Biodegradation of p-xylene – A comparison for three Cold-active *Pseudomonas* strains

Saba Miri

York University

Azadeh Rasooli

York University

Satinder Kaur Brar (✉ satinder.brar@ete.inrs.ca)

INRS

Tarek Rouissi

INRS: Institut national de la recherche scientifique

Richard Martel

INRS: Institut national de la recherche scientifique

Research Article

Keywords: Gene expression, p-xylene, Catechol 2, 3-dioxygenase, xylE,

Posted Date: June 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-167048/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

p-xylene is considered a recalcitrant compound despite the similar aromatic structure with BTE (Benzene, toluene, ethylbenzene). This study evaluated the biodegradation potential of p-xylene by three cold-active *Pseudomonas* strains (named *Pseudomonas putida* S2TR-01, *Pseudomonas* S2TR-20, and *Pseudomonas* S2TR-09). The catabolic genes (*xyIM*, *xyIA* and *xyIE*) and their regulatory genes (*xyIR* and *xyIS*) were investigated for the p-xylene metabolism. The biodegradation results showed that only strain S2TR-09 was able to degrade 200 mg/L of p-xylene after 60 h at 15 °C. The gene expression study indicated that *xyIE* (encoding catechol 2, 3-dioxygenase) represents the bottleneck for p-xylene biodegradation and lack of its expression leads to the accumulation of intermediates and inhibits biomass production as well as carbon recovery. The activity of xylene monooxygenase and catechol 2,3 dioxygenase was significantly high in *P. azotoformans* S2TR-09 (0.5 and 0.08 U/mg) in the presence of p-xylene. The expression of ring cleavage enzyme, its encoding genes (*xyIE*), and its activator (*xyIS*) enabled to link the differences in p-xylene metabolism and can be used as a novel biomarker for efficient p-xylene biodegradation in contaminated sites.

1. Introduction

The International Tanker Owners Pollution Federation (ITOPF) has identified p-xylene as one of the top 20 chemicals at high risks of hazardous and noxious substance (HNS) (Duan et al., 2020). p-xylene is highly mobile in the environment, through either gaseous, liquid, or solid phases due to its wide use as an industrial solvent (Boonsaner et al., 2011; Mazzeo et al., 2013). Contaminated sites in cold climate regions have received significant attention due to their susceptible natural environment. In the last decades, bioremediation techniques have been considered to be more effective with lesser undue damages to cold climate environments in comparison to other remediation techniques such as physical, chemical, and thermal approaches (Habib et al., 2018). However, biodegradation of p-xylene is not easy and classified as a persistent pollutant, in comparison with other BTE compounds. Biodegradation of BTEX compounds is generally initiated by mono or dioxygenase enzymes and they might face difficulty 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 involved catabolic enzymes and the transcriptional regulation of that pathway (de Lorenzo and Pérez-Martín, 1996), which directs the appropriate enzymatic cascades in response to the specific pollutant as a substrate (Díaz and Prieto, 2000). To make the bioremediation successful, proper microorganisms with high potential in enzyme production should be introduced to the contaminated site (Miri et al., 2019).

Several studies showed low or no degradation of p-xylene in *Pseudomonas* strains, such *Pseudomonas* B1 and X1 (Chang et al., 1993), *Pseudomonas stutzeri* OX1 (Arengi et al., 2001), *Pseudomonas* sp. BTEX-30 (Khodaei et al., 2017), *Pseudomonas putida* AQ8 (Chicca et al., 2020), even at favorable temperature (20–30 °C). While 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., 2013). To the best of the authors' knowledge, this study is the first report on the reason for the differences in p-xylene metabolism in cold-active *Pseudomonas* strains.

This study combined biodegradation experiments, gene expression analysis, and enzyme activity tests to identify the underlying mechanism for complete and effective biodegradation of p-xylene in cold-active strains. The potential of three BTEX-degrading *Pseudomonas* strains for p-xylene degradation was confirmed by carbon mass balances through the determination of produced intermediates.

2. Materials And Methods

2.1. Chemicals and culture medium

All the chemicals and culture media were 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). Mineral salts media (MSM) as carbon-free medium (1.8 g/L K_2HPO_4 , 4.0 g/L NH_4Cl , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$) were used for biodegradation tests (Rahul and Chandrajit, 2011).

2.2. Isolation of bacterial strains from soil samples

The bacterial strain used in the present study were isolated from a confidential petroleum site in Montreal, Canada with a typical aquifer temperature of 8 to 15 °C. The samples were collected from the soil at depths ranging from 5 to 7 m with a p-xylene concentration of 10,000 mg/kg. The samples were enriched by 200 ppm of BTEX mixture (1:1:1:1) and incubated at 15 ± 1 °C, 150 rpm for one month. Then, the mixture (soil and MSM) was transferred to a fresh MSM supplemented BTEX every week. After a 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 to 15 °C, which is the typical aquifer temperature in the contaminated site. Single colonies with different morphology were selected transferred to TSB to use for inoculation after incubation at 15 ± 1 °C for 3 days. The strains were characterized by amplification and sequencing of 16 s rRNA fragment (27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3'). The sequence was compared with available sequences in the National Center for Biotechnology Information (NCBI) database. The phylogenetic analysis was performed using the neighbor-joining algorithm of MEGA 7.0 software with 1000 bootstraps (Tamura et al., 2011).

