STUDY OF A BACTERIAL COCULTURE FOR BENZENE, TOLUENE, ETHYLBENZENE AND XYLENE BIODEGRADATION.

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Abstract

Currently, one-quarter of the Canadian population depends on groundwater for daily activities. However, the growing economy, the increase in anthropogenic activities, and the rising demand for petroleum hydrocarbons have led to the prevalence of high-risk pollutants such as Benzene-Toluene-Ethylbenzene-Xylene (BTEX), in the environment (Haiying et al., 2021). Various physical, thermal, and chemical methods have been employed in the remediation of BTEX in groundwater and soil, as they can break them down into smaller molecules (Hernández-Ospina et al., 2024). Conventional thermo-physical methods, such as incineration and pump-and-treat are often costly and energy-intensive (Soleimani, 2014). Chemical methods, like advanced oxidation processes (AOPs) based on peroxide oxidation and ultraviolet radiation, are often limited in their accuracy as they are not pollutant specific (Alori at el., 2022). Contrary to these methods' bio-based approaches such as bioremediation, offer biodiversity protection, cost-effectiveness, and reliability in meeting the clean-up requirements. Bioremediation employs bacteria to target BTEX compounds, converting them into safer substances like carbon dioxide, pyruvate or acetyl CoA (Wongbunmak et al., 2020). Particularly, certain strains from the *Pseudomonas*, *Streptomyces, Microbacterium, Bacillus, Acinetobacter, Serratia* and *Rhodococcus* genera have been proven as successful BTEX degraders (Chicca et al., 2020; Hocinat et al., 2020; Kaur et al., 2023; Yavas & Icgen, 2018). In this study, a microbial coculture of *Serratia fonticola* and *Microbacterium esteraromaticum* was employed to degrade BTEX compounds.

Bacterial coculture, when appropriate, can improve the slow degradation of aromatic hydrocarbons compared to chemical and physical approaches without disrupting the ecological balance. Coculturing BTEX-degrading bacteria creates a robust system that can be applied in different aquatic environments; a co-metabolism between BTEX compounds can improve the degradation and increase the tolerance to these compounds by the microorganism (Mukherjee et al., 2012). Coculture provides a green alternative for sensitive sites like conservation and urban areas. The obtained results show a

total BTEX degradation of 47%, 45% and 42% by the coculture, *M. esteraromaticum* and *S. fonticola*, respectively. Furthermore, the bacterial coculture showed a high ability to degrade individually benzene (99%) toluene (71%), ethylbenzene (85%) and xylene (62%) compared to the individual strains over 42 hours of cultivation. Likewise, *M. esteromaromaticum* achieved an individual degradation of 20%, 68%, 92% and xylene 87%, respectively. Furthermore, the individual compound degradation achieve by S. *fonticola* was 38%, 57%, 97%, and 54%, respectively. This study shows an improvement of BTEX degradation as well as for benzene and toluene (individually). Further studies focused on the use of inducers to produce BTEX-degrading enzymes and to induce the co-metabolism among BTEX compounds are recommended to enhance the degradation of BTEX. Finally, the microbial coculture can be a promising tool for in-situ bioremediation in contaminated water bodies.

Dedication

To my family, whose love has been my North Star during this journey.

'*The cosmos is within us; we are made of star-stuff. We are a way for the universe to know itself.'* – Carl Sagan

