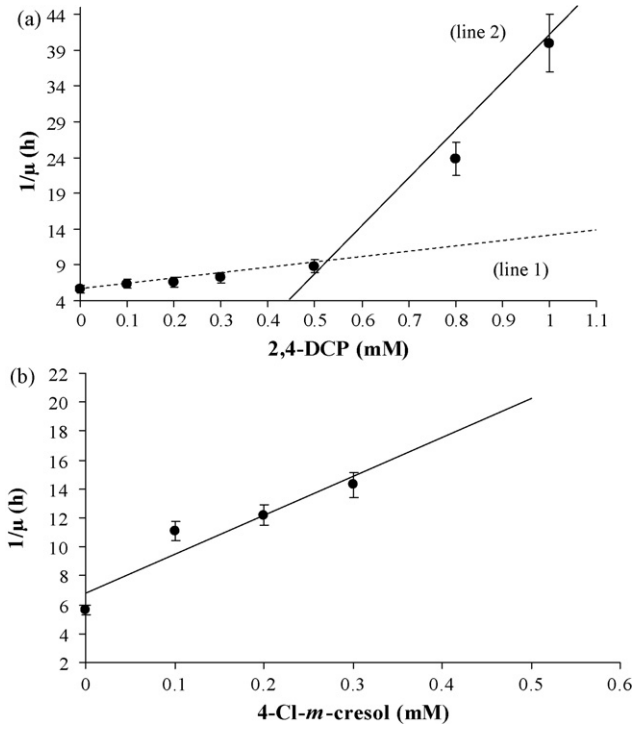

Fig. 2. Determination of K_{ig} and μ_{max} values in the presence of 2,4-DCP (a) and 4-Cl-*m*-cresol (b). Error bars indicate the deviation of triplicate experiments.

Accordingly, Table 1 shows the values of the estimated parameters.

The first corresponding K_{ig} value (concentration range 0.1–0.5 mM, line 1) characterizes mainly the sensitivity of glucose catabolism because of the inhibitory presence of 2,4-DCP on the metabolic activity of the cells. The second K_{ig} value probably has been resulted because of the death of a high percentage of microorganisms, due to high toxicity values of the medium at high concentrations 0.5–1.0 mM of the xenobiotic. *S. xylosus* growth was inhibited mainly by 4-Cl-*m*-cresol, since the estimation of growth inhibition constant shows that inhibition occurs at low values of 4-Cl-*m*-cresol initial concentration, whereas no growth was observed at higher than 0.5 mM concentrations.

Growth characteristics constitute an important indication for the degrading efficiency of *S. xylosus*, which is expected to be more efficient in the presence of 2,4-DCP, as it is closely associated to the size of bacterial population (higher number of available degrading enzymes), whereas the value of the growth inhibition constant is higher [34]. It is worth mentioning that, there is only one report

Table 1
Calculated values of μ_{max} and K_{ig}

	Concentration range	
	0.1–0.5 mM (line 1)	0.5–1.0 mM (line 2)
2,4-DCP		
μ_{max} (h^{-1})	0.179 ± 0.013	0.044 ± 0.010
K_{ig} (mM)	0.90 ± 0.09	0.37 ± 0.05
r^2	0.97	0.98
		Concentration range 0.1–0.5 mM
4-Cl-*m*-cresol		
μ_{max} (h^{-1})		0.148 ± 0.023
K_{ig} (mM)		0.25 ± 0.003
r^2		0.89

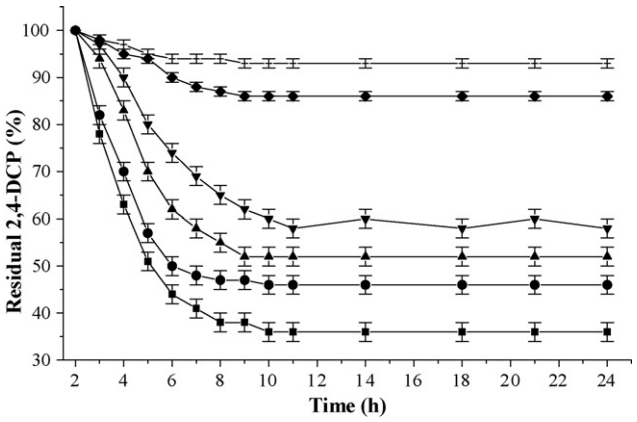

Fig. 3. Time course variations of residual 2,4-DCP percentage at concentrations (■) 0.1 mM, (●) 0.2 mM, (▲) 0.3 mM, (▼) 0.5 mM, (♦) 0.8 mM and (+) 1.0 mM 2,4-DCP, in the presence of glucose. Error bars indicate the deviation of triplicate experiments.

where the phenomenon of non-linear growth inhibition is studied and involves the degradation of 4-Cl-*m*-cresol in a system of natural-self purification using different microbial populations [3].

