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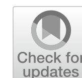
RESEARCH ARTICLE



Biodegradation of p-xylene—a comparison of three psychrophilic *Pseudomonas* strains through the lens of gene expression

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Abstract
p-Xylene is considered a recalcitrant compound despite showing a similar aromatic structure to other BTEXs (benzene, toluene, ethylbenzene, xylene isomers). This study evaluated the p-xylene biodegradation potential of three psychrophilic *Pseudomonas* strains (*Pseudomonas putida* S2TR-01, *Pseudomonas synxantha* S2TR-20, and *Pseudomonas azotoformans* S2TR-09). The p-xylene metabolism-related catabolic genes (*xylM*, *xylA*, and *xylE*) and the corresponding regulatory genes (*xylR* and *xylS*) of the selected strains were investigated. The biodegradation results showed that the *P. azotoformans* S2TR-09 strain was the only strain that was able to degrade 200 mg/L p-xylene after 60 h at 15 °C. The gene expression study indicated that the *xylE* (encoding catechol 2,3-dioxygenase) gene represents the bottleneck in *p*-xylene biodegradation. A lack of *xylE* expression leads to the accumulation of intermediates and the inhibition of biomass production and complete carbon recovery. The activity of xylene monooxygenase and catechol 2,3-dioxygenase was significantly increased in *P. azotoformans* S2TR-09 (0.5 and 0.08 U/mg, respectively) in the presence of p-xylene. The expression of the ring cleavage enzyme and its encoding gene (*xylE)* and activator (*xylS*) explained the differences in the p-xylene metabolism of the isolated bacteria and can be used as a novel biomarker of efficient p-xylene biodegradation at contaminated sites.

Keywords Gene expression · p-Xylene · Catechol 2,3-dioxygenase · *xylE*

Introduction

The International Tanker Owners Pollution Federation (ITOPF) has identified p-xylene as one of the top 20 chemicals posing a high risk among hazardous and noxious substances (HNSs) (Duan et al. 2020). p-Xylene is widely used as an industrial solvent and is highly mobile in the environment in gaseous, liquid, or solid phases (Boonsaner et al. 2011; Mazzeo et al. 2013). Contaminated sites in cold-climate regions have received significant attention due to their vulnerable natural environment. In recent decades, bioremediation techniques have been considered more effective and to cause less undue damage in cold-climate environments than other remediation techniques, such as physical, chemical, and thermal approaches (Habib et al. 2018).

Bioremediation is mainly divided into bioaugmentation (introducing competent bacteria) and biostimulation (adding exogenous oxygen and nutrients), and agreement is still limited regarding which method is the most effective for in situ bioremediation. It has been reported that the biostimulation method can effectively remediate low concentrations of BTEXs (benzene, toluene, ethylbenzene, xylene isomers) in contaminated groundwater (no more than 5 mg/L for each pollutant); however, it shows low degradation performance at high BTEX concentrations (more than 15 mg/L for each pollutant) in meeting the standards required for potable water (Kunukcu 2007; Chen et al. 2010; Yeh et al. 2010; Xin et al. 2013). Hence, at highly contaminated sites, the bioaugmentation process might achieve higher bioremediation efficiency. Some field studies conducted in marine environments have suggested that bioaugmentation using indigenous oil-degrading bacteria can be effective if these bacteria are not limited by the prevailing environmental conditions (Nikolopoulou et al. 2013).

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Microorganisms inhabiting cold environments must cope with a number of challenges, such as limited enzyme activity, slow chemical reaction rates, increased viscosity and limited availability of water (the main solvent for biochemical reactions), protein denaturation, and decreased cell membrane fluidity (Hassan et al. 2020). To date, many microorganisms participating in petroleum hydrocarbon biodegradation in cold regions that belong to the genera *Pseudomonas*, *Rhodococcus*, *Alcanivorax*, *Arthrobacter*, and *Sphingomonas* have been recognized (Miri et al. 2019). Psychrophilic bacteria have adaptive abilities, including the ability to modify the cell membrane structure, which is important in the electron transport chain, the expression of antifreeze proteins and cold-active enzymes, the production of compatible solutes, and the alteration of metabolism (Georlette et al. 2004). Thus, bioaugmentation using cold-adapted indigenous microorganisms can be applied in cold environments to increase bioremediation efficiency. Additionally, it may solve most of the challenges related to large seasonal/daily temperature variations.

To make bioremediation successful, the establishment and maintenance of physical, chemical, and biological conditions in the environment is necessary. Introducing competent microorganisms with high potential for enzyme production can be an effective approach for the bioremediation of polluted sites (Chandran et al. 2020; Bhandari et al. 2021). However, the mode of action and growth of microorganisms in polluted sites need to be further studied to achieve a better understanding (Lovley 2003), owing to the limited success of their application in contaminated environments. Indigenous microorganisms are better adapted to cold environments than any nonindigenous population. The isolation and enrichment of indigenous microbes with biodegradative potential are key steps in bioremediation (Chaudhary and Kim 2019). However, further research and field demonstrations are required to confirm the applicability of bioaugmentation under environmental conditions. The introduced microorganisms may be inhibited by the indigenous microbial community and protozoan grazers. Therefore, implementation and monitoring steps are necessary after introducing the cultured bacteria to evaluate population susceptibility as well as intrinsic bioremediation.

In addition, the biodegradation of p-xylene is not easy relative to that of other BTEX compounds, and p-xylene is classified as a persistent pollutant and non-growth substrate. The biodegradation of BTEX compounds is generally initiated by mono- or dioxygenase enzymes, which might find it difficult to attack the two methyl groups present in p-xylene (Li et al. 2014; Qu et al. 2015). The successful activation of targeted metabolic genes in specific pathways relies mainly on the catabolic enzymes involved and the transcriptional regulation of the pathways (de Lorenzo and Pérez-Martín 1996) to direct the appropriate enzymatic cascades in response to the specific pollutant as a substrate (Díaz and Prieto 2000).

Several studies have shown low or no degradation of p-xylene by *Pseudomonas* strains, such as *Pseudomonas* B1 and X1 (Chang et al. 1993), *Pseudomonas stutzeri* OX1 (Arenghi et al. 2001a), *Pseudomonas* sp. BTEX-30 (Khodaei et al. 2017), *Pseudomonas putida* AQ8 (Chicca et al. 2020), and *Pseudomonas putida* (Miri et al. 2021a), even at favorable temperatures (20–30 °C). There have been a few studies specifically addressing the bioremediation of p-xylene. For example, Rotaro et al. (2010) identified microorganisms in enrichment cultures obtained from a wastewater treatment plant for *p*-xylene degradation. They reported that the dominant bacterial strain was related to *Denitratisoma oestradiolicum* in enriched cultures (Rotaru et al. 2010). Kermanshahi pour et al. (2006) studied the biodegradation kinetics of p-xylene in a new type of biofilm reactor (immobilized soil bioreactor). The most predominant microbial communities in the bioreactor were populations of *Pseudomonas acidovorans* and *Chryseobacterium indologenes* (Kermanshahi pour et al. 2006). The TOL plasmid contains the genes encoding the enzymes necessary to aerobically degrade BTEXs and has been extensively studied in *Pseudomonas putida*. However, a few studies have been conducted on TOL plasmid expression in the presence of p-xylene in *Pseudomonas* strains (Worsey and Williams 1975; Choi et al. 2013a). Moreover, *Pseudomonas* species have been extensively identified in petroleum hydrocarbon–contaminated soil and water in cold regions (Miri et al. 2019). However, the potential of psychrophilic strains for the bioremediation of p-xylene as well as gene expression aspects of biodegradation in cold climates is still unknown.

