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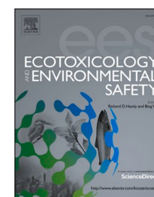

Adaptation of phenol-degrading *Pseudomonas putida* KB3 to suboptimal growth condition: A focus on degradative rate, membrane properties and expression of *xylE* and *cfaB* genes

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ABSTRACT

Detailed characterization of new *Pseudomonas* strains that degrade toxic pollutants is required and utterly necessary before their potential use in environmental microbiology and biotechnology applications. Therefore, phenol degradation by *Pseudomonas putida* KB3 under suboptimal temperatures, pH, and salinity was examined in this study. Parallelly, adaptive mechanisms of bacteria to stressful growth conditions concerning changes in cell membrane properties during phenol exposure as well as the expression level of genes encoding catechol 2,3-dioxygenase (*xylE*) and cyclopropane fatty acid synthase (*cfaB*) were determined. It was found that high salinity and the low temperature had the most significant effect on the growth of bacteria and the rate of phenol utilization. Degradation of phenol (300 mg L^{-1}) proceeded 12-fold and seven-fold longer at 10 °C and 5% NaCl compared to the optimal conditions. The ability of bacteria to degrade phenol was coupled with a relatively high activity of catechol 2,3-dioxygenase. The only factor that inhibited enzyme activity by approximately 80% compared to the control sample was salinity. Fatty acid methyl ester (FAMEs) profiling, membrane permeability measurements, and hydrophobicity tests indicated severe alterations in bacteria membrane properties during phenol degradation in suboptimal growth conditions. The highest values of pH, salinity, and temperature led to a decrease in membrane permeability. FAME analysis showed fatty acid saturation indices and cyclopropane fatty acid participation at high temperature and salinity. Genetic data showed that suboptimal growth conditions primarily resulted in down-regulation of *xylE* and *cfaB* gene expression.

1. Introduction

The constantly growing production of chemicals in many industries, such as pharmaceutical, petrochemical or textile, causes their systematic release into the various environment, mainly through improper disposal, leachate from landfills, as well as in waste or accidental sewage. Accumulation of these compounds in ecosystems poses a severe ecological threat due to their entry into the food chain, reducing the diversity of organisms in a given habitat and, consequently, adverse effects on human health (van den Brink et al., 2015; Guo et al., 2019). One of the many hazardous organic compounds is phenol, which finds applications in household products like detergents as a precursor for synthesizing chemical compounds, e.g., salicylic acid and a key intermediate in producing bisphenol A, disinfectants, surfactants, insecticides, and nylon. The growing automobile industry is aiding the phenol industry because phenol and its derivatives are used in tyres, coatings, adhesive, and high-performance rubber products. As epoxies find applications in circuit boards, the expanding electronics industry, is also driving the phenol market globally. According to a new report by Expert Market Research titled "Global Phenol Market Report and Forecast 2020–2025", the global phenol market reached a volume of 13.4 million tons in 2019, and it is expected to grow in the forecast period of 2020–2025 to reach 16 million tons by 2025. The global phenol market reached nearly 20 billion USD in 2018 and is further anticipated to reach 30.3 billion USD by 2025 (www.expertmarketresearch.com/pressrelease/global-phenol-market). Therefore, in this study, the choice of phenol as a carbon source was not accidental, but it resulted from its everyday use in industry and its frequent occurrence in various ecosystems, such as water, sewage, leachate, and soil (Reitzel and Ledin, 2002; Kurata et al., 2008; Michalska et al., 2020a).

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The massive use of phenol and its derivatives in industry causes their frequent occurrence in sewage and soil, where microorganisms, apart from chemical stress, are systematically exposed to various unfavorable abiotic factors, such as temperature fluctuations, changes in acidity, osmotic pressure, and limited availability of nutrients (Chandrasekaran et al., 2018; Samimi and Moghadam, 2020). One of the adaptive mechanisms of bacteria to those dynamically changing environmental parameters are alternations in the composition and fluidity of their cell membranes – the primary target of environmental stress, including saturation and length of the fatty acid chains, conversion of *cis* to *trans* isomers or vice versa, and changes in the percentages of cyclopropane and branched fatty acids (Yang et al., 2015; Bajerski et al., 2017; Guan and Liu, 2020). A more specific bacterial response to acid stress is the regulation of proton transmembrane transport by modulating the size of membrane channels (Guan and Liu, 2020). In turn, under hyperosmotic salt stress, bacteria accumulate polyprenyl quinones (Sévin et al., 2016). Common adaptive mechanisms also increase enzyme concentration and activity, compensating for inhibition imposed by adverse conditions (van den Berg et al., 2017). For example, acid-tolerant bacteria have high active urease or arginine deaminase systems involved in neutralizing protons and regulating pH in the cytoplasm (Mols and Abee, 2011; Guan and Liu, 2020). On the other hand, the accumulation of osmoprotectants like proline, trehalose, glutamate or carnitine ensures adaptation to a wide range of salinity and osmolality (Bremer and Krämer, 2019).

Changes in the expression pattern of genes encoding degradative enzymes also constitute an important response of bacteria to chemical stress. Particular attention is focused on genes encoding catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, and protocatechuate 3,4-dioxygenase, key ring-breaking enzymes of various aromatic compounds (Tian et al., 2017). Although the structure and activity of these enzymes are well characterized, there is scarce information concerning corresponding gene expression levels under suboptimal conditions. The available data from Fenner et al. (2005) indicated the possibility of using *xylE* gene as a marker to monitor the phenol-degrading bacteria population during simulated summer drought in a peatland.

Another gene of great importance in the mechanisms of stress tolerance and the adaptive response of bacteria to the presence of toxic compounds such as phenol, toluene and organic solvents is the cyclopropane synthase gene (*cfaB*), encoding the main enzyme responsible for the synthesis of cyclopropane fatty acids (Pini et al., 2009). To date, Pini et al. (2011) reported that the expression of *cfaB* in *Pseudomonas putida* KT2440, contrary to *E. coli* strains, is mainly dependent on the RpoS sigma factor. Moreover, cyclopropane synthase is related to its interplay with *cis-trans* isomerase (Cti) due to the utilization of *cis*-unsaturated fatty acids as a substrate by both enzymes. Cti is active in all *Pseudomonas* and *Vibrio* strains, and its activity may be regulated post-synthetically. Membrane fluidity might limit the access of hydrophilic Cti to the *cis*-fatty acids formed hydrophobic lipid bilayer embedded in a certain depth of the membrane, and the increase in the membrane fluidity could permit access of this enzyme to double bonds in the unsaturated fatty acid molecules (Eberlein et al., 2018). Another mechanism for Cti expression control in *Pseudomonas putida* F1 indicated up-regulated *cti* transcription at a temperature in the range of 20–37 °C and the presence of octanol (2.5 mM) as well as the highest Cti expression in the stationary growth phase (Kondakova and Cronan, 2019).

