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TOXICITY OF 4-CHLOROPHENOL UNDER COMETABOLIC CONDITIONS DEPENDING ON THE BACTERIAL CELL WALL STRUCTURE?

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Abstract
4-chlorophenol belongs to the group of xenobiotics that exhibits their toxicity independently of the environment and kind of organism. This monochlorophenol is found to be very hazardous to various organisms, and still new attempts are made to find the cheapest and the safest way to remove it from the polluted environments. Biological methods using the degradation potential of natural habitants of temporary polluted niches are preferentially used.
Since not each organism is able to survive in the presence of even low 4-chlorophenol concentration level the influence of additional source of carbon and energy on its toxicity to bacteria was verified. In this research phenol, benzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate and glucose were used as the nutritious substrates for Gram-negative *Stenotrophomonas maltophilia* KB2 and Gram-positive *Planococcus* sp. S5 strain. It was found that phenol, benzoate and glucose lowered the toxicity of 4-chlorophenol to *Stenotrophomonas maltophilia* KB2 strain while benzoic acids with one or two hydroxyl groups as well as glucose diminished toxic effects of this chlorophenol on the cells of Gram-positive strain *Planococcus* sp. S5.

Streszczenie
4-Chlorofenol jest przedstawicielem grupy ksenobiotyków, które wykazują swoja toksyczność niezależnie od warunków i rodzaju organizmu. Ponieważ ten izomer monochlorofenoli okazuje się być bardzo niebezpieczny dla różnych organizmów, nieustannie podejmowane są próby poszukiwania tanich i bezpiecznych jednocześnie sposóbów jego usuwania ze środowiska. Szczególnym zainteresowaniem cieszą się metody wykorzystujące potencjał degradacyjny typowych mieszkańców, zasiedlających zanieczyszczone tereny. Jednak nie każdy (mikro)organizm jest w stanie przeżyć w obecności nawet bardzo niskich, lecz toksycznych stężeń 4-chlorofenolu. Dlatego przedmiotem badań była ocena poziomu toksyczności 4-chlorofenolu w obecności dodatkowego źródła węgla i energii. W badaniach wykorzystano naturalną zdolność szczepów środowiskowych: gramujemnego *Stenotrophomonas maltophilia* KB2 oraz gramdodatniego *Planococcus* sp. S5 do rozkładu fenolu, benzoesanu, kwasu 4-hydroksybenzoesowego oraz kwasu protokatechowego i wzrostu w obecności tych związków aromatycznych. Wykazano, że fenol, benzoesan oraz glukoza (wykorzystana w badaniach jako kontrolny substrat wzrostowy) obniżały toksyczność 4-chlorofenolu dla szczepu *Stenotrophomonas maltophilia* KB2, natomiast kwas benzoesowy i jego badane hydroksylowe pochodne, jak również glukoza, zmniejszały toksyczny efekt 4-chlorofenolu na szczep *Planococcus* sp. S5.

Keywords: **4-chlorophenol; Cometabolism; Gram-positive; Gram-negative; Growth substrate.**

1. INTRODUCTION

It's generally known that xenobiotics including chlorophenols and other aromatic compounds with halogen substituent exhibit extreme toxicity to living organisms [15] and are highly resistant to microbial degradation which is thought to be the easiest and the cheapest way to their removal from the polluted environment. Toxicity of chlorophenols correlates with their hydrophobic character expressed by the logarithm of its partition coefficient between n-octanol and water ($P_{O/W}$). Xenobiotics with log $P_{O/W}$ values between 1 and 5 are highly toxic to the whole cells [8]. 4-chlorophenol as almost all other isomers of chlorophenols characterizes this high toxicity [2].