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Abbreviations

AHs – Aromatic Hydrocarbons

AOPs – Advanced Oxidation Processes

AOSs – Active Oxygen Species

BSA – Bovine Serum Albumin

BTEX – Benzene, Toluene, Ethylbenzene and Xylene

C12O – Catechol 1,2 dioxygenase

C23O – Catechol 2,3 dioxygenase

DCW – Dry Cell Weight

DW – Drinking Water

EPA – Environmental Protection Agency

ESA – European Space Agency

EPLs – Environmental Pollution Levels

GC – Gas Chromatography

GC-MS – Gas Chromatography-Mass Spectrometry

GDP – Gross Domestic Product

GTP – Guanosine Triphosphate

H2O2 – Hydrogen Peroxyde

MSM – Minimal Saline Media

NOOA – National Oceanic and Atmospheric Administration

ORSs – Oxygen-releasing substances

ToMO – Toluene monooxygenase

TSB – Tryptic Soy Broth

TSA – Tryptic Soy Agar

TSP – Total Soluble Protein

TRL – Technology Readiness Level

XMO – Xylene monooxygenase

Publications Within this Thesis

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Diego A. Hernández-Ospina; Jean Carlos Viccari Pereira, Reema Kumar; Satinder Kaur Brar (2023). Bisphenol A and Bis (2-ethylhexyl) Adipate releases from different facilities across Canada. Retrieved from the Environment and Climate Change Canada's website: www.canada.ca/npri Environment and Climate Change Canada

Conferences

Diego A. Hernández-Ospina; Carlos S. Osorio-González, Richard. Martel, Satinder K. Brar. (2024). Bacterial Coculture as a Sustainable Tool for Benzene-Toluene-Ethylbenzene and Xylene (BTEX) Biodegradation. International Water Association (IWA) World Water Congress & Exhibition, August 11-15, Toronto, Ontario, Canada

Diego A. Hernández-Ospina (2024). Fluid Perspectives: Navigating the Future of Water - in recognition of world water day and world environmental day. Student panel discussion around Education and Awareness. Topics covered: Global water crisis, understanding water scarcity, Canadian water landscape. Life Science Building, York University, Toronto Ontario.

Diego A. Hernández-Ospina (2024). Catalyzing Interdisciplinary Research Cluster for Detection and Remediation of Water Contaminants (CIRC-DRWC) Formulation of bacterial cocultures for the degradation of aromatic hydrocarbons.

Diego A. Hernández-Ospina; Carlos S. Osorio-González, Richard. Martel, Satinder K. Brar. (2023). Assessment of a bacterial coculture for BTEX degradation. 37th EASTERN Canadian Symposium on Water Quality Research, Octobre 27-28 Octobre, 2023, University of Sherbrooke, Sherbrooke, Quebec, Canada.

Diego A. Hernández-Ospina; Carlos S. Osorio-González, Richard. Martel, Satinder K. Brar. (2023). Enzymatic biodegradation of BTEX. Oral presentation. TABES Annual Summit, York University, Toronto, Ontario, Canada.

Diego A. Hernández-Ospina; Carlos S. Osorio-González, Richard. Martel, Satinder K. Brar. (2022). Advances in the Biodegradation of BTEX. Oral presentation. NSERC CREATE ANNUAL SUMMIT, York University, Toronto, Ontario, Canada.

Chapter 1: Background

1.1 Introduction

Petroleum oil has been extensively used worldwide since the Industrial Revolution in the 18th century. The global aromatic hydrocarbon (AHs) market was worth hundreds of millions of dollars in 2022 and is expected to have an annual growth rate in the next years. AHs pollution occurs from a variety of sources, including logistic oil operations such as transportation, industrial and municipal wastewater, and accidental spills from oil and gas activities. However, accidental oil spills are the most severe cases. Benzene-toluene-ethylbenzene-xylene (BTEX) are oil-derived aromatic compounds primarily produced through the catalytic cracking of light-phase oil, like naphtha. Therefore, spills and leaks from the supply chain of the oil and gas industry, including storage tanks, pipelines, and transportation are of high importance. For example, according to the U.S. National Oceanic and Atmospheric Administration (NOOA), an average of eleven oil spills per month occur only in the United States. Other sources of BTEX contamination may include diesel leakage from gas stations and effluents of pharmaceuticals, agrochemicals, polymers, dyes, plasticizers, and automotive resin industries (Boll et al., 2018a).