Utilizing microorganisms with proven degradative potential and survivability in harsh environmental conditions is crucial for successfully bioaugmentation of contaminated environments. *Pseudomonas putida* KB3 strain used in this study is valuable from a biotechnological point of view because of the documented capability of degrading various aromatic compounds, its ability to chemotaxis, surfactant, and EPS production as well as a propensity to siderophore synthesis and *quorum sensing* (Michalska et al., 2020a). In view of this, the aim of the present study was to: (i) compare the ability of *P. putida* KB3 to degrade phenol under suboptimal temperatures, pH, and salinity, (ii) analyse the changes in membrane properties during phenol exposure, and (iii) study the expression level of catechol 2,3-dioxygenase (*xylE*) and cyclopropane fatty acid synthase (*cfaB*) genes related to degradation of phenol and the adaptation of bacteria to suboptimal conditions, respectively.

2. Materials and methods

2.1. Bacterial strain

P. putida KB3 strain was previously characterized in detail by Michalska et al. (2020a). Its genome was sequenced and deposited at DDBJ/ENA/GenBank under the accession number SPUT00000000. This strain can degrade various aromatic compounds, including phenol, its methyl and chlorine derivatives, catechol, cresols, and 4-hydroxybenzoic acid. Moreover, it carries genes encoding enzymes involved in the aromatic hydrocarbon degradation pathways, such as phenol 2-monooxygenase, catechol 2,3-dioxygenase, and benzoate 1,2-dioxygenase.

2.2. Experimental design

To assess whether different temperatures, pH and salinity influenced the phenol degradation, cell membrane properties and the expression level of *xylE* and *cfaB* genes, bacteria were grown in a mineral salts medium (MSM) (Mrozik et al., 2007) containing glucose (540 mg L^{-1}) and phenol (300 mg L^{-1}) as a sole carbon source. The physicochemical conditions and symbols of particular batch cultures are summarised in Fig. 1. The initial optical density of cultures was 0.12 ± 0.02 (OD_{600}). The phenol concentration was determined using a colorimetric assay with diazoate *p*-nitroaniline at the $\lambda = 550$ nm (Lurie and Rybnikova, 1986). Although this reaction is not phenol specific, because catechol – the first metabolic derivate of phenol also gives a positive response, it was assumed that catechol was not accumulated in the culture medium due to the confirmed activity of 2,3-dioxygenase cleaving its aromatic ring. Bacteria were incubated at different temperatures (10, 30, 39 °C), pH (6.5, 7.2, 8.5) and salinity (0, 2.5, 5%) on a laboratory shaker (130 rpm). Above and below values of tested parameters, bacteria did not grow and/or did not degrade phenol. For each analysis, bacterial cells were harvested from the exponential growth phase.

2.3. Measuring the catechol 2,3-dioxygenase activity

During the degradation experiment, the activity of catechol 2,3-dioxygenase [EC 1.13.11.2] was measured. The procedure of crude extract preparation for enzyme activity assay was performed according to Wojcieszyńska et al. (2013). The enzyme activity was estimated by the spectrophotometric method using catechol as a substrate (Hegeman, 1966). The protein concentration in the crude extract was determined by the Bradford (1976) using lysozyme as a standard.

2.4. Isolating fatty acids from bacteria

To determine the composition of the whole-cell derived fatty acids of *P. putida* KB3 under different conditions, they were directly extracted from phenol-degrading bacteria and control cells in the late exponential phase of growth. The procedure of fatty acid methyl ester extraction, gas chromatography analysis, FAME identification, and data interpretation were analogous to the methodology by Nowak et al. (2016). The mean fatty acid chain length was expressed by the following equation, according to Yang et al. (2015):

$$Mean\ fatty\ acid\ chain\ lenght = \sum(\%FA \times C)/100$$

where: %FA is the percentage of fatty acid, and C is the number of carbon atoms. To prevent the alterations caused by fatty acids occasionally detected, the analysis of FAMEs included only fatty acids with a

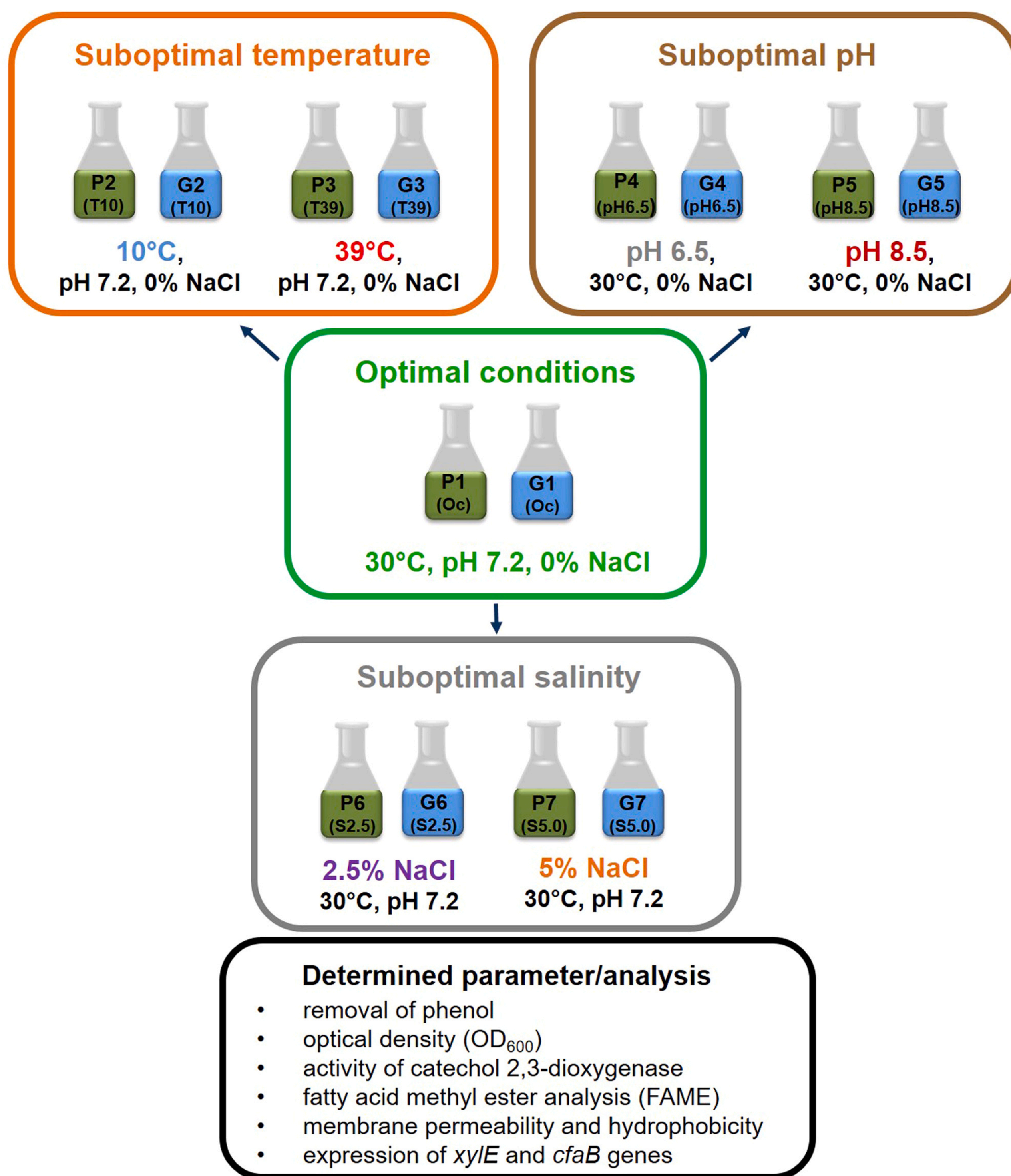

Fig. 1. The scheme of the experimental design. Abbreviations: P1 - P7 – cultures with phenol (P) as a carbon source, G1 - G7 – cultures with glucose (G) as a carbon source. The exact conditions are described below the batch cultures.

content of at least 1%.