2.3. Biodegradation tests

Experiments for growth ability and biodegradation of p-xylene were carried out in 150 ml sealed serum bottles with a concentration of 200 mg/L (high concentration). p-xylene is partially insoluble in water and its solubility is 198 mg/l at 25 °C. The value of 200 mg/l p-xylene was considered for a sufficient bioavailability of this compound to favorable biodegradation efficiency by xylene-utilizing microorganisms. Therefore, a high concentration of p-xylene was dissolved in methanol and added to the sterile flask at a selected final concentration (Li and Zhu, 2012; Li et al., 2015). The media was placed in 150 mL with a 9:1 airspace/liquid ratio sealed with a Teflon coated rubber stopper. The inocula were added after 24 h of equilibrium of p-xylene concentration between the liquid and headspace in the experimental conditions. The concentration of p-xylene was detected with GC/MS in the aquatic phase. As p-xylene is considered a highly volatile mixture; only about 50 to 60% of the concentration added can be detected in the liquid phase. The concentration in the liquid and gas phase was measured and the needed concentration was compensated to correct the results. The growth experiments indicated that *P. putida* S2TR-01 could not grow on p-xylene as a sole source of energy and carbon. To assess the effect of p-xylene, the cells were grown on TSB medium and harvested using centrifugation at $12,000 \times g$, 4 °C for 5 min, then inoculated to the MSM supplemented with 200 mg/l of p-xylene. At selected time intervals, the cells were harvested for further analyses.

The inoculation (1% v/v, OD600 = 2) was carried out using a gas-tight syringe. Inoculated cultures (OD600 = 2, correspond 6×10^7 cells/ml) were incubated at 15 °C in a rotary shaking incubator (150 rpm). A linear relationship was observed between OD600 and cell dry weight of isolates (cell dry weight (mg/L) = $308 \times OD600$, $R^2 = 0.95$).

Two sets of the control group were designed for tests; 1) Abiotic controls with autoclaved bacterial inoculation were run in parallel to monitor for abiotic processes such as volatilization and bacterial sorption for biodegradation experiments; 2) control group with isolates grown in the presence of glucose (2.5 g/L) as the sole source of carbon (del Castillo and Ramos, 2007) for intermediate, gene expression and enzyme analysis.

2.4. p-xylene analysis

For each batch study, sub-samples (100 μ L) were removed from the liquid phase of bottles using a gas-tight syringe for the OD600 and substrate concentration measurements every 6 h. The sub-samples (50 μ L) were prepared in a 40 mL standard headspace bottle containing 5 ml of ultrapure water, 10 μ L of fluorobenzene-D5 (100 mg/l diluted in methanol) as internal standard, and then sealed immediately. The concentrations of p-xylene were analyzed based on EPA 8021B and extracted using a Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler. The gas-phase of samples was quantitatively

analyzed using 7890A Gas Chromatograph (DB-1701 column 30 m×0.25 mm i.d. × 0.25 µm film thickness) at 45 °C and 300 °C for column and detector, respectively.

2.5. Gene expression analysis

Based on biodegradation and biomass kinetics, 10, 20, and 50 h were selected for gene expression study as 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).

Grown cultures were harvested by centrifugation to obtain ~ 10¹⁰ cells and then the pellets were used for RNA extraction. Total RNA was extracted from all experiments by the RNA total Kit (BioBasic Inc, Canada) and was quantified at 260 nm and 280 nm with NanoDrop 2000 spectrophotometer (Thermo Scientific). Then, the same amount of total extracted RNA (100 ng) was used to synthesize cDNA with Prime Script™ RT reagent kit and random hexamer primers (Takara Bio Inc, Japan). The quality of cDNA synthesis was assessed by agarose gel electrophoresis with DNA safe stain after PCR amplification. For each PCR reaction, a negative control (without DNA template) was performed to check possible DNA contamination (Fig. S1). Thermal cycling of PCR was 94 °C for 5 min for initial denaturation, followed by 30 cycles of 94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min; then one more 72 °C for 5 min to allow trimming of incomplete polymerizations.

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 *P. putida* (Table 1). The potential formation of primer-dimers and other secondary structures were checked in Gene Runner software Version 6.5.52 Beta.

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	AATGTCTCGGTTTCGGATCAC AGTGGTGTGCCAACTCTTCC	60	114
xylA	Xylene monooxygenase	F R	GCAGCGCTTCTATTTTCGTC 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 ATCGAGTCGGAAGTGTGTTGG	60	179
16S rRNA	Ribosomal RNA	F R	CGGAATTACTGGGCGTAAAG TCTACGCATTTACCGCTAC	59	149

Relative-expression of selected genes was carried out with a real-time PCR System (Rotor-Gene Q-QIAGEN) by SYBR Green real-time PCR Master Mix kit (QIAGEN, Germany). The reaction compositions are Taq polymerase, dNTP, MgCl₂, SYBER green I dye, 0.2 µl primers, 0.5 µl cDNA, and 10 µl H₂O. Thermocycling conditions of the real-time PCR reaction 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 RNA as housekeeping gene expression. Our preliminary results showed that the 16S expressed constitutively in p-xylene biodegradation by *isolates* (CT = 14.0 ± 0.7) so it can be used as a reference

or housekeeping gene. Mean normalized gene expression $\pm$ standard deviation (SD) was calculated from the triplicate analysis. The fold change of target genes was calculated by the $RQ = 2^{-\Delta\Delta Ct}$ as follows using Equations 1 to 3:

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

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

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

Our target genes corresponded to *xyIM*, *xyIA*, *xyIR*, *xyIE*, *xyIS*. One advantage of relative quantification method is that it does not require the use of standards curves, which makes its use faster and easier (Pfaffl, 2001). When using this method, it has to be considered that the expression level of different genes cannot be compared since absolute concentrations of each mRNA are not determined.