Therefore, this study was performed with two main objectives: (1) to evaluate the ability of three psychrophilic isolates to biodegrade p-xylene at 15 °C and (2) to investigate the reason for the differences in p-xylene metabolism through a gene expression analysis of the enzymes involved. This study combined biodegradation experiments, gene expression analysis, and enzyme activity tests to identify the mechanism underlying the complete and effective biodegradation of p-xylene by psychrophilic strains. The p-xylene degradation potential of three BTEX-degrading *Pseudomonas* strains was confirmed based on carbon mass balances through the determination of the intermediates produced.

Materials and methods

Chemicals and culture medium

All the chemicals and culture media used in this work were of analytical and microbial grade and were obtained from

Fisher Scientific, Ontario, Canada. Benzene, toluene, ethylbenzene, p-xylene (99.9%), methanol (chromatographic grade), tryptic soy broth (TSB), and tryptic soy agar (TSA) were purchased from Sigma-Aldrich (Mississauga, Ontario, Canada). Carbon-free mineral salt media (MSM) (1.8 g/L K_2HPO_4, 4.0 g/L NH_4 Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, and 0.01 g/L $FeSO_4 \cdot 7H_2O$) were used for biodegradation tests (Rahul and Mathur 2011).

Isolation of bacterial strains from soil samples

The bacterial strain used in the present study was isolated from a confidential petroleum-contaminated site in Montreal, Canada, with a typical aquifer temperature of 8 to 15 °C. The samples were collected from soil at depths ranging from 5 to 7 m where the p-xylene concentration was 10,000 mg/kg. The enrichment and adaptation steps were carried out using MSM supplemented with 50–200 mg/L BTEXs. Briefly, the samples were enriched with a 50 ppm BTEX mixture (1:1:1:1) and incubated at 15 ± 1 °C at 150 rpm for 1 month. Then, the mixture (soil and MSM) was transferred to fresh MSM supplemented with a higher concentration (100 or 200 ppm) of BTEXs. After 1 month, the culture was cultivated in TSA (Sigma-Aldrich, Canada) at 15 °C for 3 days. Isolation and biodegradation tests were carried out between 8 and 15 °C (the typical aquifer temperature range at the contaminated site). Single colonies with different morphologies were selected and transferred to TSB and incubated at 15 ± 1 °C for 3 days. The strains were characterized by the amplification and sequencing of a 16S rRNA fragment (27F: 5′-AGAGTTTGATCCTGG CTCAG-3′, 1492R: 5′-GGTTACCTTGTTACGACTT-3′). The sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was performed using the neighbor-joining algorithm of MEGA 7.0 software with 1000 bootstrap replicates (Tamura et al. 2011).

Biodegradation tests

Experiments testing growth ability and the biodegradation of p-xylene were carried out in 150-mL sealed serum bottles containing 200 mg/L p-xylene. The 200-mg/L concentration of p-xylene was selected because this compound is partially insoluble in water, with a solubility of 198 mg/L at 25 °C (Bohon and Claussen 1951). This value was considered to provide sufficient bioavailability of this compound to promote the biodegradation efficiency of xylene-utilizing microorganisms. Our previous work also showed that 200 mg/L p-xylene can induce the production of degrading enzymes as well as the expression of genes encoding enzymes relative to 50 and 100 mg/L p-xylene (low and intermediate concentrations) (Miri et al. 2021b). The medium (at pH 7.0 ± 0.2) was placed in a 150-mL serum bottle sealed with a Teflon-coated rubber stopper, with a 9:1 airspace/liquid ratio. The inoculum was added after 24 h of equilibrium of the p-xylene concentration between the liquid and headspace under the experimental conditions. Inoculation (1% v/v, OD600 = 2) was carried out using a gas-tight syringe. The inoculated cultures (Od600 = 2, corresponding to 6×10^7 cells/mL) were incubated at 15 °C in a rotary shaking incubator (150 rpm).

The concentration of p-xylene in the collected liquid phase was detected using GC/MS. As p-xylene is considered a highly volatile compound, only approximately 50 to 60% of the concentration added can be detected in the liquid phase. The concentrations in the liquid and gas phases were measured, and the necessary concentration was compensated to correct the results. Growth experiments indicated that *P. putida* S2TR-01 could not grow on p-xylene as the sole source of energy and carbon. To assess the effect of p-xylene, the cells were grown on TSB medium, harvested via centrifugation at 12,000 × *g* and 4 °C for 5 min, and then inoculated in MSM supplemented with 200 mg/l p-xylene. At selected time intervals, the cells were harvested for further analyses.

Two types of control groups were designed for the tests: (1) abiotic controls inoculated with autoclaved bacteria (121 °C for 20 min) were run in parallel to monitor abiotic processes such as volatilization and bacterial sorption in the biodegradation experiments; and (2) controls consisting of isolates grown in the presence of glucose (2.5 g/L) as the sole source of carbon (del Castillo and Ramos 2007) instead of p-xylene were used for intermediate, gene expression, and enzyme analyses.

p-Xylene analysis

In each batch study, subsamples (500 µL) were removed from the liquid phase of bottles using a gas-tight syringe for the OD600 and substrate concentration measurements every 6 h. A subsample of 50 µL was prepared in a 40-mL standard headspace bottle containing 5 mL of ultrapure water and 10 µL of fluorobenzene-D5 (100 mg/L diluted in methanol) as an internal standard, and the bottle was then sealed immediately. The concentrations of p-xylene were analyzed based on the EPA 8021B method, and p-xylene was extracted using a Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler. The gas phase of the samples was quantitatively analyzed using a 7890A gas chromatograph (DB-1701 column 30 m × 0.25 mm i.d. × 0.25 µm film thickness), at temperatures of 45 °C and 300 °C for the column and detector, respectively. The concentration of intermediates was analyzed using a CP-Wax 57 CB column (25 m × 0.25 mm × 0.20 µm, Agilent Technologies, Netherlands) connected to a Varian 2200 gas chromatograph coupled to a Saturn mass detector. Intermediates (p-cresol and

p-toluic acid) were not determined by headspace GC/MS because they are semivolatile compounds.

Gene expression analysis

Based on biodegradation and biomass kinetics (results presented in the "Biodegradation of p-xylene" section), the timepoints of 10, 20, and 50 h were selected for gene expression analysis to represent the early phase of incubation (lag period of biodegradation), exponential phase of biomass production (mid-period of biodegradation), and stationary phase of biomass (last stage of substrate biodegradation), respectively. Growing cultures were harvested by centrifugation to obtain ~ 10^{10} cells, and the pellets were then used for RNA extraction and cDNA synthesis as indicated in the supplementary material (Section S1 and Fig. S1).

The *xyl* gene sequence of *Pseudomonas putida* TOL plasmid pDK1 (GenBank accession no. AF019635.1) was used as the complete CDS (Coding Sequence) for primer design. The 16S rRNA sequence of *Pseudomonas putida* (GenBank accession no. MN625925.1) was also used as a template for primer design. Primers for PCR and real-time PCR were designed using Primer3 program version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) to target the *xylM*, *xylA*, *xylE*, *xylS*, *xylR*, and 16S rRNA genes of isolated *Pseudomonas* (Table 1). Primer-BLAST (Basic Local Alignment Search Tool) was used to check the specificity of the primers in a wide range of *Pseudomonase* strains. This online primer design platform combines BLAST with a global alignment algorithm to ensure that primers are sensitive enough to detect targets without a number of mismatches to primers (Ye et al. 2012). The potential formation of primer dimers and other secondary structures was checked in Gene Runner software Version 6.5.52 Beta. Additionally, plasmid DNA was amplified using the above primers, and the PCR products were checked for their estimated size to ensure that the primers targeted the appropriate genes in the isolated strains.