2.5. Measuring the membrane permeability

Membrane permeability was measured using a water solution of crystal violet (CV) according to the protocol described by Halder et al. (2015). Bacteria were harvested in the exponential phase of growth, centrifuged (4500 x *g*, 20 min, 4 °C), and washed with the MSM. Bacterial biomass was resuspended in the MSM to fit the optical density of approximately 1.0. Next, 0.95 mL of the obtained cell suspension was mixed with 50 μL of CV (0.1 mg mL^{-1}). The samples were incubated at 30 °C for 10 min and centrifuged (13,400 x *g*, 15 min). The optical density of the supernatant (A_{sample}) was measured at a wavelength of 590 nm. The optical density of the CV solution (0.95 mL of MSM and 50 μL of 0.1 mg mL^{-1} CV) (A_{violet}) was considered as 100%. The permeability expressed as the percentage of CV uptake was calculated according to the equation:

$$\text{uptake of CV }\% = \frac{A_{violet} - A_{sample}}{A_{violet}} \times 100\%$$

2.6. Determining the cell surface hydrophobicity

Cell surface hydrophobicity (CSH) was determined based on the modified microbial adhesion to hydrocarbons (MATH) test according to the protocol by Nachtigall et al. (2019). Bacterial cultures (50 mL) were centrifuged (5000 x *g*, 20 min, 4 °C) and washed twice with 0.9% NaCl. The pellet was resuspended in phosphate-buffered saline (PBS) to fit the optical density of approximately 0.4 (A_0). Next, 0.3 mL of *p*-xylene was added to 3 mL of cell suspension and vortexed for 1 min. The samples were incubated at room temperature for 15 min, and the optical density of the aqueous phase was measured at a wavelength of 577 nm (A_1). The blank sample (A_{blank}) was 3 mL of PBS buffer with 0.3 mL of *p*-xylene. Additionally, CSH was corrected concerning bacterial cell sedimentation in time: the bacterial cell suspension ($OD_{577} = 0.4$) was incubated for 15 min, and the optical density of this sample measured after this time was $A_{0\ norm}$. Cell surface hydrophobicity (%) was calculated as follows:

$$\text{CSH }\% = \frac{A_0 - \left(\frac{A_1}{A_{0\ norm}} - A_{blank}\right)}{A_0} \times 100$$

2.7. Analysing the expression of xylE and cfaB genes

The analysis of the expression pattern of *xylE* and *cfaB* genes was performed according to the protocol by Żur et al. (2020). Three biological and two technical replicates were performed for each treatment. The gene encoding gyrase (*gyrA*) was used as an internal control. The relative expression level was calculated according to Livak and Schmittgen (2001). The *cfaB* gene in *P. putida* KB3 was identified based on the BLASTP search with the protein sequence encoded by the *cfaB* gene from *Pseudomonas putida* DOT-T1E described by Pini et al. (2009) as a query (sequence deposited under number DQ665843). The *xylE* and *gyrA* genes were identified based on the annotation provided by the NCBI Prokaryotic Genome Annotation Pipeline and further confirmed based on the presence of characteristic domains using NCBI Conserved Domain Search and functional annotation provided by the EggNOG Database (version 5.0) (Huerta-Cepas et al., 2019). Primers used in this study were designed using Geneious Prime (version 2019.0.3) (Table S1).

2.8. Data analysis

The degradation kinetic parameters as rate constant (*k*). the average degradation rate (*V*) of phenol and the theoretical disappearance time 50 (DT_{50}) were calculated according to Nowak et al. (2016).

The obtained results were evaluated by analysis of variance, and statistical analyses were performed on three replicates of data obtained from each treatment. The statistical significance ($p < 0.05$) of differences was treated by two-way ANOVA, considering the effect of substrate and treatment, and assessed by post-hoc comparison of means using the lowest significant differences (LSD) test.

The independent samples *t*-test ($p < 0.05$) was used to compare the values of adequate pairs of obtained parameters to determine whether the carbon source or physicochemical conditions influenced the membrane permeability, hydrophobicity, unsaturation level or mean fatty acid chain length. The obtained results were also evaluated using the analysis of variance (ANOVA). The FAME profiles were both subjected to ANOVA and principal component analysis (PCA). All data were performed using the Statistica 13.3 PL software package, based on the mean values of three biological replicates.

3. Results

3.1. Phenol degradation and growth rate of P. putida KB3 at different temperatures, pH and salinity

During the experiment, differences in the kinetic parameters of phenol degradation by *P. putida* KB3 depending on the physicochemical conditions of the culture were found. Phenol was degraded in the shortest time at temperature 30 °C and pH 7.2, although the highest value of the disappearance rate (*V*) was recorded at the same temperature and pH 6.5 (Fig. 2B, Table 1). The efficiency of phenol degradation by *P. putida* KB3 was significantly influenced by the lowest temperature. At 10 °C, phenol degradation lasted 72 h, while at 30 °C it proceeded 12-fold shorter (Fig. 2A). Among suboptimal tested conditions, phenol degradation was also sharply influenced by different salinity. The presence of 2.5% and 5% NaCl in the culture medium caused a four-fold and over a seven-fold increase in the time of phenol removal, respectively, (Fig. 2C) and increase DT_{50} value from 2.12 h for control (0% NaCl) to 5.42 h for 5% NaCl (Table 1).

It was also found that low temperature and high salinity inhibited bacterial growth. When glucose was served as the carbon source, the bacterial growth was negligible in the presence of 5% NaCl during 42 h of incubation (Fig. 2I). By contrast, in the presence of phenol and high NaCl content, OD_{600} reached almost 0.5. In the presence of phenol, the temperature was the most significant parameter inhibiting the growth of bacteria. The lag phase of bacterial growth was observed at 48 h of incubation at temperature 10 °C, while at 30 °C this phase did not appear (Fig. 2D). Interestingly, the bacterial growth was not affected by pH in the tested range (6.5–8.5) (Fig. 2E, H).

3.2. The activity of catechol 2,3-dioxygenase under various growth conditions

In parallel with the biodegradation studies, the activity of catechol 2,3-dioxygenase was measured. The highest catechol 2,3-dioxygenase activity (692 ± 70 mU mg^{-1} protein) was determined at 30 °C and pH 7.2, which confirmed that these culture conditions were optimal for phenol degradation by *P. putida* KB3. The lowest activity was recorded in the presence of 5% NaCl (123 ± 14 mU mg^{-1} protein) (Table 2). Noteworthy, at 10 °C, 39 °C and 2.5% NaCl at 30 °C, the activity of the enzyme was quite similar and estimated about 200 mU mg^{-1} of protein. The high activity (473 ± 26 mU mg^{-1} of protein) was also observed at 30 °C and a slightly acidic pH value of 6.5.