Both, Gram-negative and Gram-positive bacterial strains developed various mechanisms to overcome the toxic effect of the different aromatic compounds. Some of these mechanisms such as energy-dependent active efflux pumps that export toxic organic solvents to the external medium; *cis*-to-*trans* isomerization of unsaturated membrane fatty acids and modifications in the membrane phospholipid headgroups; formation of vesicles loaded with toxic compounds as well as changes in the biosynthesis rate of phospholipids to accelerate repair processes seem to be shared among those two groups of bacteria [14]. Specific for Gram-positive bacterial cells, which are less studied than Gram-negative microorganisms, in response to organic stress is to produce stress protein with protective function, to produce the endospores or to deactivate xenobiotics by their emulsification, biodegradation or transformation [14]. Generally, Gram-negative strains because of the specific structure of their outer membrane are thought to be more resistant to toxic influence of xenobiotics [8]. In the natural environments there is no ability to expose an organism to influence of a single harmful factor. Depending on xenobiotics' structure, $P_{O/W}$ and concentration numerous compounds influence the microorganisms' cells releasing different defence mechanisms. On the other hand some of xenobiotics can serve as the source of carbon and energy. *Stenotrophomonas maltophilia* KB2 and Gram-positive *Planococcus* sp. S5 strains assimilated phenol, sodium benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, but they didn't grow in the presence of 4-chlorophenol. The aim of these studies was to verify how the presence of 4-chlorophenol influences the growth of Gram-negative *Stenotrophomonas maltophilia* KB2 and Gram-positive *Planococcus* sp. S5 strain in the presence and without additional easily assimilated source of carbon.

2. MATERIALS & METHODS

2.1. Microorganisms

Gram-negative *Stenotrophomonas maltophilia* strain KB2 and Gram-positive *Planococcus* sp. strain S5 used in these studies came from the Culture Collection of the Department of Biochemistry, University of Silesia in Katowice (Poland). Both strains were isolated from the activated sludge of sewage treatment plant in Bytom-Miechowice (Poland) [6, 9]. Bacterial strains were stored on the agar slopes at 4°C.

2.2. Preparation of inoculum

Bacterial strains were pre-grown either in the mineral salts medium [6] with 3 mM phenol as the only source of carbon and energy or in the nutrient broth. Preincubation with phenol was carried as described previously [6]. Proliferation of bacterial cells in the nutrient broth took place 48 hours. Pre-grown bacterial cells were harvested by centrifugation at 4,500 *g* for 15 min at 4°C, washed with 50 mM phosphate buffer, pH 7.0, resuspended in the same buffer, and centrifuged again at 4,500 *g* for 15 min at 4°C. The pellets were resuspended in the appropriate amounts of mineral salts medium, and such obtained culture suspensions were stored at 4°C for at most two weeks.

2.3. Culture conditions

0.2 ml of culture suspension was used to inoculate 5 ml sterile mineral salts medium supplemented with 4-chlorophenol (4-CP) and growth substrate in 15-ml tubes. The 50 mM stock solutions of phenol, sodium benzoate, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid and glucose used as a growth substrates were prepared in sterile distilled water prior to their addition to the sterilized mineral salts medium to give the appropriate final concentration of 3 mM. 20 mM 4-chlorophenol stock solution was also prepared in sterile distilled water and added to mineral medium in the amount to give the final concentration of 0.5 or 1 mM. After inoculation, culture tubes were incubated in an orbital shaker at 130 rpm at 30°C. All culture experiments were performed in triplicate.

2.4. Growth determination

The growth of bacterial cultures expressed as the optical density (OD) was determined spectrophotometrically at $\lambda = 600$ nm after 24 or 72 hours of incubation using distilled water as the blank sample. Because of

temporary yellow colour of culture suspensions each sample was centrifuged at 13,200 *g* for 5 min at 4°C and once more measured spectrophotometrically at 600 nm. The obtained difference between those two spectrophotometric measurements was the actual cell concentration. Growth percentage of bacterial cultures was calculated as follows:

$$\text{Growth } (\%) = \Delta A_{600B} \times 100 / \Delta A_{600K}$$

where ΔA_{600B} was the absorbance difference in the culture after 24h or 72h of incubation according to its t_0, and ΔA_{600K} was the absorbance difference in the control culture after 24h or 72h of incubation according to its t_0. The control culture for cometabolic cultures was carried out only in the presence of 3 mM correspondent growth substrate. For the cultures with 4-chlorophenol as the only source of carbon, the control cultures were conducted with 3 mM phenol.