One of the main concerns regarding BTEX pollution is its relatively high solubility in water and its capacity to infiltrate porous rocks reaching underground aquifers and water ecosystems, which are the primary sources of drinking water (DW) (Huang et al., 2021;

Lueders, 2017). Mono AHs compounds are well known for their negative effects on human and animal health due to their high toxicity (Wang et al., 2016). Several reports have shown that BTEX-contaminated sites are a potential source of neurotoxicity, anemia, and respiratory and cardiovascular illnesses, as well as impairments in the reproductive and immune systems of humans and animals (Latif et al., 2019). As a result, BTEX has emerged as a focal point in public health concerns and is designated as a hazardous pollutant according to the regulations set by the US Environmental Protection Agency (EPA) (Aburto-Medina and Ball, 2015; Busca, 2021; Huang et al., 2021). Another important concern is around the high volatility of BTEX. This property allows these compounds to spread further in the environment including soil and groundwater. BTEX compounds have the capacity to transition between phases, impacting their removal difficulty regardless of the cleaning strategy adopted. Currently, various physical, thermal, and chemical methods are used to remediate BTEX in groundwater and soil by breaking them down into smaller molecules like carbon dioxide and water (Ron & Rosenberg, 2014). Conventional thermal-physical techniques, such as incineration and thermal desorption, are costly, energy-intensive, and environmentally harmful, disrupting ecosystems and emitting greenhouse gases (Soleimani, 2014). Chemical approaches, including advanced oxidation processes (AOPs), often lack precision and may leave secondary pollutants or excess chemicals that harm ecosystems (Alori et al., 2022). Exothermic chemical reactions can also hinder on-site bioremediation by diffusing volatile compounds like BTEX. These methods are not permanent solutions and may merely transfer pollutants (Alori, 2015). In contrast, bioremediation offers sustainable, cost-effective, and reliable alternatives. Microorganisms can utilize BTEX as electron donors under specific environmental conditions. Aerobic bacteria like *Pseudomonas, Bacillus, Acinetobacter*, and *Rhodococcus* demonstrate significant BTEX-degrading capabilities, with

differentiation between aerobic and anaerobic processes depending on oxygen and other electron acceptors (Wongbunmak et al., 2020; Zhou et al., 2016)

Despite the capability of single bacteria to degrade BTEX, their use present important challenges such as limited activity and production of competent enzymes, and low tolerance towards environmental changes (temperature, pH, and oxygen concentration). Single strain degradation can be restricted to concentration of BTEX below actual environmental pollution levels (EPLs) in heavily polluted aquifers (>200 ppm), where the microbial growth and performance are compromised. Moreover, time is an important constraint when it comes to bioremediation, as it can take several days (up to 21 days) to degrade stable compounds in groundwater such as benzene. A potential alternative to address these challenges altogether is with the use of a coculture-based degradation. Bacterial coculture, can improve the slow degradation of AHs and creates a robust system that can be applied in different aquatic environments in sensitive sites like conservation and urban areas.

This dissertation studies a bacterial coculture of *S. fonticola* and *M. esteraromaticum* for the biodegradation of benzene, toluene, ethylbenzene and xylene (BTEX). The first chapter includes a literature review which describes the different activation mechanisms, pathways and intermediates involved in the biodegradation of BTEX as well as the research gaps and outlook in the field. In chapter 2, a detailed methodology is presented with all steps and considerations for the microorganisms growing conditions, biodegradation test and the enzyme assays.

In chapter 3, A discussion on the coculture degradation ability and tolerance towards the BTEX compounds is presented. Additionally, the chapter offers a comprehensive discussion

of the coculture performance for single-compound (SCD) and multi-compound (MCD) BTEX degradation.

In chapter 4, the thesis provides an evaluation on the effect of benzyl alcohol in the activity of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) using the bacterial coculture. Particularly, the stress-induced activity of BTEX-degrading enzymes and stress-induced BTEX co-metabolism after the supplementation of homologous monoaromatics such as benzyl alcohol is studied. Finally, Chapter 5 presents innovative hybrid approaches including different materials in which coculture can be immobilized to enhance its single-compound BTEX degradation performance while making it a promising and sustainable solution for the on-site degradation. In summary, this thesis enhances the knowledge and strategies for the decontaminating BTEX in sensitive aquatic environments.