2.6. Enzymes and Protein Assays

Bacterial cells were harvested after 30 h of incubation because the maximum enzyme activity can be assayed when the bacteria is at the early exponential growth phase (Le Meur et al., 2012).

The cells were washed twice by centrifugation at $5000 \times g$ with 20 mM phosphate buffer (pH around 7.0) and re-suspended in the same buffer. The cells were disrupted using two frequencies of ultrasounds (22 kHz and 30 kHz) for 6 min. The cellular lysates were centrifuged at $13,000 \times g$ for 20 min, and the supernatant was used for enzyme assays. Two key enzymes of *xyI* operon were selected (Xylene monooxygenase, Catechol 2, 3 dioxygenases) and their activities were measured. Based on enzyme activity tests in cold-active isolates, 15 °C was determined as an optimal temperature for enzymes obtained from isolates, so all the enzyme assayed was carried out at their optimal temperature. To determine the specific activity (units per mg of proteins), total protein concentration was determined by the Bradford method (Bradford, 1976). Briefly, an acidic solution of Coomassie dye was added to the protein solution, then the absorbance was measured at 595 nm. The concentration of total protein in the samples was determined using a standard curve with a known concentration of bovine serum albumin (BSA).

2.6.1. Xylene Monooxygenase Activity

Xylene monooxygenase activity was determined by monitoring the increase in the concentration of phenolic compounds in the medium using a colorimetric assay slightly modified from Arengi (Arengi et al., 1999). Briefly, NADPH (final concentration, 0.5 mM) and 35 μ l of 4% (vol/vol) *p*-xylene in N,N-dimethylformamide were added to the cellular lysates. At 3-min intervals after incubation at 15 °C, 1-ml samples were collected and mixed with 100 μ l of 1 M NH_4OH and 100 μ l of 2% 4-amino antipyrine. After the addition of 100 μ l of 8% $\text{K}_3\text{Fe}(\text{CN})_6$, the samples were briefly centrifuged ($14,000 \times g$), then the A_{500} of the supernatant was measured. Phenolic compound concentrations were calculated by reference to a standard curve for *p*-cresol (in the range of 0–50 $\mu\text{g/mL}$). Specific activities were reported as micromoles of compounds produced per minute per milligram of cell protein.

2.6.2. Catechol dioxygenases Assay

The reaction mixture (3.0 mL) contained 2.0 mL phosphate buffer, 0.6 mL 1 mM catechol, 0.2 mL deionized water and 0.2 mL cellular lysates. The reaction was allowed to proceed at 15 °C and the sub-samples were taken every 2 minutes. 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 (Li et al., 2014).

2.7. Carbon mass balance

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

Intermediates formed during p-xylene biodegradation were analyzed using two types of gas chromatography/mass spectrometers; 1) a GC-MS equipped with a wax capillary column (for detection of non-volatile intermediates) and; 2) a GC-MS equipped with headspace sampler (for detection of possible volatile intermediates, as mentioned in p-xylene concentration analysis).

The GC-MS is 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 from 40°C (held 1 min) to 200°C at 5°C/min (held 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 with a GC/MS by matching the retention times and ion spectra with authentic standards and NIST library data. Then the concentration was determined using the calibration curves of their standards.

The carbon recovered as C-CO₂ and the carbon recovered as biomass was determined after substrate exhaustion and the values were corrected using control flasks incubated with no carbon source. Carbon dioxide was measured chromatographically using GC-MS (7820A GC system Agilent Technologies, Netherlands) (Srivastva et al., 2016). The biomass was collected using 0.45-µm-pore-diameter filtration, placed in the oven, and the dry content was determined. The carbon recovered as biomass was calculated by assuming that 26 g of dry weight contains approximately 1 mol of carbon (Tijhuis et al., 1993). Carbon mass balance tests were conducted in triplicate for isolates and controls.

2.8. Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Tukey posthoc procedure. A probability of less than 0.05 was statistically significant.