3. RESULTS & DISCUSSION

4-chlorophenol is one of the most wide-spread isomers of chlorophenols in the environment, because it is an intermediate of biodegradation processes of other aromatic compounds with chlorine substituent(s) such as e.g. 2,4-dichlorophenoxyacetic acid or pentachlorophenol [2, 3]. Although there are some microorganisms which ability to biodegrade 4-chlorophenol was confirmed [4, 7, 17, 18], this isomer is thought to be toxic and dangerous to numerous organisms. *Stenotrophomonas maltophilia* KB2 and *Planococcus* sp. S5 were not able to degrade 1 mM 4-chlorophenol and it didn't contribute to increase of the strains' biomass (Table 1).

Table 1.
The comparison of degradation times and OD_{600} changes in the cultures of *Stenotrophomonas maltophilia* KB2 and *Planococcus* sp. S5 in the presence of 3 mM phenol, benzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate, glucose or 1 mM 4-chlorophenol

	Stenotrophomonas maltophilia KB2		*Planococcus* sp. S5	
Substrate	Degradation time, h	ΔOD_{600}	Degradation time, h	ΔOD_{600}
Phenol	5	0.318	8	0.327
Benzoate	6	0.286	6	0.374
4-HB	6	0.325	6	0.370
3,4-DHB	5	0.389	5	0.454
Glucose	7	0.398	8	0.447
4-CP	-*	0.000	-*	0.000

-* lack of degradation

Data concerning toxicity of 4-chlorophenol are very limited especially for microorganisms. Boyd and coworkers [1] showed that 4-chlorophenol at the concentration of 0.18 mM and 0.095 mM caused 50% luminescence inhibition of *Burkholderia* RASC c2 and of *Pseudomonas fluorescens* with *lux* gene, respectively. Another study on chlorophenols toxicity exhibited toxic effect of 4-chlorophenol at the concentration of 1.37 mM for *Escherichia coli* and 1.08 mM for *Klebsiella oxytoca* using MICRODEX® test [10].

Results of our studies (Fig. 1) didn't allow determining the precise value of 4-chlorophenol concentration that causes 50% growth inhibition. Regardless of preincubation both strains showed distinct sensitivity to the examined 4-chlorophenol concentration after 24 hours of incubation, although *Stenotrophomonas maltophilia* KB2 seemed to be more sensitive while the bacterial cells were pre-grown with phenol (Fig. 1A). Extension of the incubation times to 72 hours changed strains' behaviour. Although the bacterial cells pre-grown in the nutrient broth didn't exhibit the differences in the growth in the presence of various 4-chlorophenol concentrations, preincubation with phenol influenced the growth percentage of the examined strains. Gram-positive strain *Planococcus* sp. S5 appeared to be more sensitive to 4-chlorophenol (irrespective of its concentration) than Gram-negative strain *Stenotrophomonas maltophilia* KB2 (Fig. 1B). These results are in accordance with literature data showing that Gram-negative bacteria are thought to be more resistant to different xenobiotics [8, 11, 12]. Presence of the outer membrane seems to be better protection than a more extensively linked peptidoglycan of Gram-positive strains. There are some known mechanisms of organic solvent tolerance among Gram-negative bacteria. The most popular and well described are: modifications in the cell envelope to increase cell membrane rigidity and decrease permeability, special solvent-inactivating enzymes and active efflux of solvents by means of solvent efflux pumps [8, 11, 12]. It is believed that organic solvent emulsifying/deactivating/ solubilising enzymes/substances could play a very important role in diminishing solvent toxicity in Gram-positive bacteria [14].