3.3. 2,4-Dichlorophenol and 4-Cl-m-cresol degradation kinetics

Fig. 3 shows the effect of the initial concentration of 2,4-dichlorophenol (100% percentage of the initial concentration expressed) on its consumption by *S. xylosus*. It can be clearly seen that 2,4-DCP is more easily assimilated at low concentrations, since its percentages of consumption are higher (61 and 7% at 0.1 and 1.0 mM, respectively). However, with 4-Cl-*m*-cresol, the percentages of consumption are lower (34% at 0.1 mM) and the degrading ability is eliminated at higher than 0.2 mM concentrations. On the contrary, with 2,4-DCP the cells are active in a wider concentration range (0.1–1.0 mM).

Our data show that *S. xylosus* does not seem to differ from *Pseudomonas* sp., concerning 2,4-DCP percentages of consumption (65 and 11% at 0.1 and 1.0 mM, respectively) as it is reported [27]. As far as 4-Cl-*m*-cresol consumption is concerned, *Pseudomonas* sp. is more effective than *S. xylosus*, since its degrading ability is evident at higher concentrations than 0.2 mM (26% at 0.3 mM) [27]. Thus a mixed culture of *Pseudomonas* sp. and *S. xylosus* could be an attractive potential, improving 4-Cl-*m*-cresol's degrading efficiency, as it has been reported in other cases [35,36].

The assimilation of both chloro-compounds tested, is accomplished during the exponential phase of growth and is terminated after the cells enter the static phase, which indicates that degradation in this case also is closely associated with the basic cell's metabolism. As a result, degradation proceeds up to some extent in a short period of time (approx. 7 h for both 2,4-DCP and 4-Cl-*m*-cresol). These short time courses required constitute an important advantage of our system, since longer incubation periods have been reported elsewhere [37].

Fig. 4 depicts variation of initial degradation rate with the initial 2,4-DCP concentration. As it can be seen, the rate is increased with the initial 2,4-DCP content up to 0.5 mM approximately and then is decreased at higher 2,4-DCP concentrations, indicating inhibitory effects at concentrations above 0.5 mM.

Since the data presented in this figure show a typical substrate inhibition kinetics, the following equation was used to describe the batch kinetics of 2,4-DCP degradation by *S. xylosus* on the basis of initial rates as reported [38,39].

$$R = \left(\frac{R_{max} I}{K_S + I}\right)\left(\frac{K_i}{K_i + I}\right) \quad (2)$$

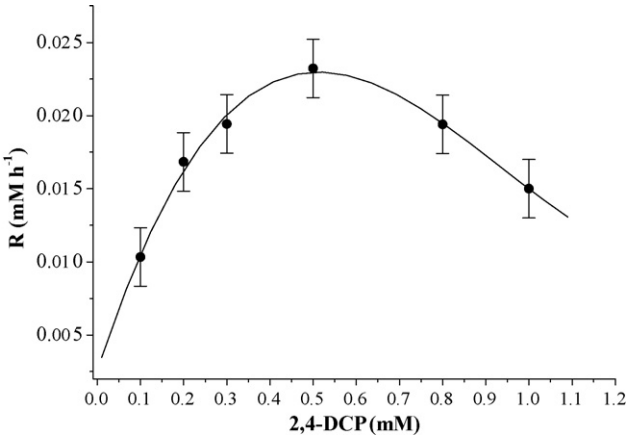

Fig. 4. Effect of initial 2,4-DCP concentration on its degradation rate with *S. xylosus* in the presence of glucose. Error bars indicate the deviation of triplicate experiments.

where R is the actual degradation rate ($mM\,h^{-1}$), calculated for the first 5 h after the final chloro-compound addition, by using the equation

$$R = \frac{\Delta I}{\Delta t} \quad (3)$$

where ΔI is $(I_2 - I_7)$ and Δt is 5 h. Also R_{max} is the maximum degradation rate ($mM\,h^{-1}$), K_S is the saturation constant (mM) and K_i is the inhibition degradation constant (mM).