3. Results And Discussion

3.1. Identification of cold-active isolates

Three cold-adapted BTEX-degrading bacteria were selected and 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 with *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 as shown in Fig. 1. Phylogenetic analysis also showed that S2TR-01, S2TR-09, and ST2TR-20 formed a phylogenetic lineage with members of the mentioned strains. BTEX degradation abilities of the three isolated were analyzed at 5–30 °C and all of them showed obvious BTEX degradation at cold temperatures (5–15 °C). The optimum temperature for growth and biodegradation was 15 °C. No growth occurred at 30 °C in presence of BTEX. These strains were interesting because they showed a high rate of BTEX degrading at low temperature (15 °C) while they exhibited different degradation and growth ability in presence of p-xylene as a sole source of carbon (Table. S1). Many mesophilic *Pseudomonas* strains have been identified as BTEX degraders, such *Pseudomonas* B1 and X1 (Chang et al., 1993), *Pseudomonas stutzeri* OX1 (Arengi et al., 2001), *Pseudomonas* sp. BTEX-30 (Khodaei et al., 2017), *Pseudomonas putida* AQ8 (Chicca et al., 2020), they also showed a low degradation rate of xylenes even at favorable temperature (20–30 °C). A study on the effect of the chemical structure of BTEX on four chemolithotrophic bacteria showed that the increase in the number of alkyl groups in a benzene ring and their size can increase the toxicity of the compound (Odokuma and Oliwe, 2003). Similarly, the results of the present study showed that p-xylene and ethylbenzene with an addition of the methyl and ethyl groups are more toxic than benzene and toluene (Table S1).

3.2. Biodegradation of p-xylene

The p-xylene biodegradation experiments were carried out at the initial concentration of p-xylene equal to ~ 200 mg/L at 15 °C. The biomass and p-xylene concentration versus time are shown in Fig. 1. Results indicate that *P. azotoformans* S2TR-09 degraded p-xylene faster than other isolates (p < 0.05) after 60 h. Biodegradation of p-xylene was completed after 5 days by *P. synxantha* S2TR-20 however, *P. putida* S2TR-01 did not degrade p-xylene even after 10 days of incubation (data not

shown). Biomass concentration confirmed that *P. putida* S2TR-01 could not use p-xylene as a sole source of carbon and energy. As shown in Fig. 2, biomass concentration of *P. azotoformans* S2TR-09 increased with time in the presence of p-xylene and followed the order: S2TR-09 > S2TR-20 > S2TR-01 but there were no changes (0.10 mg/L) during p-xylene biodegradation by *P. putida* S2TR-01. 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 54% of p-xylene degradation by *P. putida* YNS1 after 96 h incubation at 30 °C (You et al., 2013). Jeong et al. (2006) reported 90% of removal efficiency of p-xylene (5.1 mg) using *Pseudomonas* sp. NBM21 in biofilter at 30 °C after 4 h (Jeong et al., 2006). Worsey et al. (1975) showed *P. putida* mt-2 has the ability to degrade p-xylene as a carbon source for growth (Worsey and Williams, 1975). This indicates that each strain has a different degradation ability. The result of this study also showed that these cold-adapted isolates have different biodegradative abilities.

Several researchers have reported that toluene was degraded more readily and the slowest degradation rate was observed for biodegradation of p-xylene (sometimes no-biodegradation was reported) when the microbial strains were grown in basal mineral media and p-xylene alone at ambient temperature (Alvarez and Vogel, 1991; Zhou et al., 2016; Meyer-Cifuentes et al., 2017). You et al. (2018) reported the low biodegradation of p-xylene by *Rhodococcus* sp. ZJUT312 compared to other compounds of BTE (You et al., 2018). Wang et al. (2015) reported that *Pandoraea* sp. strain WL1 could degrade 16.6 ~ 99.4 mg L⁻¹ of p-xylene as a 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 for biological treatments and it may require more time (ranging from 1 to 10 years) to meet cleanup standards (Miri et al., 2019; Davoodi et al., 2020). The result of this study showed that more time is needed for complete degradation of p-xylene, however, the concentration of contaminants should be considered as an important factor because of their toxicity and inhibitory effects.

3.3. Effect of p-xylene on catabolic gene expression

Generally, biodegradation of BTEX occurs via two pathways (*tod*, and *tol*) in *Pseudomonas* (Yu et al., 2001). In the *tod* pathway, the bacteria used xylene dioxygenase as a primary enzyme for p-xylene degradation. Based on our preliminary results, the xylene dioxygenase showed no activity in the presence of p-xylene (data not shown); hence the isolates did not use the *tod* pathway in this case. Therefore, TOL plasmid gene expression was studied in the presence of p-xylene (Fig. 3). The genetic organization of the catabolic genes in TOL plasmid reflects the biochemical operation, so the catabolic genes are organized in two operons; *upper* and *meta*-cleavage operons (Fig. 4). The upper operon comprises 5 genes (*xyICMABN*) that encode the enzymes needed for mono oxidation of aromatic hydrocarbons to their corresponding carboxylic acids. *xyIM* and *xyIA* encode two subunits of xylene monooxygenase as a first and important enzyme in the biodegradation pathway of BTEX (Bühler et al., 2002). The *meta*-cleavage pathway encodes *xyIXYZLTEGFJQKIH* the enzymes for the degradation of benzoate to Krebs cycle intermediates. The 13 genes are conserved as one of the largest operons in prokaryotes due to over 11 kb in size (Marqués and Ramos, 1993). *xyIE* encodes a key enzyme, catechol 2,3-dioxygenase, involves in the ring cleavage of aromatic hydrocarbons. *xyIR* and *xyIS* genes encode the specific transcriptional regulators that activate the expression of the two pathway genes, *xyIR* and *xyIS* located downstream of the *meta*-cleavage operon (Fig. 4; (Moreno et al., 2010). The genes encoding *xyIS* and *xyIR* are located at the 3' end of the *meta*-operon (Fig. 4).