The relative expression of selected genes was analyzed with a real-time PCR System (Rotor-Gene Q-QIAGEN) and a SYBR Green real-time PCR Master Mix kit (QIAGEN, Germany). The reaction components were Taq polymerase, dNTP, $MgCl_2$, SYBR green I dye (20 μL Master Mix), 0.2 μL primers, 0.5 μL cDNA, and 10 μL H_2O. The thermocycling conditions for real-time PCR are shown in Table S2. All experiments were repeated in triplicate in both biological and analytical experiments. Each mRNA expression value was normalized against the threshold cycle of 16S rRNA expression as a housekeeping gene. Our preliminary results showed that 16S rRNA was constitutively expressed during p-xylene biodegradation by the tested *isolates* (CT = 14.0 ± 0.7), indicating that it could be used as a reference or housekeeping gene. Mean normalized gene expression ± standard deviation (SD) values were calculated from triplicate analyses. The fold changes of target genes were calculated as $RQ = 2^{-\Delta\Delta ct}$ values using Eqs. 1, 2, 3:

$$\triangle C_t = (C_t \text{ of targeted gene}) - (C_t \text{ of housekeeping gene}) \quad (1)$$

$$\triangle\triangle C_t = (\triangle C_t \text{ of experimental test}) - (\triangle C_t \text{ of control test}) \quad (2)$$

$$RQ = 2^{-\triangle\triangle Ct} \quad (3)$$

Our target genes were *xylM*, *xylA*, *xylR*, *xylE*, and *xylS*. Our housekeeping gene was the 16S RNA gene.

One advantage of the relative quantification method is that it does not require the use of standard curves, which makes it faster and easier than other approaches (Pfaffl 2001). The melting curve of the real-time PCR products was checked by raising the temperature in increments of 0.3 °C to ensure that only the targeted products were formed. This

Table 1 Primer sequences used for PCR and real-time PCR in this study

Target gene	Product	Orientation	Primer sequence (5′-3′)	Tm (°C)	Amplicon size (bp)
XylM	Xylene monooxygenase hydroxylase subunit	F R	AATGTCTCGGTTCGGATCAC AGTGGTGTGCCAACTCTTCC	60	114
xylA	Xylene monooxygenase	F R	GCAGCGCTTCTATTTCGTC GAGAGCAATGCGACAATGG	58	166
xylE	Catechol 2,3-dioxygenase	F R	CTTCAAGGTGACCGACGATG GGTGTACTCCTTCTCGGCATAG	60	172
xylS	Transcriptional activator	F R	ACCACAGAATCTTCGGATGC GGCGAAAAATAGTGCTCCTG	60	157
xylR	Transcriptional activator	F R	ACCCGCTCTAGCTCTCCTTC ATCGAGTCGGAACTGTTTGG	60	179
16S rRNA	Ribosomal RNA	F R	CGGAATTACTGGGCGTAAAG TCTACGCATTTCACCGCTAC	59	149

study applied qPCR to achieve more accurate and quantitative results than are obtained with other analytical methods (such as protein and biochemical analyses) for the TOL plasmid study.

Enzyme and protein assays

Bacterial cells were harvested after 50 h of incubation because the maximum enzyme activity can be assayed when the bacteria are in the stationary phase of growth (Le Meur et al. 2012). The extraction of intracellular products is dependent on the growth phase (Harrison 1991), and it is well documented that the cell lysis effect can be negligible when bacterial cells reach the stationary growth phase (Nagy et al. 2001). Our preliminary results also confirmed that 50 h of incubation was the best time for the extraction of intracellular enzymes. These preliminary results were obtained based on the xylene monooxygenase assay in cell suspensions according to Arenghi et al. (1999) and in cell lysates after ultrasonication. Briefly, the cells were washed twice by centrifugation at 5000 × *g* with 20 mM phosphate buffer (pH approximately 7.0) and were resuspended in the same buffer. The cells were disrupted by applying two frequencies of ultrasound (22 kHz and 30 kHz) for 6 min. The cellular lysates were centrifuged at 13,000 × *g* for 20 min, and the supernatant was used for enzyme assays. Two key enzymes encoded by the *xyl* operon were selected (xylene monooxygenase and catechol 2,3-dioxygenase), and their activities were measured (Miri et al. 2021b). Based on enzyme activity tests in psychrophilic isolates, 15 °C was determined to be the optimal temperature for enzymes obtained from the isolates, so all of the enzymes assays were carried out at this optimal temperature. To determine specific activity (units per mg of proteins), the total protein concentration was determined by the Bradford method (Brasford 1976). Briefly, an acidic solution of Coomassie dye was added to the protein solution, and the absorbance was then measured at 595 nm. The total protein concentration in the samples was determined using a standard curve with known concentrations of bovine serum albumin (BSA).

Xylene monooxygenase activity

Xylene monooxygenase activity was determined by monitoring the increase in the phenolic compound concentration in the medium using a colorimetric assay slightly modified from Arenghi et al. (1999). Briefly, 0.5 mM NADPH (final concentration) and 35 μL of 4% (vol/vol) *p*-xylene in N,N-dimethylformamide were added to the cellular lysates. After incubation at 15 °C, 1-mL samples were collected at 3-min intervals and mixed with 100 μL of 1 M NH_4OH and 100 μL of 2% 4-amino antipyrine. After the addition of 100 μL of 8% $K_3Fe(CN)_6$, the samples were briefly centrifuged (14,000 × *g*), and the A500 of the supernatant was then measured. Phenolic compound concentrations were calculated by reference to a standard curve for p-cresol (in the range of 0–50 μg/mL). Specific activity was defined as micromoles of phenolic compounds produced per minute per milligram of cell protein.

Catechol dioxygenase assay

The reaction mixture (3.0 mL) contained 2.0 mL phosphate buffer, 0.6 mL of 1 mM catechol, 0.2 mL deionized water, and 0.2 mL cellular lysates. The reaction was allowed to proceed at 15 °C, and subsamples were taken every 2 min. Catechol 2,3-dioxygenase activities were determined by measuring the production of 2-hydroxymuconic semialdehyde at 375 nm. One unit of enzyme activity was defined as the amount of enzyme that formed 1 μmol of muconic acid per minute per milligram of cell protein (Li et al. 2014).

Carbon mass balance

The carbon mass balance of each isolate was determined by measuring substrate consumption using GC-head space (as mentioned previously), intermediate, biomass growth, and carbon dioxide production measurements.

Intermediates formed during p-xylene biodegradation were analyzed using two types of gas chromatography/mass spectrometry systems: (1) a GC–MS system equipped with a wax capillary column (for the detection of nonvolatile intermediates) and (2) a GC–MS system equipped with a headspace sampler (for the detection of possible volatile intermediates, as indicated for p-xylene concentration analysis).

The GC–MS system was equipped with a CP-Wax 57 CB column of 25 m × 0.25 mm × 0.20 μm (Agilent Technologies, Netherlands). The temperature of the GC oven was programmed to increase from 40 °C (held for 1 min) at 5 °C/min to 200 °C (held for 10 min). The injector temperature was set at 180 °C, and the MS interface temperature was set at 250 °C and 280 °C for the ionization source. All metabolites were identified via GC/MS by matching the retention times and ion spectra with authentic standards and NIST library data. Then, the concentrations were determined using the calibration curves of the corresponding standards.

The amounts of carbon recovered as C–CO_2 and the carbon recovered as biomass were determined after substrate exhaustion, and the values were corrected using control flasks incubated with no carbon source. Carbon dioxide was measured chromatographically via GC–MS (7820A GC system Agilent Technologies, Netherlands) (Srivastva et al. 2016). Biomass was collected via 0.45-μm-pore-diameter filtration and placed in an oven, and the dry biomass content was determined. A linear relationship was observed between the OD600 and the cell dry weight of the isolates (cell dry

weight (mg/L) = 308 × OD600, $R^2 = 0.95$). The amount of carbon recovered as biomass was calculated by assuming that 26 g dry weight contains approximately 1 mol of carbon (Tijhuis et al. 1993). Carbon mass balance tests were conducted in triplicate for isolates and control samples.