When $I \ll K_i$ the inhibition term can be neglected and Eq. (2) may be written as

$$R = \frac{R_{max} I}{K_S + I} \quad (4)$$

Eq. (4) is relevant to the well known Monod equation. In double reciprocal form Eq. (4) can be written as

$$\frac{1}{R} = \frac{1}{R_{max}} + \frac{K_S}{R_{max}} \frac{1}{I} \quad (5)$$

A plot of $1/R$ versus $1/I$ yields a line with a slope of K_S/R_{max} and intercept of $1/R_{max}$.

Experimental data presented in Fig. 4 (for $I < 0.5$ mM) were plotted in form of $1/R$ versus $1/I$ (data not shown). From the slope and intercept of the best-fit line the following values were found:

$$R_{max} = 0.037\,mM\,h^{-1} \text{ and } K_S = 0.26\,mM\,(r^2 = 0.99)$$

For high initial 2,4-DCP concentrations ($I \gg K_S$) the inhibition term becomes the determining factor. Therefore, the rate expression takes the following form, by analogy with Eq. (1), showing the inhibition effect to biodegradation.

$$R = \frac{R_{max} K_i}{K_i + I} \quad (6)$$

In double reciprocal form Eq. (6) can be written as

$$\frac{1}{R} = \frac{1}{R_{max}} + \frac{1}{R_{max} K_i} I \quad (7)$$

A plot of $1/R$ versus I yields a line with a slope of $1/K_i\,R_{max}$ and intercept of $1/R_{max}$.

Experimental data presented in Fig. 4 (for $I > 0.5$ mM) were plotted in form of $1/R$ versus I (data not shown). From the slope and intercept of the best-fit line the following values were found:

$$R_{max} = 0.053\,mM\,h^{-1} \text{ and } K_i = 0.41\,mM\,(r^2 = 0.93)$$

In the case of 4-Cl-*m*-cresol inhibition it was evident only at concentrations from 0.1 to 0.2 mM, since degradation rate was decreased with its initial concentration (data not shown). Eq. (7) was also used for the determination of both parameters R_{max} and K_i and the following values were found:

$$R_{max} = 0.028\,mM\,h^{-1} \text{ and } K_i = 0.019\,mM\,(r^2 = 0.99)$$

The above values show the strong inhibitory effect that 4-Cl-*m*-cresol performs to its own degradation, contrary to 2,4-DCP. That difference in resistance to biodegradation is probably due to the different substituents of the aromatic nucleus, which has resulted in low affinity of the participating enzymes. There are some reports showing the negative impact of a methyl-substituent introduction in the aromatic nucleus. More specifically, Bergauer et al. [40] studied the biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts and found that *m*-cresol was more resistant to biodegradation comparing to other aromatic compounds, including phenol, showing a relation between the chemical structure of the compounds and their exerted toxicity.

In contrast to 4-Cl-*m*-cresol, 2,4-DCP is more susceptible to biodegradation as the degradation rates and the above estimated parameters R_{max} and K_i indicate. The results of this study suggest that 2,4-DCP degradation activity is less inhibited comparing to growth, since the specific growth rate is only reduced in the presence of various initial concentrations of 2,4-DCP. *S. xylosus* preserved its degradation potential despite the non-optimum growth conditions. It is interesting to be mentioned, that the value of the degradation inhibition constant K_i (0.41 mM) coincides with the growth inhibition constant K_{ig} (0.37 mM at 0.1–0.5 mM 2,4-DCP initial concentration), which indicates the strong relation between growth and degrading activity. In addition, the substrate-inhibited kinetic model used, in order to correlate the experimental data, showed that strong inhibition on degradation activity is evident at high concentrations of 0.8–1.0 mM. This is a very important notice leading to the conclusion that a wastewater process industry may effectively operate at a wide range of concentrations (from 0.1 to 0.5 mM).

The estimation of the saturation constant K_S, gave high value of this parameter, which is associated with the low specificity of the bacterial system, indicating that cells use glucose as carbon source and co-metabolize 2,4-dichlorophenol [41]. In most reports, the values of this parameter reported are significantly lower (<100 $\mu g\,l^{-1}$), which is closely associated with highest specificity ability of the bacteria adopted [42]. Nevertheless the high value of K_S estimated in this case showed that this system may operate in a wider concentration pollutant range under dynamic conditions, which is very important since modeling and design of an activated sludge system require knowledge of kinetic expression over a wide concentration range [42,43].