The results showed that *xyIM* and *xyIA* were induced in all isolates in the presence of p-xylene because the biodegradation pathway is initiated by the xylene monooxygenase enzyme. The results are consistent with the gene expression profile of *xyIR* that showed up-regulation in presence of p-xylene. Xylene can induce transcription of *xyIR* and then upper operon without the need of their conversion to their carboxylic acid form (Marqués and Ramos, 1993; Moreno et al., 2010). Whereas, the genes coding the enzymes that convert benzoate or methyl benzoates into Krebs cycle intermediates are located in the *meta*-cleavage operon and are induced by *xyIS* and the converted form of p-xylene. Our results showed that the *xyIS* was induced only in the isolates (*P. azotoformans* S2TR-09 and *P. synxantha* S2TR-20) that have the ability to grow in the presence of p-xylene but in *P. putida* S2TR-01, *xyIS* and *meta*-cleavage operon accordingly did not induce (Fig. 5). The level of the *meta*-cleavage operon expression was consistent with p-xylene degradation and biomass in order of *P.* S2TR-09 > *P.* S2TR-20 > *P.* S2TR-01. Growth on p-xylene requires the expression of *meta*-cleavage operon because the Krebs cycle serves

as the main source of cellular energy for aerobic microorganisms (Seo et al., 2009). Transcription stimulation of the *meta*-cleavage operon activated by the *xylS* regulator with the interaction of benzoate and toluates as effectors (Marqués and Ramos, 1993). Figure 3. shows the gene expression profiles followed in time of *P. putida* growing on xylene and differences in the gene expression were observed depending on the time of degradation. As can be seen in Fig. 3., when the bacteria degraded the xylene, the inducer of the upper pathway and carbon source was not presented anymore and bacteria (S2TR-09 and S2TR-20) decreased the expression of *xylM*. Velazquez et.al attributed the higher relative expression of the gene in the middle of experiment to a certain delay in induction of the meta-operon genes. However, in the case of *xylE*, genes remained up-regulated during the complete experiment until the end of experiment (Velázquez et al., 2005). Moreover, xylene is able to induce also the meta-pathway due to the recognition of Ps1 by the regulator of the upper pathway, XylR,. Therefore, an up-regulation of *xylE* was observed since the beginning of the experiment and when metabolites accumulate due to the xylene degradation an even higher relative expression of the gene was observed at the middle of the experiment. (Fig. 3. E.). The slight decrease on the upper-pathway enzymes for S2TR-20 might be because of the presence of xylene more than those for S2TR-09.

Our results showed that the *xylS* was NOT induced in the isolates (S2TR-02) that does not have the ability to grow in the presence of p-xylene. After 20 hours slight up-regulation of *xylR* and *xylS* was observed later on it seems that the energetic status of cells growing on xylene allowed *P.putida* cells to initially up-regulate *xylM*. (Fig. 3. A, D).

3.4. Enzymes activity detection

In the aerobic system, oxygenase is the key enzyme of microbial degradation. The key enzymes in the metabolism of p-xylene were xylene monooxygenase and catechol 2,3 dioxygenase (Miri et al., 2020). Figure 5 shows that the activity of xylene monooxygenase and catechol 2,3 dioxygenase was significantly high in *P. azotoformans* S2TR-09 (0.5 and 0.08 U/mg respectively) in the presence of p-xylene ($P < 0.05$). Whereas these two enzymes showed low or no activity in *P. putida* S2TR-01 (0.3 U/mg for xylene monooxygenase and 0.08 U/mg for catechol 2,3 dioxygenase) that were consistent with p-xylene degradation ability. As xylenes have two methyl side-chains on their aromatic nucleus, it resulted in the inability of xylenes as a growth substrate for some BTEX-degrading microorganisms and intermediates accumulated in the culture medium so that their toxicity inhibited the growth of microorganisms. This can be attributed to a decrease in the efficiency of catechol 2,3 dioxygenases that will cleave the aromatic nucleus (Yu et al., 2001). Arengi et al. showed that *Pseudomonas stutzeri* OX1 was able to transform toluene and o-xylene by toluene-o-xylene monooxygenase (ToMO), but the ToMO-encoding operon did not contain genes involved in ring-cleavage enzymes. Therefore, these genes may be clustered in one or more operons which were independently, but coordinately regulated (Arengi et al., 1999). Our result also showed that all isolates produced xylene monooxygenase in presence of p-xylene but two of them produced low ring-cleavage enzyme (catechol 2,3 dioxygenase) that was consistent with gene expression and biodegradation results.