1.2 Literature Review

New perspectives on the anaerobic degradation of BTEX: mechanisms, pathways, and intermediates

(Review Article published in Chemosphere)

1.2.1 Types of biodegradation: Aerobic and Anaerobic

Depending on the compartment, budget constraints, the target compound, and the technical and environmental features of the contaminated site, BTEX biodegradation can have different approaches. Under common atmospheric conditions, aerobic degradation occurs naturally in the site until oxygen is gradually depleted in any hydrocarbon-contaminated site providing an opportunity for an anaerobic process. Moreover, the occurrence of metals and organic and inorganic nutrients in the contaminated site could determine the growth and thriving of anaerobic and aerobic BTEX-degrading microorganisms. Likewise, the ability of these microorganisms to utilize nutrients, and various molecules as electron acceptors including oxygen, or metals such as iron oxide and manganese dioxide, will make the difference between an aerobic and an anaerobic degradation process (Hidalgo et al., 2020; Kalantary et al., 2014).

Table 1 outlines crucial distinctions between aerobic and anaerobic biodegradation of BTEX. Factors like flexibility, versatility, and operational challenges, which are pivotal in choosing the most suitable option are essential to make an informed decision between aerobic and anaerobic degradation. It's important to grasp the feasibility, ease of process monitoring, maintenance needs, environmental and social impact, technology maturity, and available alternatives.

Table 1. Aerobic and Anaerobic Biodegradation of BTEX *(permission obtained from Chemosphere)*

	Aerobic		**Anaerobic**	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Versatility	Use of atmospheric oxygen. Appropriate for sites with multiple contaminants (e.g. different hydrocarbons) Combined with multiple technologies: Biostimulation, bioaugmentation, in-situ bioventing, air sparging, oxygen-releasing substances (ORSs), pump and treat, ozone injection, soil vapor extraction.	Not suitable for volatile and light-weight petroleum hydrocarbons such as BTEX Its use of electron acceptors is limited to only oxygen. Reliance on dissolved oxygen for the degradation of BTEX in water bodies.	Enhancement mature technologies available: Bioaugmentation, bioventing and biostimulation. Use of multiple electron acceptors: nitrate, sulfate, metals, humic, etc. Anaerobes can growth in wide ranges of pH (5 – 9).	Reduce enzymatic capabilities. Anaerobes such as methanogens are quite sensitive towards pH changes (7 – 7.5). Some activation mechanisms of degradation are not as well-understood.
Technology maturity	A high mature alternative with 37 active patents created during the last 10 years, with 30% involving	Only 3 patents are focused on groundwater BTEX bioremediation	With 18 active patents during the last century. Anaerobic BTEX bioremediation technology has	Important gaps on anaerobic pathways, involved enzymes and applicability.

	Aerobic		Anaerobic	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
	physical, chemical or hybrid bioremediation systems. Likewise, 50% of the patents are in a pilot or industrial stage, mainly focused on BTEX removal from wastewater and soil.		several opportunity areas to improve. In summary: - 20% combined bioremediation with bioventing or biostimulation. - 33% are chemical approaches - 47% are biological approaches.	62.5% of the existing patents are currently in the bench scale stage, with no patent reported at industrial scale. Used mainly for soil and groundwater BTEX biodegradation.
Feasibility	Feasible in surface-sensitive and urban areas Feasible in cold and surface-contaminated areas	Inadequate to address free mobile products and petroleum hydrocarbons (PHs) in low permeability soil (e.g. clay)	Good for remote and difficult to access sites (e.g. contaminants buried underground). Less energy intensive than aerobic processes Feasible in surface-sensitive and urban areas	Lower biodegradation rates and lower energy yield per molecule of BTEX degraded. Less feasible for urgent clean up operations.