Statistical analysis

Statistical analysis was performed via one-way analysis of variance (ANOVA) followed by Tukey's post hoc procedure. A probability of less than 0.05 was considered statistically significant. All analytical and biological tests were performed in triplicate. Error bars indicate SD, $n = 3$. The p-xylene removal and target gene expression correlations were analyzed using the two-tailed Pearson correlation test.

Results and discussion

Identification of psychrophilic isolates

Among all obtained isolates, three psychrophilic bacteria were selected based on their ability to grow on BTEXs and the differences in their behavior regarding p-xylene degradation as the sole source of carbon. The three BTEX-degrading bacteria were named S2TR-01, S2TR-09, and ST2TR-20. The 16S rRNA gene sequences indicated that S2TR-01 was most closely related to the type of strain of *Pseudomonas putida* ATCC 12633 ^{T}, with 99% sequence similarity. S2TR-09 and ST2TR-20 showed more than 98% similarity to *Pseudomonas azotoformans* DSM 18862 ^{T} and *Pseudomonas synxantha* ATCC 9890 ^{T}, respectively. The 16S rRNA gene sequences of each isolate were deposited in the NCBI GenBank database and assigned the accession numbers shown in Fig. S2. Phylogenetic analysis also showed that S2TR-01, S2TR-09, and ST2TR-20 formed a phylogenetic lineage with members of the mentioned strains. *Pseudomonas* spp. is an excellent microorganism for use in bioremediation because of the plasticity and flexibility of its metabolic pathways and its ability to live under diverse environmental conditions (Palleroni et al. 2009). Kabelitz et al. (2009) reported that *Pseudomonas* species are the dominant microbial community in ecosystems that are highly contaminated with kerosene and BTEXs. Their results showed that *Pseudomonas* not only survives under harsh conditions, such as low oxygen levels, high loads of aromatic carbon pollutants, and high solvent concentrations, but also replicates under these conditions, which is an important indicator of efficient bioremediation (Kabelitz et al. 2009).

The BTEX degradation abilities of the three isolated bacterial strains were analyzed at 5–30 °C, and all of them showed obvious BTEX degradation at cold temperatures (5–15 °C). The optimum temperature for bacterial growth and biodegradation was 15 °C, and no growth was observed at 30 °C in the presence of BTEXs (Table S1). These strains showed interesting results such as a high rate of BTEX degradation at a low temperature (15 °C). However, they exhibited different degradation and growth abilities in the presence of p-xylene as the sole source of carbon (Table S1). Many mesophilic *Pseudomonas* strains have been identified as BTEX degraders, such as *Pseudomonas* B1 and X1 (Chang et al. 1993), *Pseudomonas stutzeri* OX1(Arenghi et al. 2001a), *Pseudomonas* sp. BTEX-30 (Khodaei et al. 2017), and *Pseudomonas putida* AQ8 (Chicca et al. 2020). These strains have also shown low xylene degradation rates even at favorable temperatures (20–30 °C). A study on the effect of the chemical structure of BTEXs on four chemolithotrophic bacteria showed that increases in the number of alkyl groups in a benzene ring and the size of the compounds can increase the toxicity of these compounds (Odokuma and Oliwe 2003). Similarly, the isolated bacteria showed different p-xylene degradation rates (as described in the following section), which could be due to the additional methyl group in p-xylene and its toxic effect relative to other BTE compounds. However, a more detailed toxicity assay is needed to confirm that p-xylene and ethylbenzene, with additional methyl and ethyl groups relative to benzene and toluene, respectively, are more toxic than the latter pair of compounds.

Biodegradation of p-xylene

The p-xylene biodegradation experiments were carried out at an initial p-xylene concentration equal to ~ 200 mg/L at 15 °C. The changes in biomass and the p-xylene concentration versus time are shown in Fig. 1. The results indicated that *P. azotoformans* S2TR-09 degraded p-xylene faster than the other isolates after 60 h ($p < 0.05$). The biodegradation of p-xylene by *P. synxantha* S2TR-20 was complete after 5 days (120 h). However, *P. putida* S2TR-01 showed no p-xylene degradation even after 10 days of incubation (Fig. 1). The biomass results confirmed that *P. putida* S2TR-01 could not use p-xylene as the sole source of carbon. Although a small decrease in the p-xylene concentration (~ 30 mg/L) was observed in the presence of *P. putida* S2TR-01, this change was not attributed to biomass production (Fig. 1). This was due to the accumulation of intermediates, as mentioned in the following section on carbon mass balance. As shown in Fig. 1, the concentration of *P. azotoformans* S2TR-09 biomass increased with time in the presence of p-xylene in the following order: S2TR-09 > S2TR-20 > S2TR-01 ($p < 0.05$). Based on the reported literature, the degradation of p-xylene can be expected to vary among *Pseudomonas* strains. For instance, You et al. (2013) reported that 54% p-xylene degradation was achieved by *P. putida* YNS1 after 96 h of incubation at 30 °C (You et al. 2013). Jeong et al. (2006) reported