3.5. Carbon mass balance

Carbon mass balances in each isolate were determined by measuring the amounts of consumed C substrate (p-xylene) that recovered as C-CO₂ and biomass. Initially, the carbon balances were determined including only the amounts of C-CO₂ and C-biomass after 60 h of incubation. The carbon recovery was 91%, 97%, and 85% and in *P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20 respectively (Table 2). To evaluate the reason for this incomplete carbon recovery, the intermediates were detected and then the concentration of them was measured. The results from GC/MS showed that p-xylene firstly converted to p-toluic acid then to p-cresol (Fig. S2) that accumulated in the media in *P. putida* S2TR-01 and *P. synxantha* S2TR-20, while no intermediate was detected in the presence of *P. azotoformans* S2TR-09. As shown in Table 2, when the concentration of p-cresol as a central intermediate and other intermediate were taken into account carbon recoveries increased from 91–94% for *P. putida* S2TR-01 and 97–99% for *P. azotoformans* S2TR-09 and 85–96% for *P. synxantha* S2TR-20. The result was consistent with p-xylene degradation and biomass results that showed the growth ability of *P. azotoformans* S2TR-09 and production of ring cleavage enzyme in presence of p-xylene. The carbon recovery value in isolates with a low p-xylene degradation rate (S2TR-01 and 20) was lower (94 and 96%) than *P. azotoformans* S2TR-09 (99%). Similarly, Prenafeta-Boldú *et al.* reported a higher carbon recovery value together with the lack of accumulation of

intermediates points to mineralization for p-xylene biodegradation in *Cladophialophora* sp. strain T1. While the components that were partially oxidized to dead-end products showed lower carbon recovery value (Prenafeta-Boldú et al., 2002).

Table 2
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
<i>P. putida</i> S2TR-01	2,080 ± 65	1,870 ± 50	6 ± 0.4	10 ± 2	25 ± 0.8	50 ± 2	91	94
<i>P. azotoformans</i> S2TR-09	2,120 ± 87	< 0.1	689 ± 21	1,388 ± 76	ND	15 ± 0.5	97	99
<i>P. synxantha</i> S2TR-20	2,090 ± 63	1467 ± 31	85 ± 12	227 ± 18	31 ± 0.8	198 ± 5	85	96

The metabolism of a substrate involves multiple enzymatic steps. As mentioned previously, the aerobic biodegradation of aromatic hydrocarbon has been divided into the *upper* pathway, which started from the original compound (hydrocarbon compound without any modification) to central intermediates (modified hydrocarbons), and the lower pathway, which transitions from the ring cleavage of intermediates down to the needed molecules for biomass (Moreno et al., 2010).

Tsai et al. reported that catechols are formed during aerobic biodegradation of a variety of aromatic compounds (Tsai and Li, 2007). Arengi et al., also reported that most of the monoaromatic catabolic pathways give rise to (methyl) catechols further processed through *meta* cleavage pathways (Arengi et al., 2001). The reaction products can be easily converted to tricarboxylic acid cycle (TCA cycle) intermediates, which are further broken down into CO₂ and water which provided energy for biomass production (Yu et al., 2001). However, the result of this study showed that p-cresol was the central intermediate and correlated well with those obtained from gene expression (*xyIE* and *xyIS* in presence of p-xylene). The results suggest that the ability of growth on p-xylene is correlated with the expression of ring cleavage enzymes and lack of ring cleavage enzymes or low concentration of them may lead to the accumulation of toxic intermediates.

4. Conclusion

p-xylene as one of the high risks of hazardous and noxious substance is a persistent pollutant in terms of biodegradation. Preferential utilization of a substrate depends on the genetic regulation in bacteria rather than a simple adaptation. In this study, three bacterial strains namely *P. putida* S2TR-01, *P. azotoformans* S2TR-09, and *P. synxantha* S2TR-20 were evaluated for p-xylene degradation. Only *P. azotoformans* S2TR-09 catabolized p-xylene after 60 h at 15 °C while two other strains did not degrade p-xylene even after 5 days of incubation. The results from gene expression and enzyme assays showed that the expression of *xyIE* (encoded ring cleavage enzyme) and its activator (*xyIS*) determined the metabolic rate of p-xylene. The expression of *xyIE* and *xyIS* can be considered as a biomarker of efficient biodegradation of p-xylene. The GC-MS analysis confirmed the formation of p-toluic acid and p-cresol in isolates with lower p-xylene degradation rates (S2TR-01 and S2TR-20) that was consistent with the lack of ring cleavage enzymes. The strain *P. azotoformans* S2TR-09 could be useful for

bioremediation of p-xylene in the contaminated site and the expression of *xy/E* and *xy/S* could be considered as a biomarker of efficient biodegradation of p-xylene in isolated bacteria. Monitoring the expression of these genes can also provide site management with immediate feedback on the system after biodegradation.

Declarations

Acknowledgments and Funding

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254, CRD Grant and Strategic Grant 447075) and Techno-Rem Inc. for financial support. The support from James and Joanne Love Chair in Environmental Engineering is equally appreciated.

Data Availability

Data are available upon reasonable request.

Authors' contributions

-Study conception and design and acquisition of data: SM

- Analysis and interpretation of data: SM, AR

-Drafting of manuscript: SM, AR, SKB,TR, RM

-Critical revision: SKB, TR, RM

All authors approved the final version to be submitted.

Compliance with ethical standards

Competing interests: The authors declare no conflicts of interest.

Ethical approval: Not applicable

Consent to participate: Not applicable

Consent to publish: Not applicable

References

Alvarez, P., and Vogel, T.M. (1991) Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Applied and Environmental Microbiology* **57**: 2981-2985.

Arengi, F.L., Pinti, M., Galli, E., and Barbieri, P. (1999) Identification of the *Pseudomonas stutzeri* OX1 toluene-o-xylene monooxygenase regulatory gene (*touR*) and of its cognate promoter. *Applied and environmental microbiology* **65**: 4057-4063.