3.3. Changes in bacterial membrane properties under various growth conditions

Phenol and suboptimal growth conditions visibly influenced the permeability of cell membranes, cell surface hydrophobicity, and FAME profiles. In bacteria cultured with phenol, except for the pH 6.5% and 2.5% NaCl, membrane permeability values were significantly different from adequate values obtained for the cells grown on glucose (Table 3). Interestingly, the membranes of bacteria incubated with phenol at 39 °C, pH 8.5% and 5% salinity were less permeable than membranes of cells incubated with glucose, while at 10 and 30 °C they were more permeable. Regardless of the introduced carbon source, the most permeable were membranes of bacteria incubated at the highest temperature and salinity. At 39 °C and 5% NaCl, cell membranes were permeable in 65–66% and 81–87% in the presence of phenol and glucose, respectively (Table 3).

In parallel with studies on membrane permeability, the CSH of *P. putida* KB3 was analysed. The results revealed that phenol did not significantly influence the CSH at different temperatures (Table 3). By comparison, phenol and suboptimal acidity caused an increase in the CSH value, while salinity induced a decrease in CSH compared to corresponding values calculated for cells cultured with glucose.

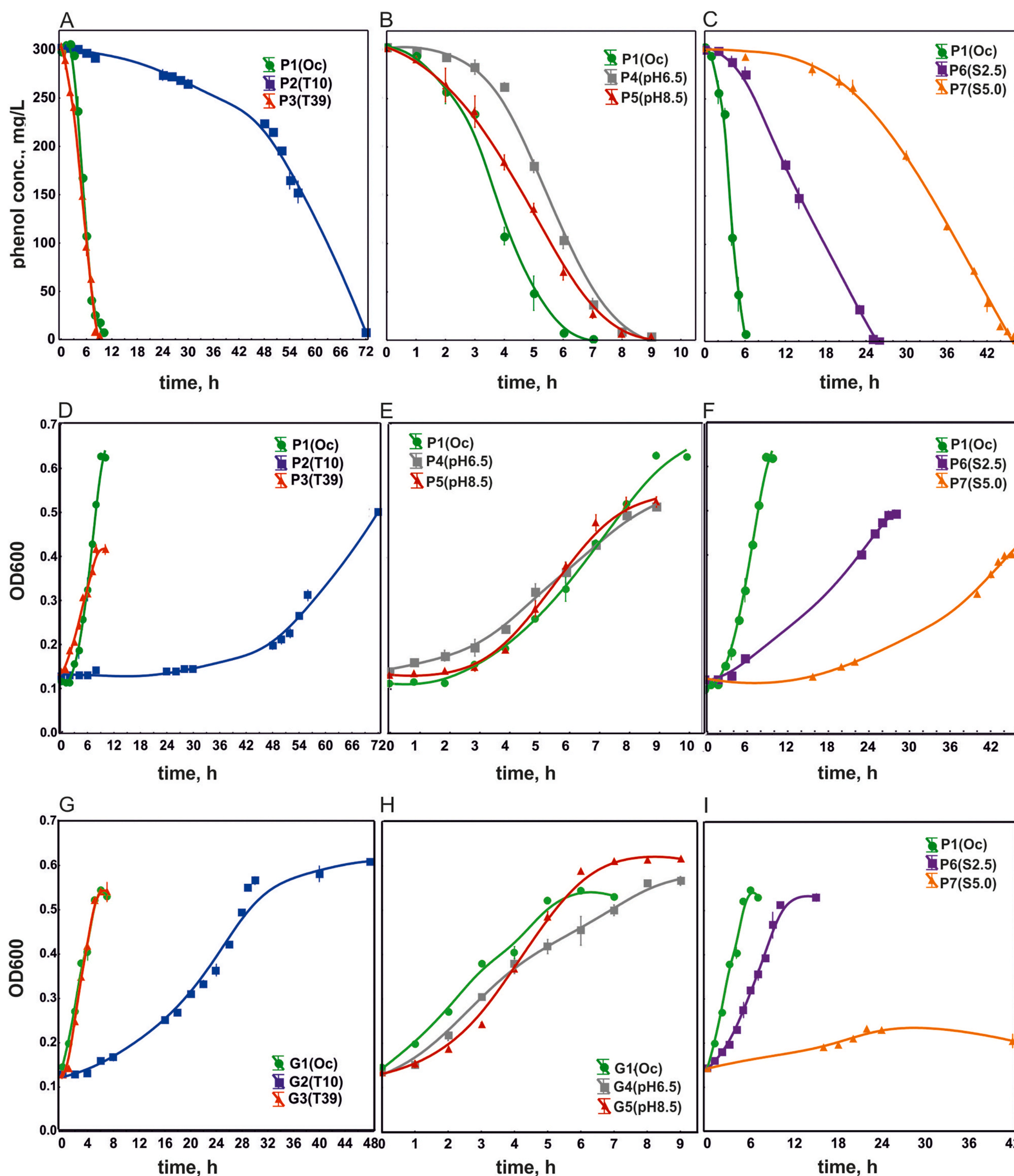

Fig. 2. Degradation of phenol (300 mg L^{-1}) (A–C) and the growth of *P. putida* KB3 in the presence of phenol (D–F) and glucose (G–I) under different physicochemical conditions.

Interestingly, pH 8.5 resulted in the largest reduction in the CSH of the cells, both incubated in the presence of phenol and glucose.

Analysing the changes in the FAME profiles, the different responses of *P. putida* KB3 to various growth conditions were observed. The low temperature (10 °C) caused the decrease in the straight-chain contents and a simultaneous increase in the unsaturated fatty acid participation in the FAME profiles, resulting in the lower SAT/UNSAT ratio, while 39 °C and 5% salinity generated the opposite effect (Fig. 3A, B, Table 3). Additionally, the higher temperature, pH fluctuations, and salinity caused the increase in cyclopropane fatty acid percentages in the bacterial membrane during culture on glucose. The influence of phenol on fatty acid composition was mainly observed as the increase in the cyclopropane fatty acid participation in the cells incubated at 10 °C, 30 °C, and 2.5% NaCl. The PCA analysis revealed that the 17:0 *cyclo*, 19:0 *cyclo,* and 16:0 were the most abundant fatty acids in the cells incubated at the higher salinity, 16:1 ω7*c* and 18:1 ω7*c* were common in the cells exposed to 10 °C; however, 12:0 and 18:0 were present in the cells incubated at the elevated temperature (Fig. 3C, D). The

Table 1
The degradation rate constant (k), disappearance time 50 (DT_{50}), and disappearance rate (V) of phenol in batch cultures of *P. putida* KB3 under different culture conditions.

Symbol of treatment	k, h^{-1}	DT_{50}, h	V, mg L^{-1} h^{-1}
P1(Oc)	0.327 ± 0.007^{b}	2.12 ± 0.01^{b}	52.1 ± 0.1^{b}
P2(T10)	0.151 ± 0.025^{a}	4.69 ± 0.72^{a}	9.0 ± 0.2^{a}
P3(T39)	0.371 ± 0.024^{b}	1.88 ± 0.12^{b}	38.3 ± 0.8^{c}
P4(pH6.5)	0.647 ± 0.058^{c}	1.08 ± 0.10^{c}	75.2 ± 3.4^{d}
P5(pH8.5)	0.438 ± 0.036^{d}	1.59 ± 0.13^{bc}	47.5 ± 4.2^{e}
P6(S2.5)	0.148 ± 0.001^{a}	4.68 ± 0.02^{a}	13.1 ± 0.2^{f}
P7(S5.0)	0.129 ± 0.012^{a}	5.42 ± 0.47^{d}	10.1 ± 0.3^{af}

In each column, the means of k, DT50 and V with different letters in superscript are significantly different ($p < 0.05$, post-hoc LSD test) considering the effect of treatment (different temperatures, pH and salinity). Using post-hoc LSD test, the means with the same letters in each column are statistically equal at the significance level $p < 0.05$.