	Aerobic		Anaerobic	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Maintenance and monitoring	Heavily contaminated areas and recalcitrant pollutants Requires high maintenance. Easy monitoring is easier: Contaminated areas being often open access. Multiple regulated field and analytical methods developed	Clean-up progress can be directly track by monitoring oxygen levels in soil or water. Measurable degradation products such as carbon dioxide can facilitate real-time monitoring. Monitoring techniques are well-established and easier to implement.	Feasible in inaccessible underground areas Low maintenance compared to aerobic systems. More appropriate for sites with limited access or where monitoring is more challenging. Multiple alternatives of stable in situ indicators specific to BTEX anaerobic degradation. (e.g. benzylsuccinates /phenylitaconates and benzoates).	Monitoring relies on advanced tools such as molecular probes, proteomic analysis and stable isotope probing and Moreover, its monitoring relies on more the identification of bacterial strains and complex anaerobic microbial communities.
Operation	Optimal temperature between 20 – 45 °C		Optimal temperature between 28 °C and 40 °C	Slow metabolic rates compared to aerobes.

	Aerobic		Anaerobic	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Environmental and social impact	Faster process with higher degradation rates and intrinsically facilitated with a more robust degradation to target different levels of pollution. Works well for BTEX when it is present with diverse contaminants.	Require human labour and demand logistics.	Improved by natural attenuation such as sorption and volatilization from groundwater. Anaerobes have a great resistance to changes in pH.	Prone to nutrient limitation in soil.
	Less formation of harmful intermediates with a direct conversion to CO2 and H2O.	Less contaminant-specific	More intermediate by-products compared to aerobic due to slower metabolism.	Anaerobic pathways are not clearly understood.
	Accepted by Regulatory authorities and the public.	Can disrupt the ecosystem and native microbes.	Requires minimal equipment and labour work and it is accepted by regulatory authorities and the public.	Public acceptance is not as good as it is for aerobic.
	Create employment and improve local economies, and it is aligned			No knowledge about the toxicity of anaerobic intermediates

	Aerobic		Anaerobic	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
	with indigenous traditions and land rights.	Requires more equipment and labour work than anaerobic biodegradation.	More sustainable with less carbon footprint and energy demands. Create employment and improve local economies, and it is aligned with indigenous traditions and land rights.	It might result in methane emissions, a greenhouse gas.

CO2: Carbon dioxide H2O: Water

Aerobic biodegradation surpasses anaerobic biodegradation in versatility. Its capability to concurrently address a broad spectrum of contaminants in complex mixtures and at varying concentration levels sets it apart. The microbial diversity in aerobic microorganisms outpaces that of the anaerobic counterparts, primarily because bacteria utilize molecular oxygen in aerobic biodegradation—a process prevalent in surface water. Likewise, aerobic biodegradation boasts enzymes with significant potential for BTEX degradation. Some of these enzymes include monooxygenases like toluene monooxygenase (ToMO), benzene monooxygenase and xylene monooxygenases (XMO), dioxygenases such as catechol 1-2 dioxygenase (C12O), ethylbenzene dioxygenase and catechol 2,3-dioxygenase (C23O), among others (Olajire and Essien, 2014).

On the other hand, the anaerobic biodegradation process has less microbial diversity with reduced enzyme capabilities. The BTEX-degrading reported anaerobes are limited. Table 2 shows some studies performed with anaerobic microorganisms able to utilize BTEX along with the operational conditions, and their degradation ability. These studies have been limited to a few families of bacteria and archaea include *Desulfobulbaceae*, *Rhodocyclaceae*, and *Peptococcaceae* (Lueders, 2017). Even though most of the microorganisms employed in research are found as a consortium, single strains like the metal-reducing microbes (DMRM); *Aromatoleum aromaticum* EbN1 and *Georgfuchsia toluolica* G5G6 are capable of degrading toluene and ethylbenzene after the reduction of iron (Fe (III)) and manganese (Mn (IV)) as electron acceptors (Dorer et al., 2016; Tremblay and Zhang, 2020). Studies of BTEX biodegradation have been limited to contaminated soil and groundwater and innovative approaches have been proposed. For example, Rahman et al., (2018) who used glucose and acetate as co-substrates to enhance the benzene biodegradation and increase CO2 and CH4