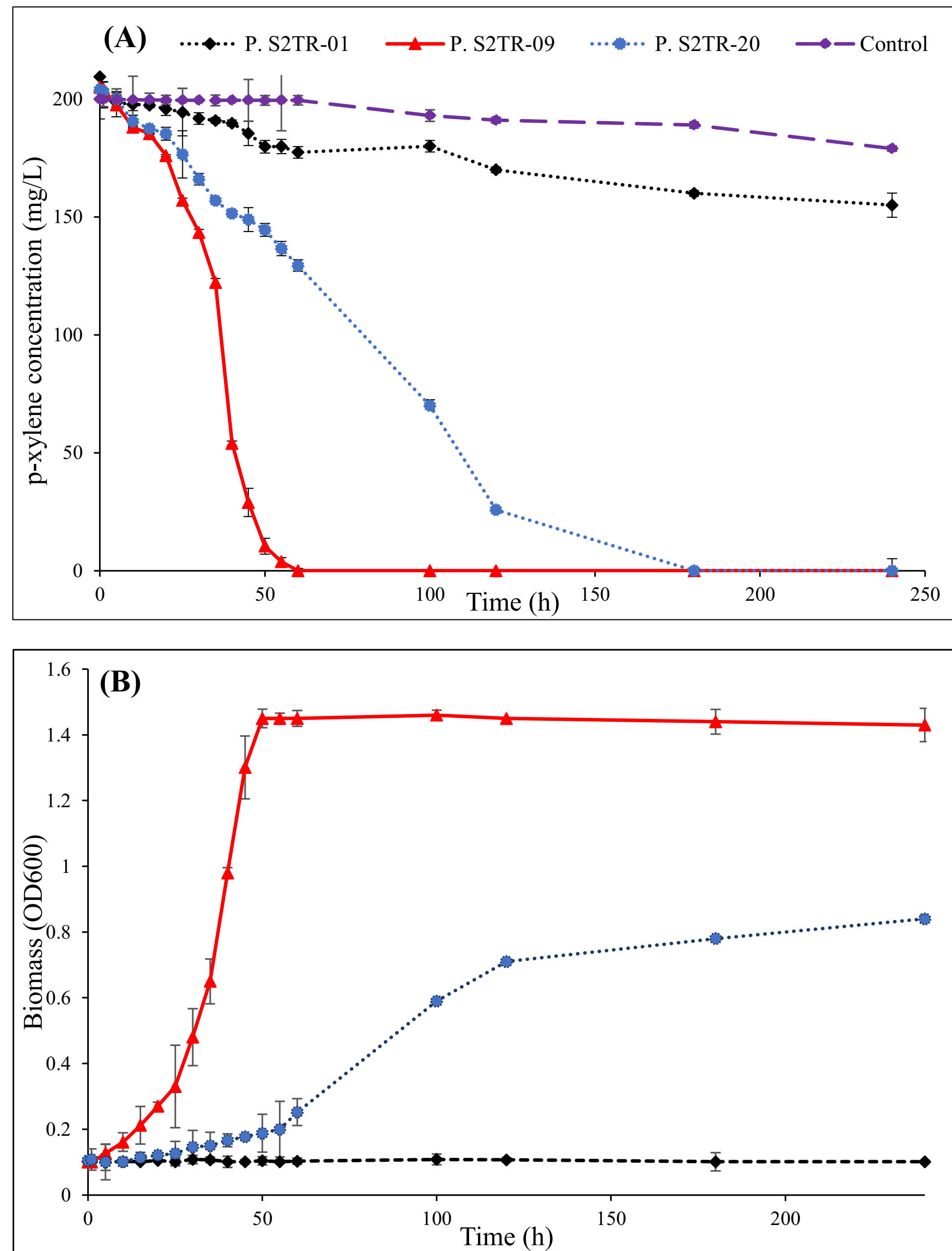

Fig. 1 p-xylene biodegradation (A) and biomass production (B) by *P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20 with an initial concentration of 200 mg/L. Error bars indicate standard deviation (SD); $n=3$

90% removal of p-xylene (5.1 mg) using *Pseudomonas* sp. NBM21 in a biofilter at 30 °C after 4 h (Jeong et al. 2006). Worsery et al. (1975) showed that *P. putida* mt-2 could degrade p-xylene as a carbon source for growth (Worsey and Williams 1975). These results indicated that each strain has a different degradation ability. The results of this study also showed that the examined psychrophilic isolates present different biodegradative abilities in the presence of p-xylene.

Several researchers have reported that when microbial strains are grown in basal mineral media or media containing p-xylene alone at ambient temperature (20–30 °C), toluene is degraded more readily, and the slowest biodegradation rate is observed for p-xylene (sometimes no biodegradation is reported) (Alvarez and Vogel 1991; Zhou et al. 2016; Meyer-Cifuentes et al. 2017). You et al. (2018) reported lower p-xylene biodegradation by *Rhodococcus* sp. ZJUT312 relative to its degradation of other BTE compounds (You et al. 2018). Wang et al. (2015) reported that *Pandoraea* sp. strain WL1 could degrade 16.6~99.4 mg/L p-xylene as the sole source of carbon in the liquid phase within 6 to 18 h at 30 °C (Wang et al. 2015). In the case of cold-climate environments, low temperatures (e.g., below 10 °C) pose an additional challenge to biological treatments, and it may require more time (ranging from 1 to 10 years) to meet cleanup standards in such areas (Miri et al. 2019; Davoodi et al. 2020). The results of this study showed that more time is needed for the complete

degradation of p-xylene compared to the times indicated by previous studies. However, the concentration of contaminants should be considered an important factor because of their toxicity and inhibitory effects.

Effect of p-xylene on catabolic gene expression

The TOL plasmid is the best-studied catabolic plasmid as a broad-host-range plasmid (Benson and Shapiro 1978; Smets et al. 1994). Generally, BTEX biodegradation occurs via two pathways (*tod* and *tol*) in *Pseudomonas* strains (Yu et al. 2001). In the *tod* pathway, bacteria use xylene dioxygenase as a primary enzyme for p-xylene degradation. Based on our preliminary results and a previous study (Miri et al. 2021b), xylene dioxygenase shows no activity in the presence of p-xylene in the exponential growth phase (data not shown); hence, the isolates did not use the *tod* pathway in this case. As bacteria use one of these pathways, we conclude that these strains contain a TOL-like pathway. We thus followed the expression of TOL-type genes in the presence of p-xylene (Fig. 2). The genetic organization of the catabolic genes in the TOL plasmid reflects the biochemical pathways, so the catabolic genes can be organized into two operons: *upper* and *meta*-cleavage operons (Fig. 3). Notably, the proposed genetic map of the TOL plasmid in isolates (Fig. 3) was described based on isolates with the same catabolic phenotype, and the archetypal TOL plasmid pWWO originated in *P. putida* mt-2. Many other TOL plasmids have been identified that have the same prototype but show different sizes because of gene duplications encompassing the entire catabolic operons or parts of catabolic operons (Williams et al. 2004). The comparative sequencing of the TOL plasmids of isolates could be carried out to provide a wealth of information about the role of TOL plasmids and the history of bacterial evolution.

The *upper* operon comprises 5 genes (*xylCMABN*) that encode the enzymes responsible for the monooxidation of aromatic hydrocarbons to their corresponding carboxylic acids. *xylM* and *xylA* encode two subunits of xylene monooxygenase, which is the first and most important enzyme in the biodegradation pathway of BTEXs (Bühler et al. 2002). The *meta*-cleavage operon (containing *xylXYZLTEGFJQKIH*) encodes the enzymes responsible for the degradation of benzoate to Krebs cycle intermediates. These 13 genes are conserved as one of the largest operons found in prokaryotes, at over 11 kb (Marqués and Ramos 1993). The *xylE* gene encodes a key enzyme, catechol 2,3-dioxygenase, involved in the ring cleavage of aromatic hydrocarbons. The *xylR* and *xylS* genes encode specific transcriptional regulators that activate the expression of two pathway genes, *xylR* and *xylS*, located downstream of the *meta*-cleavage operon (Fig. 3; (Moreno et al. 2010). In pWW0, the genes encoding XylS and XylR are located at the 3′ end of the *meta*-cleavage

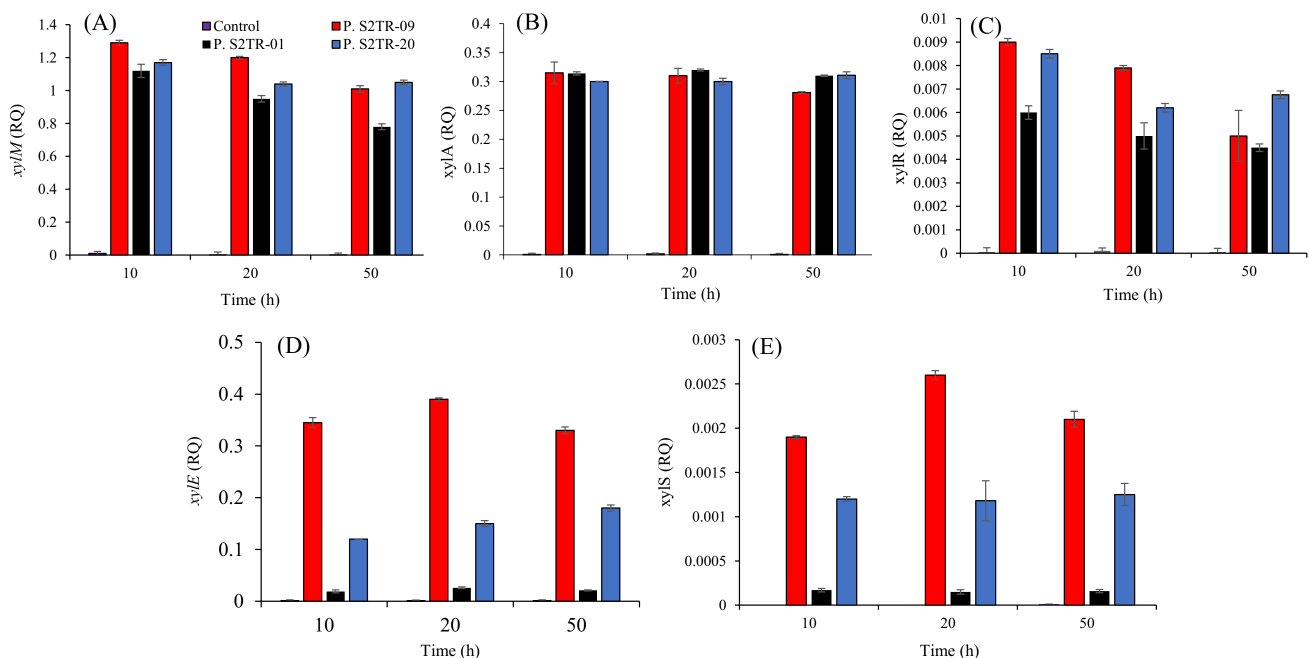

Fig. 2 Gene expression of *upper* and *meta* operons and their transcriptional regulatory genes in different isolated bacteria (*P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20) grown on p-xylene; RQ, relative quantification; Control, the sample form the bacteria grow on media without p-xylene (the presented data results from S2TR-09 due to similar data from other isolates). Error bars indicate standard deviation (SD); $n = 3$