Table 2
The activity of catechol 2,3-dioxygenase of *P. putida* KB3 during phenol degradation under different culture conditions.

Symbol of treatment	Catechol 2,3-dioxygenase activity, mU mg^{-1} protein
P1(Oc)	692 ± 70^{a}
P2(T10)	203 ± 7.0^{b}
P3(T39)	204 ± 78^{b}
P4(pH6.5)	473 ± 26^{c}
P5(pH8.5)	327 ± 10^{d}
P6(S2.5)	215 ± 68^{b}
P7(S5.0)	123 ± 14^{e}

The means of catechol 2,3-dioxygenase activity with different letters are significantly different from others ($p < 0.05$, LSD test) considering the effect of treatment (pH, temperature and salinity). The differences between the means sharing the same letters are not statistically significant (they are equal at significance level $p < 0.05$).

temperature 39 °C also caused a decrease in hydroxy acid (10:0 3OH, 12:0 2OH and 12:0 3OH) abundance. These changes were reflected in high fatty acid saturation indices, but only in the case of 5% salinity, while SAT/UNSAT ratio was statistically significant compared to other conditions (Table 3). Noteworthy, analysed growth factors did not cause substantial changes in the average length of fatty acid chains in the presence of glucose. By comparison, the simultaneous effect of phenol and high temperature or suboptimal pH caused a statistically significant increase in the mean fatty acid chain length (Table 3).

3.4. The influence of growth conditions on the cfaB and xylE gene expression profiles

To complete the overall picture of the influence of various conditions on the bacterial growth and phenol degradation, the expression profiles of *xylE* and *cfaB* genes were analysed (Fig. 4). The *cfaB* gene (E4L40_22805) was identified based on the alignment with the *cfaB* gene from *P. putida* DOT-T1E. The pairwise identity of proteins encoded by these genes equals 98.7%. The functional annotation also enabled the identification of the reference gene, *gyrA* (E4L40_23765). In the genome of *P. putida* KB3 strain, only one gene encoding catechol 2,3-dioxygenase (*xylE*) was identified (E4L40_22805).

In the cultures with glucose, the highest expression of the *xylE* gene was observed in the control cells (Fig. 4C). In the presence of phenol, the expression level of the *xylE* gene mainly remained unchanged in response to the varying temperature and pH (Fig. 4D). The down-regulation of the *xylE* gene expression was observed in response to the increased salinity, showing ten-times and five-times lower expression levels compared to the control cells cultured with 2.5% NaCl and 5% NaCl, respectively.

In the presence of glucose, the highest expression of the *cfaB* gene was detected in the cells grown under optimal conditions (Fig. 4A). A similar - but lower than in the control cells - expression level was confirmed in other cultures. Under phenol exposure, the expression of the *cfaB* gene was more diverse, with the highest up-regulation in response to the elevated salinity of 5% NaCl (Fig. 4B). Under 2.5% NaCl exposure, the expression was 2.5-fold lower than in the control cells. A significant difference in the gene expression was also noticed at the selected pH value, with the most pronounced down-regulation observed at 6.5 pH. The difference between pH 8.5 and optimal 7.2 was not statistically significant.

4. Discussion

The variation of the physicochemical conditions is one of the most common stress factors affecting the survival of microorganisms in the environment. This is also the reason for bioaugmentation failure because microbial activity and the associated biodegradation efficiency of contaminants depend on external factors such as the complexity of the local edaphic and climate conditions.

Table 3
Membrane permeability, cell surface hydrophobicity (CSH), the ratio of saturated to unsaturated fatty acids (SAT/UNSAT) and mean fatty acid chain length of *P. putida* KB3 grown on phenol or glucose under various culture conditions.

Symbol of treatment	Membrane permeability, %	CSH, %	SAT/UNSAT	Mean fatty acid chain length
P1(Oc)	$38.87 \pm 0.37^{II,a}$	37.70 ± 4.50^{a}	1.42 ± 0.01^{ab}	15.72 ± 0.01^{ab}
G1(Oc)	$19.90 \pm 3.22^{II,b}$	43.17 ± 4.14^{a}	1.28 ± 0.18^{a}	15.62 ± 0.08
P2(T10)	$40.33 \pm 1.36^{I,a}$	39.88 ± 4.16^{a}	$1.07 \pm 0.04^{I,a}$	15.55 ± 0.12^{a}
G2(T10)	$25.28 \pm 1.44^{I,a}$	41.73 ± 2.16^{a}	$0.87 \pm 0.08^{I,b}$	15.79 ± 0.02
P3(T39)	$64.84 \pm 0.89^{III,b}$	24.41 ± 6.04^{bd}	1.76 ± 0.08^{b}	16.00 ± 0.07^{c}
G3(T39)	$81.44 \pm 0.64^{III,c}$	29.62 ± 0.62^{b}	1.84 ± 0.01^{c}	15.91 ± 0.19
P4(pH6.5)	16.03 ± 2.00^{c}	$62.78 \pm 1.28^{I,c}$	$1.33 \pm 0.01^{II,ab}$	15.85 ± 0.06^{bc}
G4(pH6.5)	18.41 ± 0.37^{b}	$49.13 \pm 7.02^{I,c}$	$1.42 \pm 0.01^{II,ad}$	15.80 ± 0.02
P5(pH8.5)	$30.91 \pm 0.53^{IV,d}$	$17.69 \pm 2.76,^{II,d}$	1.49 ± 0.02^{ab}	15.98 ± 0.57^{c}
G5(pH8.5)	$35.34 \pm 0.84^{IV,d}$	$2.64 \pm 0.18^{II,d}$	1.57 ± 0.27^{ac}	15.76 ± 0.02
P6(S2.5)	27.00 ± 3.38^{e}	$40.86 \pm 2.05^{III,a}$	$5.58 \pm 0.40^{III,c}$	15.79 ± 0.17^{ac}
G6(S2.5)	30.91 ± 4.43^{e}	$46.13 \pm 1.19^{III,ac}$	$1.70 \pm 0.01^{III,cd}$	15.79 ± 0.01
P7(S5.0)	$66.46 \pm 0.74^{V,b}$	$29.36 \pm 4.47^{IV,b}$	3.91 ± 0.01^{d}	15.70 ± 0.09^{ad}
G7(S5.0)	$86.53 \pm 0.34^{V,f}$	$42.98 \pm 2.26^{IV,a}$	2.52 ± 0.66^{e}	15.72 ± 0.01

In each column, the means with the same carbon source -phenol (P1-P7) and glucose (G1-G7) with different letters are significantly different ($p < 0.05$, LSD test) considering the effect of treatment (pH, temperature and salinity). It means that one test was done for samples P1-P7 and the second for samples G1-G7. Results without any letters are statistically equal at a significance level $p < 0.05$. Samples with the same treatment (the same temperature, salinity and pH) but with a different carbon source (phenol and glucose) with the same Roman numerals are significantly different ($p < 0.05$, the independent samples t-test). It means that Roman numerals denote pairs of means (e.g. P1 - G1) that are not equal at the significance level $p < 0.05$. Differences between pairs of means P1-G1, etc., without Roman numerals, are not statistically significant ($p < 0.05$).