One way of 4-chlorophenol toxicity reduction can be its transformation under cometabolic conditions. Cometabolism takes place in the presence of additional source of carbon and energy and it allows to convert 4-chlorophenol to its less toxic intermediate such as e.g. 4-chlorocatechol. Previous studies (Guzik et al. 2009) showed that *Stenotrophomonas maltophilia* KB2

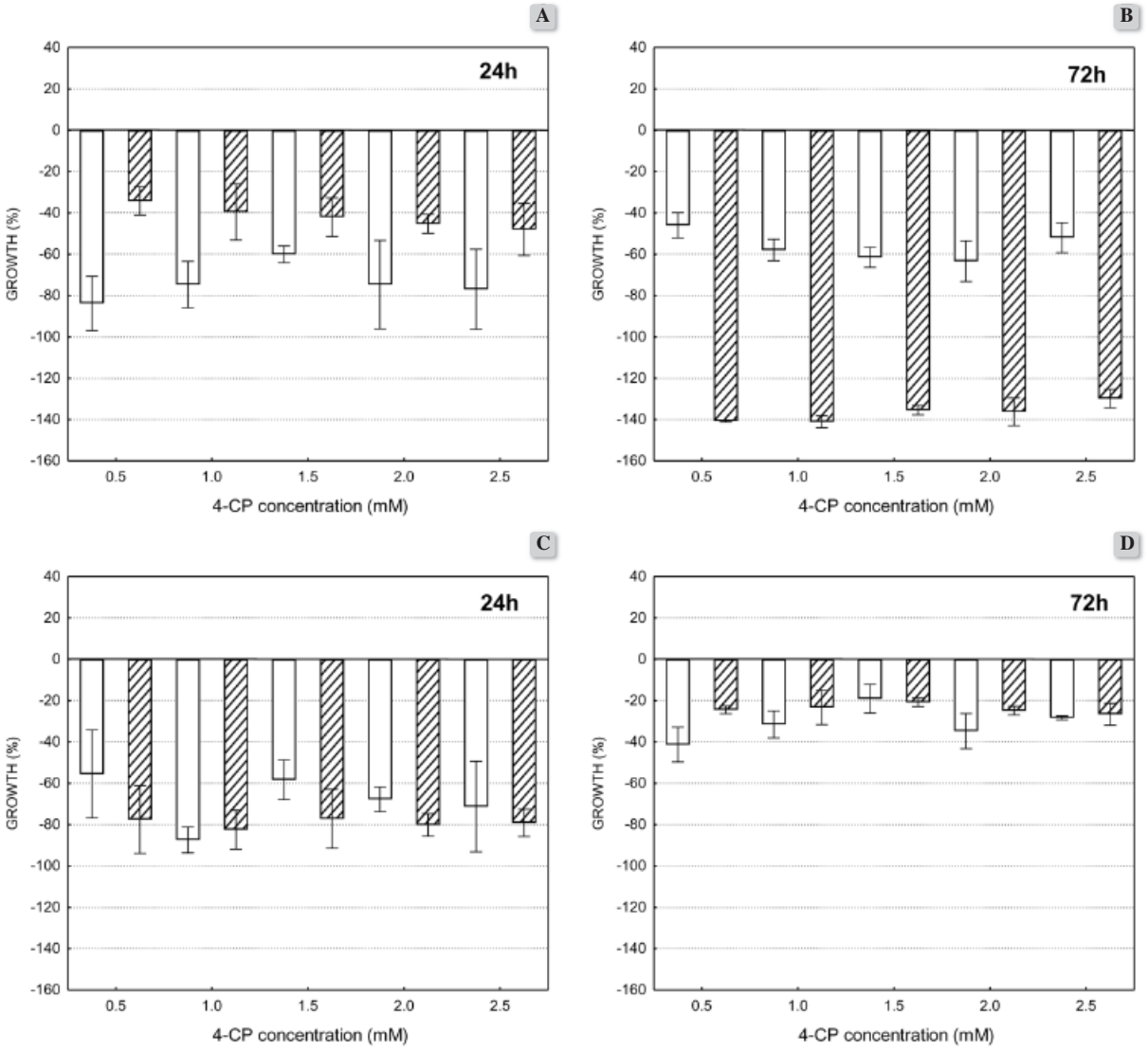

Figure 1.
Growth percentage of *Stenotrophomonas maltophilia* KB2 (white) and *Planococcus* sp. S5 (dashed) preincubated with phenol (A and B) and pre-grown in nutrient broth (C and D) in the presence of various 4-chlorophenol concentrations after 24 hours and 72 hours of incubation, according to the appropriate control cultures (see Material and methods). Data are displayed as mean and standard deviation