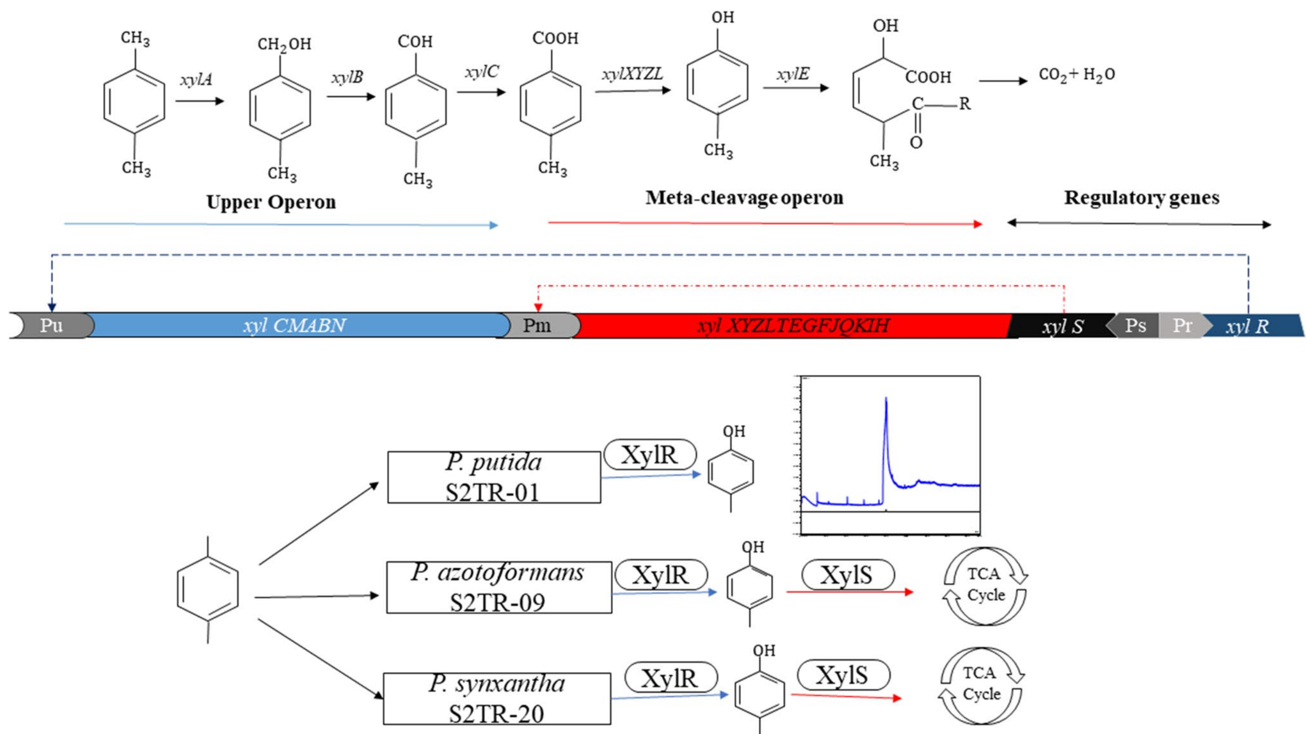

Fig. 3 Genetic map of the TOL pathway in biodegradation of p-xylene. Biodegradation of p-xylene requires the induction of *xylS* that regulates the meta-cleavage enzymes in isolated strains. The enzymes encoded upper operon transform p-xylene to p-cresols. Then, p-cresol is transformed into TCA cycle intermediates by encoded meta-cleavage enzymes. The structural genes, *xylCMABN*, and *xylXYZLTEGFJOKIH*; regulatory gene, *xyls*, and *xylR*; and promoters, Pu, Pm, Ps, and Pr

operon, which controls the expression of the genes in this pathway (Fig. 3).

The results presented in Fig. 2 show that *xylM* and *xylA* were upregulated in all isolates in the presence of p-xylene because xylene monooxygenase is needed to initiate the degradation pathway. The results obtained for *xylM* and *xylA* were consistent with the *xylR* expression profile, showing upregulation in the presence of p-xylene. Although there was no significant difference in the gene expression of *xylM* and *xylA* between the isolates, *xylR* showed a decreasing trend after 10 h of incubation (active time of biodegradation). This may correspond to a decrease in the p-xylene concentration and the negative autoregulation of *xylR*, as reported by de Las Heras et al. (2012).

However, Bertoni et al. (1998) and Marqués (1194) reported that *xylR* expression in pWW0 was immediately downregulated after the addition of p-xylene to cells growing on mineral salt media. Our results showed the upregulation of *xylR* in the presence of p-xylene and growth phase–dependent *xylR* expression. Xylene can induce the expression of *xylR* and its encoded protein (XylR), and this protein can bind aromatic compounds and regulate the expression of the genes encoded by the *upper* operon in the TOL plasmid (Marqués and Ramos 1993; Moreno et al. 2010; de Las Heras et al. 2012). Further investigation of the expression of this gene in different strains would provide a wealth of information on this gene.

Pearson's correlation indicated a statistically significant relationship between the expression of target genes and the p-xylene removal rate in *P. azotoformans* S2TR-09 (Table 2). The p-xylene degradation rate was calculated as the change in the p-xylene concentration divided by the change in time, with 10, 20, and 50 h representing the *lag* period of biodegradation, mid-period of biodegradation, and last stage of biodegradation, respectively. The expression of *xylE* and p-xylene removal were significantly correlated in all isolates ($p < 0.05$), which was consistent with p-xylene degradation in the order of *P.* S2TR-09 $>$ *P.* S2TR-20 $>$ *P.* S2TR-01 ($p < 0.05$). The growth of bacterial strains on p-xylene requires the expression of a *meta*-cleavage operon because this pathway funnels the intermediate benzoate derivative into the Krebs cycle, which provides the main source of cellular energy for aerobic microorganisms (Seo et al. 2009). Transcriptional stimulation of the *meta*-cleavage operon is activated by the *xylS* regulator, with the interaction of benzoate and methyl benzoate as effectors (Marqués and Ramos 1993). As shown in Fig. 2, gene expression levels differed depending on the degradation ability of the strain and the time of degradation. Our results showed that *xylS* was induced only in

Table 2 Correlations between fold change of target gene and p-xylene removal rate

		p-Xylene removal	*xylM*	*xylA*	*xylR*	*xylE*	*xylS*
P. putida S2TR-01	10 h	−1.16922	1.12	0.314	0.006	0.019*	0.00017
	20 h	−0.1956	0.95	0.32	0.005	0.026*	0.00015
	50 h	−0.2	0.78	0.31	0.0015	0.021*	0.00016
P. azotoformans S2TR-09	10 h	−1.7	1.29**	0.315**	0.009**	0.345*	0.0019*
	20 h	−1.2	1.2**	0.31**	0.0079**	0.39*	0.0026*
	50 h	−3.52	1.01**	0.280**	0.005**	0.33*	0.0021*
P. synxantha S2TR-20	10 h	−1.35	1.17	0.3	0.0085**	0.12*	0.0012
	20 h	−1.87	1.04	0.3	0.0062**	0.15*	0.00118
	50 h	−1.98	1.05	0.311	0.0067**	0.18*	0.00125

*Symbolize the significant difference between p-xylene concentration and each targeted gene at $p < 0.05$

**Symbolize the significant difference between p-xylene concentration and each targeted gene at $p < 0.01$

the isolates (*P. azotoformans* S2TR-09 and *P. synxantha* S2TR-20) that could grow in the presence of p-xylene, whereas in *P. putida* S2TR-01, *xylS* and the *meta*-cleavage operon were not induced (Fig. 2).