Comparing the degrading ability of *S. xylosus* with other microorganisms used for the same purpose, it seems that *S. xylosus* can be used in a wider concentration range of 2,4-DCP for its degradation [44]. Kargi and Eker [38,39] estimated high values of saturation and inhibition constant, using *Pseudomonas putida* cells in order to degrade 2,4-DCP, in the presence of glucose as carbon source, due to higher concentrations values studied (up to 1.8 and 4.6 mM, respectively). However, maximum degradation rates exhibited lower values due to extended degradation periods (8 and 10 days), comparing with the value reported here. It has to be mentioned that because of the high variability in the reported values of parameters, generalizations are difficult to be made, since each microbial system is unique, regarding strain characteristics, culture history and environmental conditions.

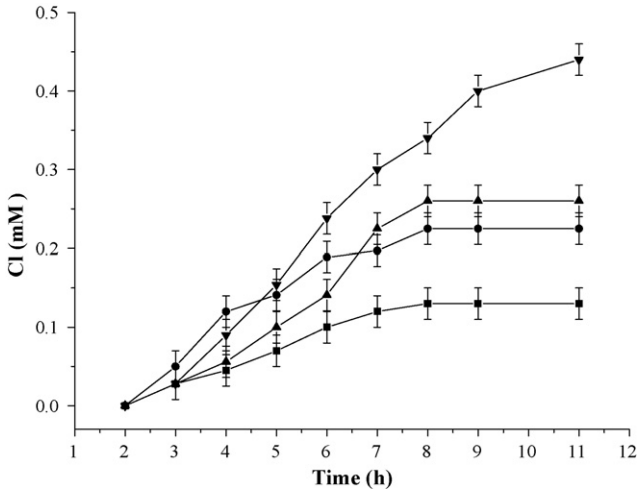

Fig. 5. Effect of 2,4-DCP at concentrations (■) 0.1 mM, (●) 0.2 mM, (▲) 0.3 mM and (▼) 0.5 mM on chloride ions liberation in the presence of glucose.

3.4. The effect of glucose

Glucose consumption and depletion of the two aromatic compounds tested, take place in parallel during the exponential phase of growth. Interesting, when glucose has been almost consumed, there is still 2,4-DCP or 4-Cl-*m*-cresol left in the medium, indicating that both xenobiotics degradation is a co-metabolic phenomenon, since the non-growth (secondary) substrate, can only be transformed in the obligate presence of a growth substrate as it is reported [45].

In order to study better the effect of the initial chloro-compound concentration on glucose depletion rate, the respective kinetic constants were calculated. The plot of time course variations with glucose concentration gave a straight line, revealing a zero-order reaction rate. In general, the biodegradation of an easily assimilated carbon sources, such as glucose or acetic acid, follows zero-order reaction kinetics due to the higher concentrations of these compounds, comparing with the lower concentrations of the toxic pollutants [46].

According to this, higher values of maximum degradation rate were estimated at low pollutant's concentrations (0.57 and 0.61 mM h^{-1} at 0.1 mM 2,4-DCP and 4-Cl-*m*-cresol, respectively), in contrary to higher concentrations where the degradation rate of glucose has lower values (0.21 mM h^{-1} at 1.0 mM 2,4-DCP), showing thus the inhibitory effect of both aromatic compounds on cell's metabolism.

From the above results it is evident that there is strong interaction between glucose and the recalcitrant pollutants used here, since the presence of glucose enhances pollutant's degradation, whereas the presence of the pollutant affects negatively the biochemical reactions associated with cell's main metabolism (growth inhibition reported before), slowing down the consumption of glucose, which is used as main carbon and energy source.

It is well known that the majority of wastewater treatment plants involve not only mixtures from agrochemicals industries, but also mixtures from domestic activities. It has been postulated that the presence of a conventional carbon source stimulates bacterial growth and alters the toxicity effects of hazardous chemicals on bacterial growth, which has been also observed here [47]. However, most of the studies involve cases where the toxic chemical can be used as the main carbon source by the microorganisms [48].

Another report concerning biodegradation of 2,4,6-trichlorophenol in the obligatory presence of glucose as a carbon source has been carried out [49]. In this case, where no structural similarity is evident between the two organic compounds, glucose is used for the production of cofactors such as NADH, which is participating in the degradation pathway of the organic pollutant as electron donor [50]. The concurrent utilization of glucose and xenobiotic in this study and the partial degradation reported is probably the result of their "complementary" activity.