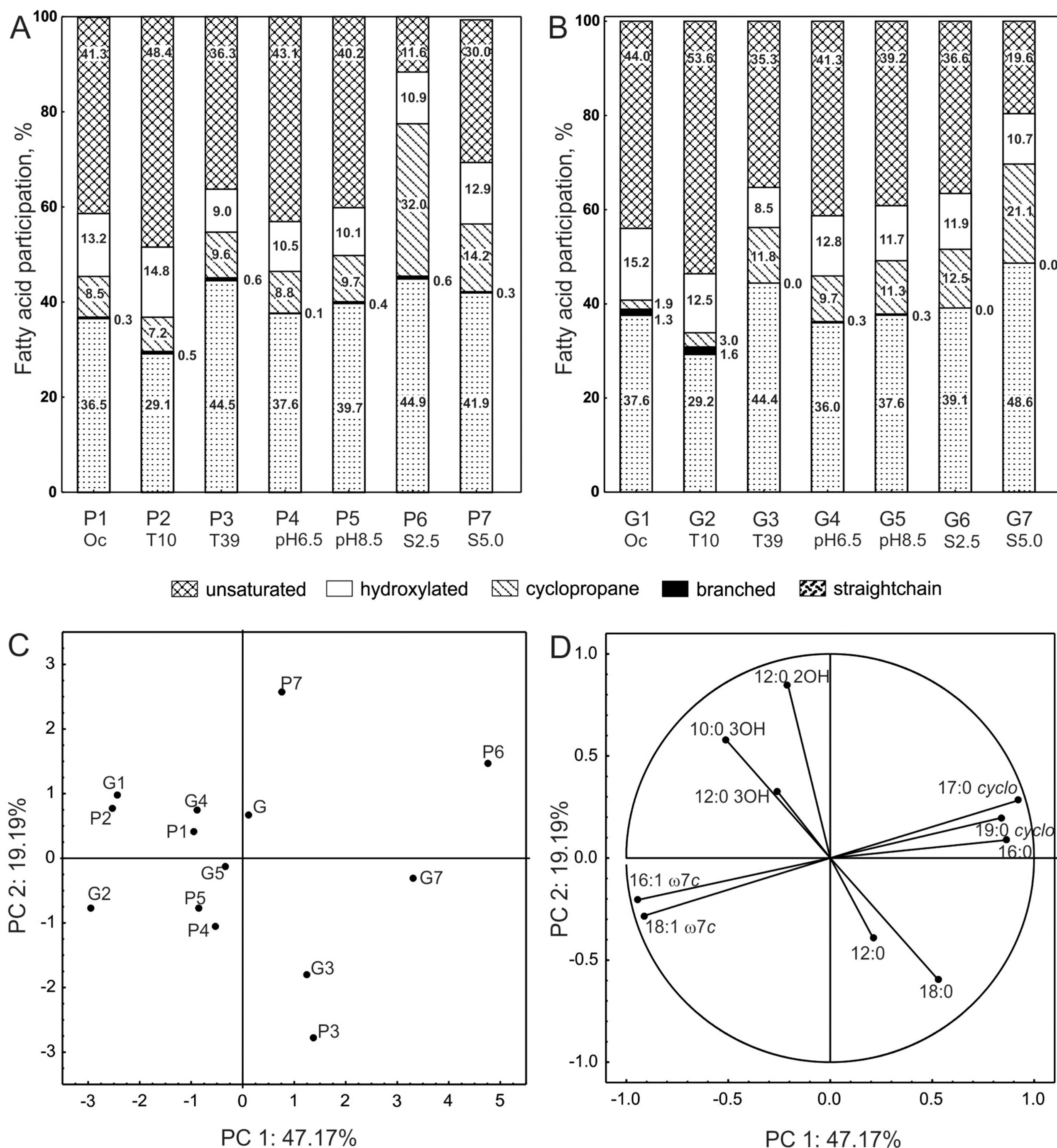

Fig. 3. Proportions of different groups of fatty acids and PCA analysis for *P. putida* KB3 growing under various culture conditions in the presence of phenol (A) and glucose (B). The projection of FAMEs isolated from bacteria on the plane defined by PC1 and PC2 (C), and the correlation of fatty acids with PC1 and PC2 (D).

The effect of phenol on cellular fatty acid composition, the proteome and transcriptome of various *Pseudomonas* strains has been described in a few studies. The main variations in membrane composition were attributed to the *cis-trans* isomerization of unsaturated fatty acids as a short-term adaptive response (Heipieper et al., 2003), while a long-term response was mainly related to the formation of cyclopropane fatty acids (Nowak and Mrozik, 2016). Quantitative proteome and transcriptome analyses enable complementing the picture of the global reaction of *Pseudomonas* to phenol-induced stress. For example, Santos et al. (2004) documented that the proteins involved in the fatty acid biosynthesis (FabB, FabBx1 and AccC-1) and cell envelope biosynthesis (LpxC, VacJ and MurA) in *P. putida* KT2440 exposed to phenol were up-regulated, which confirmed the modifications within the cell envelope. In another study, Wierckx et al. (2008) indicated the up-regulation of genes in *P. putida* S12 involved in homogentisate and protocatechuate degradation pathways confirming that surplus tyrosine was metabolized into 4-hydroxyphenylpyruvate and further incorporated in the tricarboxylic acid cycle via homogentisate pathway. Moreover, an increased expression of genes encoding amino acid transporters (AroP-type transporters) indicated an important role of these proteins in regulating intracellular pools of 4-hydroxyphenylpyruvate – the tyrosine precursor. However, it is worth emphasizing that *P. putida* S12 is a phenol-producing strain, and the redirection of metabolism to protocatechuate or homogentisate degradation pathways was due to the low activity of tyrosine-phenol