Velazquez et al. (2005) showed that upper *xyl* operon gene expression was quickly and strongly induced upon exposure to *m*-xylene, while a certain delay in the induction of the *meta*-cleavage operon genes was observed. They showed that the expression of the *xylE* gene remained upregulated in all growth phases until the end of the experiment. They also mentioned that the most evident change after 3 h of contact of *P. putida* mt-2 with *m*-xylene was the expression of the entire complement of *lower* operon *xyl* genes (Velázquez et al. 2005). Our results showed an upregulation of *xylE* in S2TR-09 during the *lag* period of biodegradation, when metabolites had been accumulated. Even higher relative expression of the gene was observed in the exponential phase of biomass production (Fig. 2(D)).

Generally, the expression of the *meta*-cleavage operon is controlled in two ways: (1) the XylS protein activates the promotors of the operon, which is positively regulated by intermediates such as benzoate derivatives; and (2) the toluene/xylene-activated XylR protein in combination with a sigma factor stimulates transcription from the xylS gene promoter via a cascade regulatory system (Ramos et al. 1987). Our correlation analysis between the gene expression of *xyl* genes and the p-xylene removal rate showed that a small increase in *xylS* in *P. azotoformans* S2TR-09 had a significant effect on p-xylene removal ($p < 0.05$). Previous studies showed that the expression of the *xylR* gene is constitutively high in *Pseudomonas putida* (Inouye et al. 1985), while the *xylS* gene appears to generally be expressed at a low basal level. *xylS* expression seems to be required to activate the *meta*-cleavage operon via its promotor (*Pm*) (Ramos et al. 1987). Our results showed that *xylS* was NOT induced in S2TR-01, and this result emphasizes that S2TR-01 cannot degrade p-xylene to use it as a sole carbon source. The differences in the expression of the genes of the *meta*-cleavage operon and its regulatory gene (*xylS*) can explain the differences in p-xylene degradation among the isolates.

Enzyme activity detection

In aerobic systems, oxygenases are key enzymes in microbial degradation. The key enzymes in the metabolism of p-xylene are xylene monooxygenase and catechol 2,3-dioxygenase (Miri et al. 2021a). Previous research showed that the oxidation of xylene may be initiated by the direct oxidation of the aromatic ring or the oxidation of one or two methyl groups of the aromatic nucleus by xylene monooxygenase. This enzyme can catalyze multistep oxidation and produce catecholic or noncatecholic derivatives (Choi et al. 2013b). Then, in the ring dearomatization of central intermediates, *ortho*-, *meta*-, or *para*-cleavage by catechol dioxygenases occurs as the crucial step in detoxification (Arenghi et al. 2001b; Tsai and Li 2007a). Catechol 2,3-dioxygenase is an iron-containing enzyme that is able to cleave the rings of oxidized derivatives from p-xylene to achieve the complete detoxification of this contaminant (Miri et al. 2021b). Mass spectra results showed that p-xylene is initially oxidized to central intermediates such as p-toluic acid and p-cresol by xylene monooxygenase (Fig. S3), and in the second step of biodegradation, the dominant intermediate p-cresol is finally decomposed into inert intermediates by catechol 2,3-dioxygenase.

Based on our preliminary time-course experiments, the best time for enzyme activity detection was the stationary phase of bacterial growth, when the cell lysis effect was minimal. Nagy et al. (2001) also reported that when growth reached the stationary phase, the activity of intracellular enzymes approached the maximum, and the cell lysis effect could be negligible at this time (Nagy et al. 2001). Xylene monooxygenase and catechol 2,3-dioxygenase activities are shown in Fig. 4. The results showed that xylene monooxygenase was produced in all isolates, which may support the results of the expression analysis of *xylA* and *xylM* as well

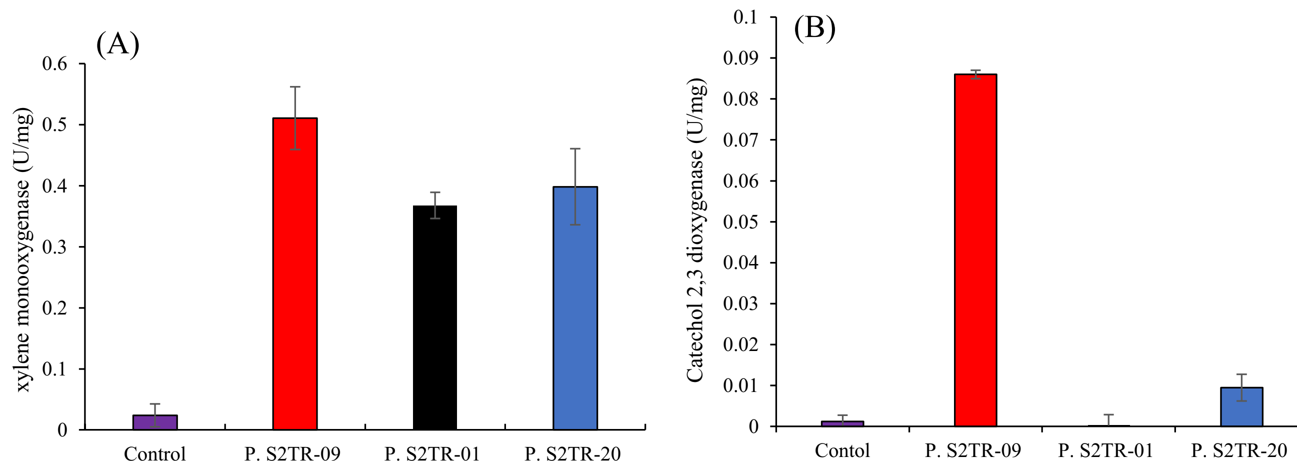

Fig. 4 Xylene monooxygenase and catechol 2,3-dioxygenase activity in different isolated bacteria (*P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20) grown on p-xylene. Control, the sample from the bacteria grow on media without p-xylene (the presented data results from S2TR-09 due to similar data from other isolates). Error bars indicate standard deviation (SD); $n = 3$

as intermediate production in the following section. Figure 4 shows that the activity of xylene monooxygenase and catechol 2,3-dioxygenase was significantly increased in *P. azotoformans* S2TR-09 (0.5 and 0.08 U/mg, respectively) in the presence of p-xylene ($p < 0.05$). Catechol 2,3-dioxygenase showed low or no activity in *P. putida* S2TR-01 and *P. synxantha* S2TR-20, which was consistent with the observed p-xylene degradation rate. This result indicated that the ring cleavage enzyme is necessary for the biodegradation of p-xylene in the isolates. Arenghi et al. (1999) showed that in *Pseudomonas stutzeri* OX1, toluene and o-xylene could be transformed by toluene-*o*-xylene monooxygenase (ToMO), but the ToMO-encoding operon did not contain genes related to ring-cleavage enzymes. Therefore, these genes may be clustered in one or more operons that are independently but coordinately regulated (Arenghi et al. 1999). Our results also showed that all isolates produced xylene monooxygenase in the presence of p-xylene, but catechol 2,3-dioxygenase was only produced in the S2TR-09 isolate, which was consistent with the gene expression and biodegradation results.