Arengi, F.L., Berlanda, D., Galli, E., Sello, G., and Barbieri, P. (2001) Organization and regulation of metaCleavage pathway genes for toluene and o-xylene derivative degradation in *Pseudomonas stutzeri* OX1. *Applied and environmental microbiology* **67**: 3304-3308.

Boonsaner, M., Borriukwisitsak, S., and Boonsaner, A. (2011) Phytoremediation of BTEX contaminated soil by *Cannax generalis*. *Ecotoxicology and environmental safety* **74**: 1700-1707.

- Brasford, M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.
- Bühler, B., Witholt, B., Hauer, B., and Schmid, A. (2002) Characterization and application of xylene monooxygenase for multistep biocatalysis. *Applied and environmental microbiology* **68**: 560-568.
- Chang, M.K., Voice, T.C., and Criddle, C.S. (1993) Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and p-xylene by two *Pseudomonas* isolates. *Biotechnology and Bioengineering* **41**: 1057-1065.
- Chicca, I., Becarelli, S., Dartiahl, C., La China, S., De Kievit, T., Petroni, G. et al. (2020) Degradation of BTEX mixture by a new *Pseudomonas putida* strain: role of the quorum sensing in the modulation of the upper BTEX oxidative pathway. *Environmental Science and Pollution Research* **27**: 36203-36214.
- Choi, E.J., Jin, H.M., Lee, S.H., Math, R.K., Madsen, E.L., and Jeon, C.O. (2013) Comparative genomic analysis and benzene, toluene, ethylbenzene, and o-, m-, and p-xylene (BTEX) degradation pathways of *Pseudoxanthomonas spadix* BD-a59. *Applied and environmental microbiology* **79**: 663-671.
- Davoodi, S.M., Miri, S., Taheran, M., Brar, S.K., Galvez-Cloutier, R., and Martel, R. (2020) Bioremediation of Unconventional Oil Contaminated Ecosystems under Natural and Assisted Conditions: A Review. *Environmental Science & Technology* **54**: 2054-2067.
- de Lorenzo, V., and Pérez-Martín, J. (1996) Regulatory noise in prokaryotic promoters: how bacteria learn to respond to novel environmental signals. *Molecular microbiology* **19**: 1177-1184.
- del Castillo, T., and Ramos, J.L. (2007) Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. *Journal of bacteriology* **189**: 6602-6610.
- Díaz, E., and Prieto, M.a.A. (2000) Bacterial promoters triggering biodegradation of aromatic pollutants. *Current Opinion in Biotechnology* **11**: 467-475.
- Duan, W., Du, S., Meng, F., Peng, X., Peng, L., Lin, Y. et al. (2020) The pathways by which the marine diatom *Thalassiosira* sp. OUC2 biodegrades p-xylene, combined with a mechanistic analysis at the proteomic level. *Ecotoxicology and Environmental Safety* **198**: 110687.
- Habib, S., Ahmad, S.A., Wan Johari, W.L., Shukor, M.Y.A., and Yasid, N.A. (2018) Bioremediation of Petroleum Hydrocarbon in Antarctica by Microbial Species: An Overview. *Pertanika Journal of Science & Technology* **26**.
- Jeong, E., Hirai, M., and Shoda, M. (2006) Removal of p-Xylene with *Pseudomonas* sp. NBM21 in biofilter. *Journal of Bioscience and Bioengineering* **102**: 281-287.
- Khodaei, K., Nassery, H.R., Asadi, M.M., Mohammadzadeh, H., and Mahmoodlu, M.G. (2017) BTEX biodegradation in contaminated groundwater using a novel strain (*Pseudomonas* sp. BTEX-30). *International Biodeterioration & Biodegradation* **116**: 234-242.
- Le Meur, S., Zinn, M., Egli, T., Thöny-Meyer, L., and Ren, Q. (2012) Production of medium-chain-length polyhydroxyalkanoates by sequential feeding of xylose and octanoic acid in engineered *Pseudomonas putida* KT2440. *BMC biotechnology* **12**: 53.
- Li, F., and Zhu, L. (2012) Effect of surfactant-induced cell surface modifications on electron transport system and catechol 1, 2-dioxygenase activities and phenanthrene biodegradation by *Citrobacter* sp. SA01. *Bioresource technology* **123**: 42-48.
- Li, F., Zhu, L., Wang, L., and Zhan, Y. (2015) Gene expression of an arthrobacter in surfactant-enhanced biodegradation of a hydrophobic organic compound. *Environmental science & technology* **49**: 3698-3704.