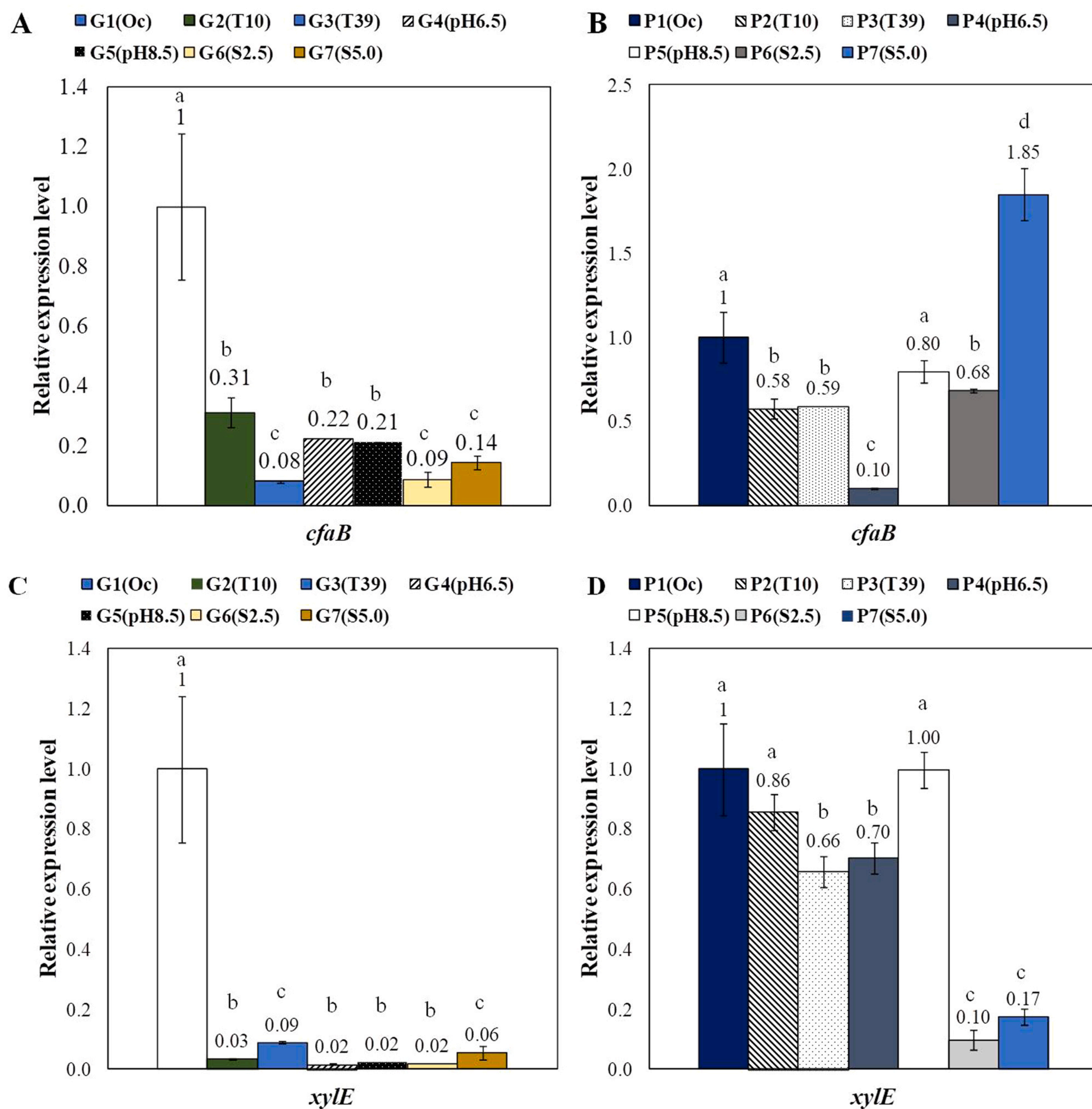

Fig. 4. Relative level of transcript accumulation of the *cfaB* (A,C) and *xylE* (B,D) genes in *P. putida* KB3 growing in the presence of glucose (A,B) and phenol (C,D) under various culture conditions. The same letters indicate no statistically relevant difference between the treatments (N = 3), as determined by two-way ANOVA, followed by Tukey's multiple comparisons post hoc test, $p < 0.05$.

lyase converting tyrosine into phenol. Here, we discuss the simultaneous influence of phenol and suboptimal growth conditions on membrane properties and genes related to the degradation of phenol and the adaptation of bacteria to suboptimal conditions.

The ability of *P. putida* KB3 to degrade phenol across a wide range of temperatures (10–39 °C) indicated its potential use in bioremediation processes in temperate climates with apparent annual temperature fluctuations. It is worth emphasizing that the operating temperature of most wastewater treatment plants ranges between 10 and 20 °C, and for large parts of the year, it remains low, especially in cold geographic regions (Funamizu and Takakuwa, 1999). Although *P. putida* KB3 degraded phenol nine-times longer and the substrate disappearance rate (DT_{50}) value was almost six-times higher at 10 °C compared to corresponding values at 30 °C, the final biomass estimated as optical density was comparable in cultures incubated at both temperatures. It suggests that bacterial metabolism was slowed down but not inhibited at low temperature. Also, phenol-induced catechol 2,3-dioxygenase activity in bacteria incubated at 10 °C compared to its activity at 30 °C may prove the prolongation of phenol degradation time. This was probably due to the thermodynamics of enzymatic processes since the temperature-sensitive step is the binding of the substrate to the enzyme through weak binding site-ligand interactions that define the catalytic constant (Struvay and Feller, 2012). At low temperatures, these processes occur more slowly, which may explain the observed relationships.

The factor that most influenced phenol degradation and bacterial growth was salinity. It is estimated that salt-affected soils represent about 40% of the world lands, and about 5% of industrial wastewater is either saline or hyper-saline (Guo et al., 2015; Praveen et al., 2015). The

salt content in the wastewater produced by chemical, pharmaceutical, food industry, as well as mining and mineral industries varies from 2 up to 40% (Castillo-Carvajal et al., 2014). Contamination of saline ecosystems with toxic aromatic compounds is a serious problem worldwide, especially in recent years (Castillo-Carvajal et al., 2014; Guo et al., 2015; Chandrasekaran et al., 2018). Salinity affects the biodegradation of xenobiotics in several ways: through cell dehydration by osmotic differences across cell membranes, a decrease in the availability of dissolved oxygen, and a decrease in aromatic compound solubility in water by salting-out effect (Castillo-Carvajal et al., 2014). In our studies, when glucose was used as a carbon source, bacterial growth was almost completely inhibited in the presence of 5% NaCl, and the expression of the *cfaB* gene was strongly affected. Interestingly, *P. putida* KB3 was able to degrade phenol in saline conditions, although its growth was visibly inhibited compared to growth in the control sample. It suggests that phenol stimulates defence mechanisms of the cells, which enable survival in a culture with high osmotic pressure. One explanation of this phenomenon could be that phenol acts as a compatible solute, similar to ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) synthesized by phototrophic *Alpha-* and *Gammaproteobacteria* such as *Halomonas elongata* (Imhoff et al., 2021; Vandrich et al., 2020). These bacteria are able both to synthesize and accumulate this osmoprotectant by uptake from the surrounding through osmoregulated transporter TeaABC (Vandrich et al., 2020). The biosynthesis of ectoine and hydroxyectoine under osmotic stress was also indicated in *P. stutzeri* A1501 (Stöveken et al., 2011), which may confirm the assumption that compounds with the ring structure can act as compatible solute.

Down-regulation of the expression of the *cfaB* gene in the lowest pH did not confirm the supposition that cyclopropane fatty acids are responsible for bacterial resistance to low pH (Kim et al., 2005; Pini et al., 2009); however, the suboptimal conditions may not be harsh enough to induce such response. Similar observations were made by Pini et al. (2009) who found no significant differences in survival of *cfaB* mutant and wild-type *P. putida* DOT-T1E strain at pH 6.0, 5.5, 5.0 and 4.0. On the other hand, Budin-Verneuil et al. (2005) revealed that the *Lactococcus lactis* MG1363 *cfa* gene was highly induced by acidity. Furthermore, CFA defective mutants of *E. coli* and *S. enterica* sv. *typhimurium* were highly sensitive to acid stress. Here, the effect of salinity was also observed in the relative expression level of *xylE* gene, which was down-regulated in high salinity. Genetic data align with the catechol 2,3-dioxygenase activity measurements, especially for 5% salinity when the enzyme activity was the lowest. In another study, Heinaru et al. (2016) reported that the *xylA*-specific mRNA in *P. stutzeri* 2A20 strain was approximately 16-fold and six-fold up-regulated as compared to the non-induced cells, respectively.