3.5. Chloride ions liberation and metabolites detection

Fig. 5 shows the effect of 2,4-DCP initial concentrations on its dechlorination. Chloride ions liberation, which is nearly stoichiometric, is carried out during the exponential phase of growth and its final concentration in the medium is proportional to the initial concentration of 2,4-DCP. More specifically, at 0.5 mM 2,4-DCP the final chloride ions concentration of 0.45 mM is the highest estimated at all the concentrations range studied. This is evident after 5 h, since earlier chloride ions release is lower than the previous observed at 0.1 mM 2,4-DCP, probably due to increased medium toxicity values, which have caused a lag phase in the liberation of chloride ions. At 0.8 and 1.0 mM 2,4-DCP, chloride ions concentrations were not determined due to the low percentages of consumption (13 and 7%, respectively).

The stoichiometric chloride ions release is the result of the aromatic cleavage of 2,4-dichlorophenol, which leads in complete

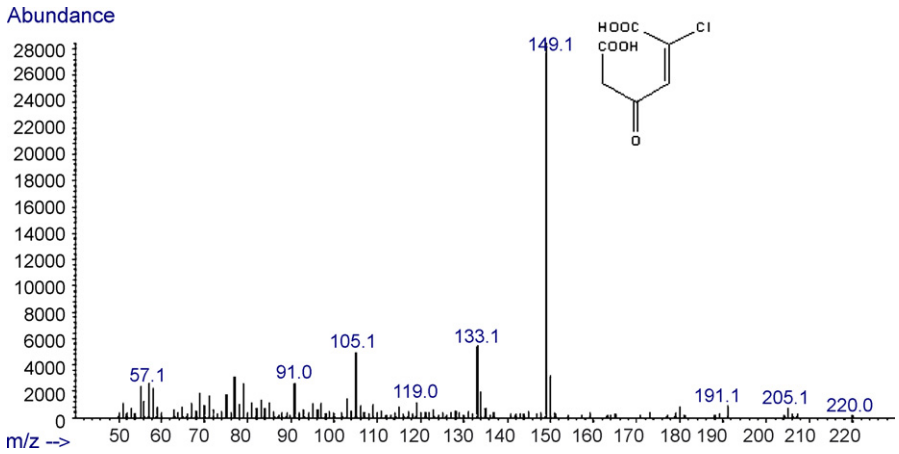

Fig. 6. Mass spectrum of 2-Cl-maleylacetic acid isolated from *S. xylosus* culture in 0.1 mM 2,4-DCP in the presence of glucose.

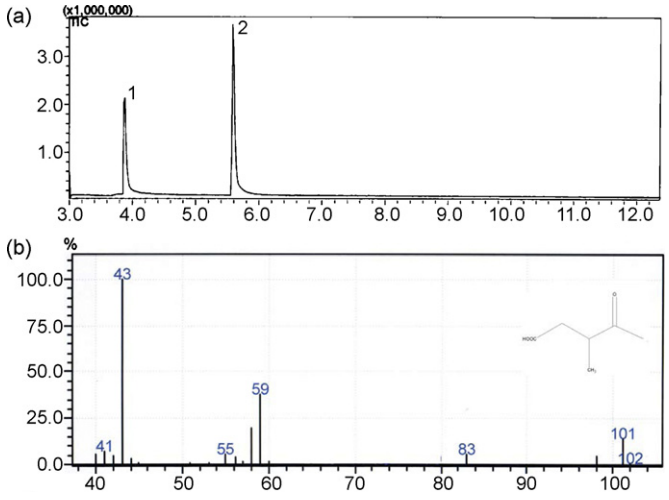

Fig. 7. (a) GC analysis of the culture extract obtained with *S. xylosus* in 0.1 mM 4-Cl-*m*-cresol in the presence of glucose (peak 1 solvent, peak 2 metabolic product of 4-Cl-*m*-cresol) and (b) MS spectrum of *S. xylosus* metabolic product.

degradation, according to previous reports [51]. The elucidation of the degradation pathway has showed the formation of 3,5-dichlorocatechol first [52].

GC–MS analysis of the culture extract obtained after 24 h showed the absence of 3,5-dichlorocatechol, which is also supported by the chloride ions release reported. The ring cleavage of 2,4-dichlorophenol is further supported by the identification of one ring cleavage product, which is 2-Cl-maleylacetic acid [52]. The mass spectrum of 2-Cl-maleylacetic acid is characterized by the presence of its molecular ion peak at *m*/*z* 191.1 (Fig. 6). Loss of carboxy group forms the base peak at *m*/*z* 149.1. Other fragments were formed at *m*/*z* 133.1 [CO–CH=CCl–COOH], *m*/*z* 105.1 [CH=CCl–COOH], *m*/*z* 91 [CCl–COO] and *m*/*z* 57.1 [C–COOH].