is able to grow in the presence of phenol, benzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate and glucose in the concentration of 3 mM. Ability to use those organic compounds as the only source of carbon and energy for *Planococcus* sp. S5 has been verified (Table 1). The comparison of the degradation times and OD_{600} changes showed that glucose and 3,4-dihydroxybenzoic acid contributed to the largest increase of both bacterial strains' biomass, and degradation times of benzoic acid and its hydroxylated derivatives were the briefest ones.

Under cometabolic conditions the best nutritious substrates reducing the toxicity of 0.5 mM 4-chlorophenol appeared to be 3,4-dihydroxybenzoate and glucose for both examined strains regardless of incubation time (Fig. 2). After 24 hours of incubation 50% and larger growth of Gram-positive strain *Planococcus* sp. S5 was also observed in the presence of 3 mM 4-hydroxybenzoate. When incubation time was prolonged to 72 hours toxicity of 0.5 mM 4-chlorophenol was diminished in the presence of all growth substrates for Gram-negative *Stenotrophomonas maltophilia* KB2 strain pre-grown

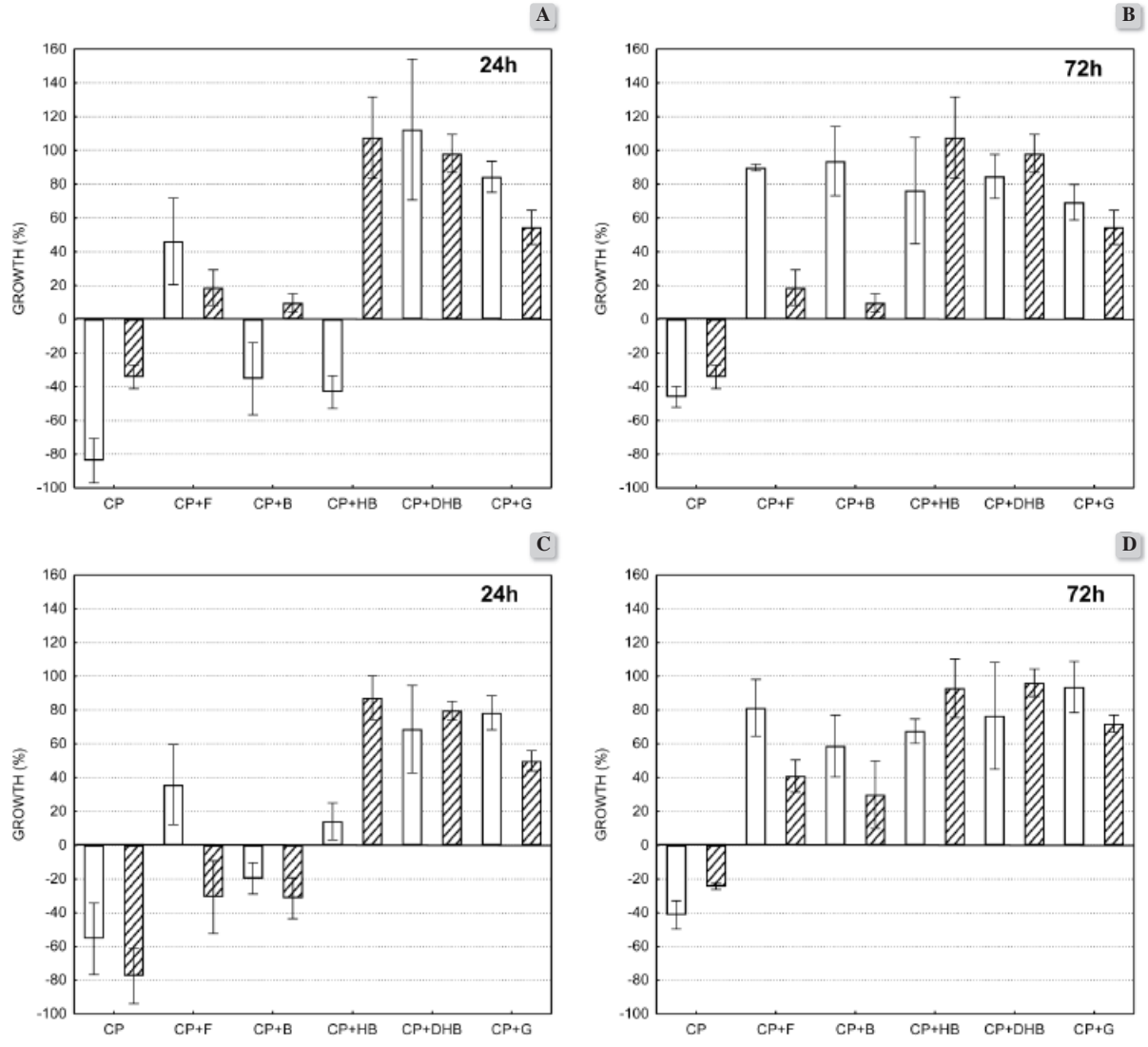

Figure 2.