Carbon mass balance

The carbon mass balances in each isolate were determined by measuring the amounts of consumed C (p-xylene) that were recovered as C–CO_2 and biomass. Initially, the carbon balances were determined based only on the amounts of C–CO_2 and C-biomass after 60 h of incubation (the late stage of p-xylene biodegradation). The carbon recovery rates were 91%, 97%, and 85% in *P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20, respectively (Table 3). To investigate the reason for this incomplete carbon recovery, the intermediates were detected at 60 h (based on the p-xylene biodegradation results), and their concentrations were then measured. The GC/MS results showed that p-xylene was converted first to p-toluic acid and then to p-cresol, which accumulated in the media of *P. putida* S2TR-01 and *P. synxantha* S2TR-20, while no intermediates were detected in the presence of *P. azotoformans* S2TR-09 (Fig. S3). The examination of p-cresol biodegradation also confirmed that *P. azotoformans* S2TR-09 and *P. synxantha*

Table 3 Carbon balance in three isolated bacteria after 60 h of incubation at 15 °C

Isolate	Initial concentration of p-xylene (μmol)	Concentration of p-xylene (μmol)	C-Biomass (μmol)	C-CO_2 (μmol)	Concentration intermediates (μmol)		C-recovery (%)	
					p-Toluic acid	p-Cresol	Substrate, biomass, CO_2	Substrate, biomass, intermediates, CO_2
P. putida S2TR-01	2,080 ± 65	1,870 ± 50	6 ± 0.4	10 ± 2	25 ± 0.8	50 ± 2	91	94
P. azotoformans S2TR-09	2,120 ± 87	< 0.1	689 ± 21	1,388 ± 76	ND	15 ± 0.5	97	99
P. synxantha S2TR-20	2,090 ± 63	1467 ± 31	85 ± 12	227 ± 18	31 ± 0.8	198 ± 5	85	96

S2TR-20 could degrade 200 mg/l p-cresol within 10 h and 45 h, respectively, while *P. putida* S2TR-01 showed no p-cresol degradation after 60 h (Fig. S4). The formation of toluic acid and p-cresol in the presence of *P. putida* S2TR-01 indicated a small decrease in p-xylene concentration (Fig. 1). This was attributed to p-xylene transformation and NOT to degradation and biomass production. As shown in Table 3, when the concentrations of p-cresol, as the central intermediate, and other intermediates (p-toluic acid) were taken into account, the carbon recovery rates increased from 91 to 94% for *P. putida* S2TR-01, 97 to 99% for *P. azotoformans* S2TR-09, and 85 to 96% for *P. synxantha* S2TR-20. The results were consistent with the growth ability and production of ring cleavage enzymes of strains in the presence of p-xylene. The significantly high percentages of p-xylene conversion to biomass (~33%) and CO_2 (~66%) indicated the predominant fate of p-xylene in the presence of S2TR-09 was incorporation into cell materials and mineralization. The carbon recovery rates in isolates with a low p-xylene degradation rate (S2TR-01 and 20) were lower (94 and 96%) than those in *P. azotoformans* S2TR-09 (99%). Similarly, Prenafeta-Boldú et al. (2002) reported a higher carbon recovery rate together with a lack of accumulation of intermediates, pointing to mineralization for BTE biodegradation in *Cladophialophora* sp. strain T1, while o- and m-xylene isomers were partially oxidized to dead-end products and showed lower carbon recovery values. These authors showed that toluene and ethylbenzene were both used for growth; approximately 67% of the carbon source was converted to CO_2, and 21% was recovered as biomass (Prenafeta-Boldú et al. 2002). Moreover, Zhang et al. (2013) reported that *Mycobacterium cosmeticum* byf-4 converted o-xylene to CO_2 (65%) and biomass (27%), which resulted in 92% carbon recovery, while the removal efficiency was 99.99% (Zhang et al. 2013). These results highlight the importance of the carbon mass balance assay in the evaluation of microorganisms for the biodegradation of contaminants.

The metabolism of a substrate involves multiple enzymatic steps. As mentioned previously, the aerobic biodegradation of aromatic hydrocarbons has been divided into the *upper* pathway, which starts with the original compounds (hydrocarbon compounds without any modification) and produces central intermediates (modified hydrocarbons), and the lower pathway, beginning with the ring cleavage of intermediates and producing the molecules needed for biomass production (Moreno et al. 2010).

Tsai et al. reported that catechols are formed during the aerobic biodegradation of a variety of aromatic compounds (Tsai and Li 2007b). Arenghi et al. (2001a) also reported that most of monoaromatic catabolic pathways give rise to (methyl) catechols, which are further processed through *meta*-cleavage pathways. The reaction products can be easily converted to tricarboxylic acid cycle (TCA cycle) intermediates, which are further broken down into CO_2 and water, providing energy for biomass production (Yu et al. 2001). However, the results of this study showed that p-cresol was the central intermediate and correlated well with the observations regarding gene expression (*xylE* and *xylS* in the presence of p-xylene). The results suggest that the ability to grow on p-xylene is correlated with the expression of ring cleavage enzymes and that a lack of ring cleavage enzymes or low concentrations of these enzymes may lead to the accumulation of toxic intermediates.

Conclusion

p-Xylene, which belongs to the high-risk hazardous and noxious substances, is a persistent pollutant in terms of its recalcitrance to biodegradation. The preferential utilization of a substrate depends on genetic regulation in bacteria rather than a simple adaptation. In this study, three psychrophilic bacterial strains, *P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20, were evaluated for their p-xylene degradation abilities. *P. azotoformans* S2TR-09 catabolized p-xylene after 60 h, while the two other strains showed lower p-xylene degradation at 15 °C. The biomass results also followed the order S2TR-09 > S2TR-20 > S2TR-01. The gene expression study showed no significant differences in the expression of the *xylM* and *xylA* genes among all isolates, while *xylE* and *xylS* genes were induced only in isolates *P. azotoformans* S2TR-09 and *P. synxantha* S2TR-20, which could grow in the presence of p-xylene. Therefore, the expression of *xylE* (encoding a ring cleavage enzyme) and its activator (*xylS*) was correlated with the p-xylene removal rate. Enzyme activity tests also confirmed that all isolates produced xylene monooxygenase in the presence of p-xylene, while a ring-cleavage enzyme (catechol 2,3-dioxygenase) was produced only in the isolate that could grow on p-xylene as the sole source of carbon. The expression of *xylE* and *xylS* or the production of catechol 2,3-dioxygenase can be considered a biomarker of complete, efficient biodegradation of p-xylene. The analysis of intermediates confirmed the formation of p-toluic acid and p-cresol as dead-end products in isolates with lower p-xylene degradation rates (S2TR-01 and S2TR-20), which was consistent with the lack of ring cleavage enzymes. *P. azotoformans* S2TR-09 may be useful for the bioremediation of p-xylene at contaminated sites, and the expression of *xylE* and *xylS* may be considered a biomarker of the efficient biodegradation of p-xylene in isolated bacteria. Monitoring the expression of these genes can also provide persons responsible for site management with immediate feedback on the complete degradation of p-xylene after bioaugmentation. However, further studies and field demonstrations are required to confirm the applicability of the

bioaugmentation method using *P. azotoformans* S2TR-09 at cold-climate sites.

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- Analysis and interpretation of data: S. M., A. R.
- Drafting of manuscript: S. M., A. R.
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Data availability Data are available upon reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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