- Li, Y., Li, B., Wang, C.-P., Fan, J.-Z., and Sun, H.-W. (2014) Aerobic degradation of trichloroethylene by co-metabolism using phenol and gasoline as growth substrates. *International journal of molecular sciences* **15**: 9134-9148.
- Marqués, S., and Ramos, J.L. (1993) Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Molecular microbiology* **9**: 923-929.
- Mazzeo, D.E.C., Matsumoto, S.T., Levy, C.E., de Angelis, D.d.F., and Marin-Morales, M.A. (2013) Application of micronucleus test and comet assay to evaluate BTEX biodegradation. *Chemosphere* **90**: 1030-1036.
- Meyer-Cifuentes, I., Martinez-Lavanchy, P.M., Marin-Cevada, V., Böhnke, S., Harms, H., Mueller, J.A., and Heipieper, H.J. (2017) Isolation and characterization of *Magnetospirillum* sp. strain 15-1 as a representative anaerobic toluene-degrader from a constructed wetland model. *PloS one* **12**: e0174750.
- Miri, S., Naghdi, M., Rouissi, T., Kaur Brar, S., and Martel, R. (2019) Recent biotechnological advances in petroleum hydrocarbons degradation under cold climate conditions: A review. *Critical Reviews in Environmental Science and Technology* **49**: 553-586.
- Miri, S., Davoodi, S.M., Brar, S.K., Rouissi, T., Sheng, Y., and Martel, R. (2020) Psychrozymes as novel tools to biodegrade p-xylene and potential use for contaminated groundwater in the cold climate. *Bioresource Technology*. 124464.
- Moreno, R., Fonseca, P., and Rojo, F. (2010) The Crc global regulator inhibits the *Pseudomonas putida* pWW0 toluene/xylene assimilation pathway by repressing the translation of regulatory and structural genes. *Journal of Biological Chemistry* **285**: 24412-24419.
- Odokuma, L., and Oliwe, S. (2003) Toxicity of substituted benzene derivatives to four chemolithotrophic bacteria isolated from the New Calabar River. *Global Journal of Environmental Sciences* **2**: 72-77.
- Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.
- Prenafeta-Boldú, F., Vervoort, J., Grotenhuis, J., and Van Groenestijn, J. (2002) Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora* sp. strain T1. *Applied and Environmental Microbiology* **68**: 2660-2665.
- Qu, D., Wang, C., Wang, Y., Zhou, R., and Ren, H. (2015) Heterotrophic nitrification and aerobic denitrification by a novel groundwater origin cold-adapted bacterium at low temperatures. *Rsc Advances* **5**: 5149-5157.
- Rahul, M.A.K., and Chandrajit, B. (2011) Biodegradation of waste gas containing mixture of BTEX by *B. Sphaericus*. *Research Journal of Chemical Sciences* _____ ISSN **2231**: 606X.
- Seo, J.-S., Keum, Y.-S., and Li, Q.X. (2009) Bacterial degradation of aromatic compounds. *International journal of environmental research and public health* **6**: 278-309.
- Srivastva, N., Singh, R.S., Upadhyay, S.N., and Dubey, S.K. (2016) Degradation kinetics and metabolites in continuous biodegradation of isoprene. *Bioresource technology* **206**: 275-278.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**: 2731-2739.
- Tijhuis, L., Van Loosdrecht, M.C., and Heijnen, J.v. (1993) A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnology and bioengineering* **42**: 509-519.

- Tsai, S.-C., and Li, Y.-K. (2007) Purification and characterization of a catechol 1, 2-dioxygenase from a phenol degrading *Candida albicans* TL3. *Archives of microbiology* **187**: 199-206.
- Velázquez, F., Parro, V., and de Lorenzo, V. (2005) Inferring the genetic network of m-xylene metabolism through expression profiling of the xyl genes of *Pseudomonas putida* mt-2. *Molecular microbiology* **57**: 1557-1569.
- Wang, X., Wang, Q., Li, S., and Li, W. (2015) Degradation pathway and kinetic analysis for p-xylene removal by a novel *Pandoraea* sp. strain WL1 and its application in a biotrickling filter. *Journal of Hazardous Materials* **288**: 17-24.
- Worsey, M.J., and Williams, P.A. (1975) Metabolism of toluene and xylenes by *Pseudomonas* (putida (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of Bacteriology* **124**: 7-13.
- You, J., Du, M., Chen, H., Zhang, X., Zhang, S., Chen, J. et al. (2018) BTEX degradation by a newly isolated bacterium: Performance, kinetics, and mechanism. *International Biodeterioration & Biodegradation* **129**: 202-208.
- You, Y., Shim, J., Cho, C.H., Ryu, M.H., Shea, P.J., Kamala-Kannan, S. et al. (2013) Biodegradation of BTEX mixture by *Pseudomonas putida* YNS 1 isolated from oil-contaminated soil. *Journal of Basic Microbiology* **53**: 469-475.
- Yu, H., Kim, B.J., and Rittmann, B.E. (2001) The roles of intermediates in biodegradation of benzene, toluene, and p-xylene by *Pseudomonas putida* F1. *Biodegradation* **12**: 455-463.
- Zhou, Y., Huang, H., and Shen, D. (2016) Multi-substrate biodegradation interaction of 1, 4-dioxane and BTEX mixtures by *Acinetobacter baumannii* DD1. *Biodegradation* **27**: 37-46.

Figures

Figure 1

Phylogenetic tree of S2TR-01, S2TR-09, and S2TR-20 (red underline) and other related bacterial genera. Bootstrap values are shown from 1,000 replicates. GeneBank accession numbers for the 16S rRNA sequence are shown in the parentheses.

Figure 2

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.

Figure 3

Genes 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

Figure 4

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.

Figure 5

XMO and catechol 2,3 dioxygenase activity in *P. Putida* grown in presence of Benzene (B), Toluene (T), ethylbenzene (E), p-xylene (X) and their mixture.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.docx](#)