Because the adaptation of bacteria to suboptimal growth conditions combined with the degradation of the aromatic compound has not been studied extensively, this research aimed to investigate this phenomenon as well as to find out and explain adaptive mechanisms of bacteria involved in the alterations of their cellular fatty acid composition and membrane properties. It was indicated that the higher temperature and salinity caused a significant increase in the membrane permeability from 20 to 81–86% when glucose was served as a carbon source. Because *P. putida* KB3 did not grow in the presence of 5% NaCl, it might suggest that the membrane permeability above 85% is lethal for these microorganisms. According to Gandhi and Shah (2015), the membrane disruption in the saline conditions was probably connected with the alternation in the degree of cell hydration and exosmosis. Literature data indicate that microorganisms can modify the surface of the cells, absorbing available sources of carbon and energy with different physicochemical properties, which ensures them to colonize various ecosystems (Kaczorek et al., 2013). The presence of phenol caused an increase in membrane permeability compared to the cells grown on glucose, or these changes were not significant. These shifts could be connected with the passive transport of phenol inside the bacterial cell – the phenomenon postulated by Gallert and Winter (1993) and Vermaas et al. (2019). The phenol molecule with dimensions of 0.54 × 0.46 nm is rather of considerable size for bacterial cells, and its transport inside the cell probably requires a loosening of the membrane structure. Although the uptake of hydrocarbons could have a generally passive nature, the adaptations increasing these compounds uptake rates into the cell have been evolved (Sikkema et al., 1995). An example is the transport of phenol into the cell via the OprB1 glucose porin postulated by Kivistik et al. (2006) or by porin B suggested by Wierckx et al. (2005). In our work, the exceptions were bacteria exposed to phenol and high temperature or salinity, in which the permeability of membranes decreased by 20%. It can be assumed that the reduction in membrane permeability in the presence of phenol increased cell survival at saline conditions. Bacteria did not grow effectively under saline exposure compared to other tested conditions, resulting from the high energy demand for cell maintenance.

Apart from permeability, the CSH can be considered a sensitive and valuable parameter for assessing the influence of external factors on bacterial cells. In our study, the presence of phenol caused a decrease in CSH of *P. putida* KB3 cells in almost all analysed culture conditions. Literature data concerning the relationship between bacterial cell hydrophobicity and exposure to aromatic compounds are ambiguous and related to the properties of both substances and species of bacteria. For example, Kaczorek et al. (2010) reported an increase in CSH of *Aeromonas hydrophila* under toluene, propylbenzene, and tertbutyl benzene exposure by over 50%. In a more detailed study by Lahesaare et al. (2006), an essential role of adhesin LapF was demonstrated in increasing the hydrophobicity of *P. putida* cells in the stationary growth phase, which increased their survival in the presence of methanol as an example of hydrophilic solvent. Moreover, the CSH value can be modified by biosurfactants and exopolysaccharides secreted outside by the cells (Michalska et al., 2020b). Comparing here both, permeability and CSH parameters, it can be pointed that the increase in the CSH was closely correlated with the increase in membrane permeability. A similar phenomenon was observed by Smułek et al. (2017), who investigated the influence of alkyl xylosides on membrane properties of *Pseudomonas* strains.

The composition of fatty acids also determines cell membrane properties. Reduced cell permeability caused by changes in lipid composition is only partial, and the solvents that enter the periplasmic space and cytoplasm denature proteins (Udaondo et al., 2012). Here, in the presence of glucose, the higher membrane permeability measured at 5% salinity correlated with a higher content of cyclopropane fatty acids in bacterial cells. The cyclopropane ring reduces the order and reactivity of the membrane compared to a membrane with high levels of unsaturated fatty acids – precursors of cyclopropane fatty acids. Comparing FAME profiles of *P. putida* KB3 incubated under optimal conditions (30 °C, pH 7.2) when glucose and phenol were added as a carbon source, it was found that the presence of aromatic compound generated an increase in the participation of 17:0 *cyclo* and 19:0 *cyclo* fatty acids. A similar phenomenon was observed in *Stenotrophomonas maltophilia* KB2 cultured under the same conditions (Nowak et al., 2016). Noteworthy, there was no clear correlation between the high CSH and high content of hydroxylated fatty acids, which might suggest other mechanisms involved in maintaining the hydrophilic-hydrophobic balance of the cell, like an increase in hydrophilic extracellular polymeric substances (EPS) production. Regardless of the introduced carbon source, higher salinity and temperature caused an increase in the participation of straight-chain fatty acids, while lower temperature generated an increase in the percentage of unsaturated fatty acids. Such adaptive mechanisms of bacteria to various external conditions were previously reported in the literature. For example, Bajerski et al. (2017) observed the higher content of unsaturated fatty acids in *Chryseobacterium frigidisoli* PB4^T during incubation at temperatures in the range of 0–10 °C compared to bacterial cells incubated at 14–20 °C, when saturated, branched fatty acids were more abundant. In another study, Chihib et al. (2005) proved that the saturation of cellular fatty acids was the adaptive

response of different *Aeromonas* species to the temperatures in the range of 5–35 °C. Additionally, a significant increase in the saturated, straight-chain 16:0 fatty acid content was detected in the presence of high salt concentration.

5. Conclusions

Due to its ability to degrade phenol under various culture conditions, i.e., temperature, salinity and pH, *P. putida* KB3 seems to be a valuable strain in bioremediation processes. Among the tested conditions, salinity (5%) and low temperature (10 °C) were found as the most critical factors affecting phenol utilization. Moreover, degradation ability was coupled with relatively high catechol 2,3-dioxygenase activity measured for all treatments. The expression level of *xylE* gene indicated that increased salinity caused its down-regulation, while other conditions had little effect on the expression profile. Phenol also stimulated defence mechanisms of the cells concerning changes in the membrane permeability, hydrophobicity, fatty acid saturation level and content of the cyclopropane fatty acids. Integration of biochemical analyses with the relative expression level of *xylE* and *cfaB* genes allowed to complete information on the physiology of *P. putida* under various growth conditions and strengthened its position as a prospective candidate for bioaugmentation of phenol-contaminated environments. However, the final verification of the suitability of the KB3 strain for the treatment of phenol-contaminated areas must be carried out under field conditions. One of the environmental challenges in field trials that were not assessed in the laboratory study is competition with indigenous microorganisms and the risks associated with protozoan grazing.

CRediT authorship contribution statement

Agnieszka Nowak - Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft preparation, Visualization. **Joanna Żur-Pińska –** Investigation, Methodology, Writing - original draft preparation. **Artur Piński –** Investigation, Formal analysis, Visualization. **Gabriela Pacek –** Investigation, Validation. **Agnieszka Mrozik –** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112431.

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