In our study, co-metabolic degradation of 2,4-DCP seems to follow mainly the metabolic pathway of 2,4-DCP degradation, when this is used as a sole carbon source, by the identification of 2-Cl-maleylacetic acid. No difference in the metabolic intermediates observed, in the presence of a conventional carbon source, as it was previously reported in the case of 1,2-dichlorobenzene co-metabolic degradation [53].

In the presence of 0.1 mM 4-Cl-*m*-cresol chloride ions release was lower comparing to 2,4-dichlorophenol at the same concentration, due to the presence of only one chlorine atom in the molecule. GC analysis of the culture extract obtained after 24 h, revealed the presence of only one peak (Fig. 7a, peak 2). MS spectra showed the presence of two characteristic mass fragments at *m*/*z* ratios 43 and 59 (Fig. 7b). From the study of MS spectra it is obvious that no aromatic products are formed, which shows that aromatic cleavage has been occurred. Assuming that *ortho*-fission of aromatic nucleus has been carried out, according to degradation pathways previously studied in chloro-toluenes, one of the degradation products possible to be observed after subsequent dechlorination and decarboxylation is 3-methyl-4-oxo-pentanoic acid [54]. The basic fragment at *m*/*z* 43 may correspond to [CH_3CO–], whereas the second major mass fragment at *m*/*z* 59 may correspond to [HOOC–CH_2–]. No molecular ion was observed in this MS spectrum, probably due to extensive molecular fragmentation that electronic ionization method can cause. However, the fragmentation pathway of the proposed 3-methyl-4-oxo-pentanoic acid is in accordance with the general fragmentation pathway observed in most aliphatic ketones [55].

4. Conclusions

The design of a wastewater process is complicated, depending on many factors. Kinetic parameters can be strongly influenced from growth conditions, culture history of biomass and also from the presence of other organic pollutants, which may slow down the overall efficiency and produce unwanted dead-end products. In addition, if more than one bacterial species are present may collaborate in order to relieve industrial effluents from hazardous substances. We recognize that further research is needed to fully account for all of these factors in expressions of microbial kinetics.

Here, we examined the potential of using a Gram-positive bacterium, in order to remove two priority organic pollutants (2,4-DCP and 4-Cl-*m*-cresol), which cannot be used as sole carbon and energy source. In literature most of the studies are dealing with organic pollutants that can be used by the microorganism for their metabolic needs, which is more than ideal, given the present conditions. In real wastewater treatment plants, an organic pollutant cannot be used always directly as a carbon source. This study was carried out in an attempt to solve this problem, establishing the fact that these strains may also contribute to the detoxification of industrial effluents.

Adaptation as well as the presence of glucose as a conventional carbon source enhanced *S. xylosus* tolerance towards 2,4-DCP and 4-Cl-*m*-cresol and improved its degradation efficiency during subsequent cultivations.

2,4-DCP and 4-Cl-*m*-cresol caused a sequential decrease on specific growth rate. The application of a non-competitive inhibition equation showed that 4-Cl-*m*-cresol influenced more bacterial multiplication, revealing lower values of growth inhibition constant comparing to 2,4-DCP.

Despite the inhibitory action on bacterial growth, *S. xylosus* maintained its degrading ability. 2,4-DCP was found to be more susceptible to biodegradation comparing to 4-Cl-*m*-cresol, as the estimation of higher degradation rate and percentages of consumption values indicated. In addition, in the presence of 2,4-DCP strong inhibition was evident only at higher concentrations than 0.5 mM, whereas in the presence of 4-Cl-*m*-cresol, *S. xylosus* reduced only the degradation rate.

2,4-DCP and 4-Cl-*m*-cresol were co-metabolically degraded, up to an extent, in the obligate presence of glucose during exponential growth in a short period of time. The inhibitory effect of 2,4-DCP and 4-Cl-*m*-cresol on glucose consumption is more pronounced at higher concentrations, where glucose kinetic constants have lower values.

In the presence of 2,4-DCP, the nearly stoichiometric release of chloride ions and the identification of 2-Cl-maleylacetic acid in the medium showed that aromatic nucleus cleavage has taken place, since no 3,5-dichlorocatechol was identified in the medium. Aromatic cleavage has also taken place in the presence of 4-Cl-*m*-cresol, since the only metabolic product identified was 3-methyl-4-oxo-pentanoic acid.

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