Growth percentage of *Stenotrophomonas maltophilia* KB2 (white) and *Planococcus* sp. S5 (dashed) preincubated with phenol (A and B) and pre-grown in nutrient broth (C and D) in the presence of 0.5 mM 4-chlorophenol (CP) and 3 mM additional source of carbon after 24 hours and 72 hours of incubation, where: F – phenol, B – benzoate, HB – 4-hydroxybenzoate, DHB –3,4-dihydroxybenzoate, G – glucose. Data are displayed as mean and standard deviation

both, with phenol and in the nutrient broth. Phenol and benzoate in the concentration of 3 mM didn't support 50% growth of *Planococcus* sp. S5 in comparison to the control cultures.

When the concentration of 4-chlorophenol was increased to 1.0 mM 50% growth of the examined strains was observed only after 72 hours of incubation (Fig. 3).

Simultaneously as in the cometabolic cultures of *Stenotrophomonas maltophilia* KB2 with 0.5 mM 4-CP, all tested nutritious substrates supported 50% or more growth of this Gram-negative strain. Toxicity of 4-chlorophenol in concentration of 1.0 mM for *Planococcus* sp. S5 was reduced only in the presence of benzoic acid with one or two hydroxyl group(s) and glucose.

Irrespective of the bacterial cell wall structure hydroxybenzoic acids and glucose seem to be the important factors that reduce toxicity of 4-chlorophenol. Biodegradation of 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid demands activity of protocatechuate dioxygenases in the contrary to 4-chlorophenol degradation which requires presence

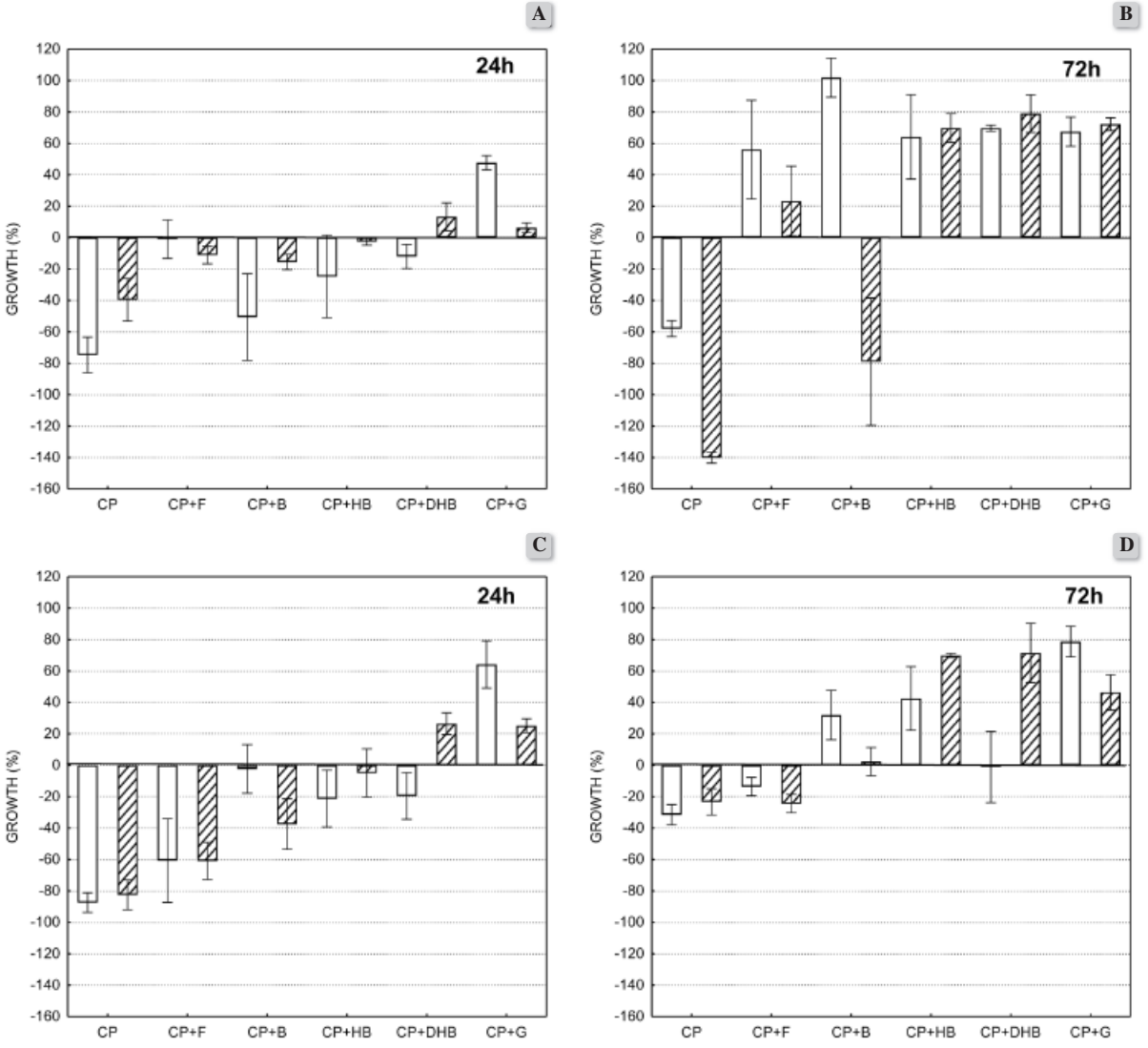

Figure 3.
Growth percentage of *Stenotrophomonas maltophilia* KB2 (white) and *Planococcus* sp. S5 (dashed) preincubated with phenol (A and B) and pre-grown in nutrient broth (C and D) in the presence of 1.0 mM 4-chlorophenol (CP) and 3 mM additional source of carbon after 24 hours and 72 hours of incubation, where: F – phenol, B – benzoate, HB – 4-hydroxybenzoate, DHB – 3,4-dihydroxybenzoate, G – glucose. Data are displayed as mean and standard deviation

of catechol dioxygenases [16]. Results of those growth tests indicate that dioxygenase of 3,4-dihydroxybenzoic acid are resistant to toxic activity of 4-chlorophenol or even its transformation products. Interestingly harmful effect of 4-CP didn't influence the enzymes of basic metabolism because glucose also supported growth of the examined strains even in the presence of 1 mM 4-chlorophenol. Diminished sensitivity of Gram-negative *Stenotrophomonas maltophilia* KB2 to 4-chlorophenol in the presence of phenol or sodium benzoate especially after elongated incubation may result not only from the features of its cell wall structure, but also from the ability if this strain to complete degradation of 4-chlorophenol under these conditions (data not shown).

Results of this research imply that the cell wall structure is not the only agent determining the susceptibility to toxic activity of 4-chlorophenol, but also type and degradation pathway of the additional source of carbon and energy under cometabolic conditions. Simultaneously Gram-negative *Stenotrophomonas maltophilia* KB2 seems to be the better degrader to use while removal of this monochlorophenol isomer is needed.

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