production during anaerobic digestion. Table 3 highlights the genes and enzymes encoded for some BTEX biodegradation tests under anaerobic conditions. The number of enzymes and genes reported for anaerobic degradation is as limited as the microbial diversity of anaerobes. Genes encoding benzylsuccinate synthase (K07540), 4-hydroxybenzoyl-CoA (K01075) and benzoyl-CoA reductase (K04114) are often most reported. However, these genes are more specific to toluene and ethylbenzene anaerobic degradation and are often focused only on microbial community analysis. Overall, anaerobic bioremediation is an emerging area of exploration that demands important advancements in metagenomics and enzyme-based technologies to assess its potential for in situ application for all the compounds including benzene and xylene which are known to a lesser extent (Miri et al., 2019).

Table 2. Studies of BTEX biodegradation under anaerobic conditions *(permission obtained from Chemosphere)*

Microorganism	**Compartment**	**Contaminant**	**Initial Concentration (µM)**	**Electron Acceptor**	**Conditions: time (days), Temperature (°C)**	**Total Degradation (%)**	**Reference**
Bacterial consortium	Soil	Toluene	900	Humic Substances	15 days, 25 °C	55	(Jiang et al., 2023)
Bacterial consortium	Soil	Benzene	120	Sulfate	210 days, 28 °C	50	(Wu et al., 2022)
		Toluene	90	Nitrate	90 days, 28 °C	100	
		Ethylbenzene	100	Iron	90 days, 28 °C	100	
		p-xylene	100		210 days 28 °C	30	
		m-xylene	100		210 days, 28 °C	30	
		o-xylene	200		210 days, 28 °C	55	
Bacteria consortium	Groundwater	Toluene	500	Iron	10 days, 20* °C	100	(Huang et al., 2021)

Microorganism	Compartment	Contaminant	Initial Concentration (µM)	Electron Acceptor	Conditions: time (days), Temperature (°C)	Total Degradation (%)	Reference
Benzene-Degrading Culture DGG-B	Groundwater	Benzene	18	Methane	70 days, 23°C	100	(Toth et al., 2021)
Bacteria consortium	Sludge	Benzene and glucose	205	Acetic Acid	22 days, 35 °C	75	(Rahman et al., 2018)
		Benzene and acetate		Carbon Dioxide			
Bacteria consortium N3	Soil and Groundwater	Benzene	1000	Nitrate	30* days, 20°C	77*	(Keller et al., 2018)
Bacteria consortium N4				Sulfate	35* days, 20°C	80*	
***Aromatoleum aromaticum* EbN1**	Surface Water	Toluene	170	Nitrate	22 days, 25°C		
		Ethylbenzene	150		7 days, 25°C		
				Manganese			
***Georgfuchsia toluolica* G5G6**	Groundwater	Toluene	190	Nitrate	3 days, 25°C	100	(Dorer et al., 2016)
			140		7 days, 25°C		

Microorganism	Compartment	Contaminant	Initial Concentration (µM)	Electron Acceptor	Conditions: time (days), Temperature (°C)	Total Degradation (%)	Reference
		Ethylbenzene		Manganese			
		Toluene	170	Nitrate	2 days, 25°C		
		Ethylbenzene					
Bacteria consortium	Soil		150	Manganese	7 days, 25°C		
Bacteria consortium	Soil	Benzene	760	Sulfate	180 days, 22°C	100	(Herrmann et al., 2010)
						100	(Laban et al., 2010)
Bacteria consortium	Soil	Benzene & Toluene	350	Iron	49 days, 30°C		
Bacteria consortium	Soil	Benzene	4.5	Manganese	80 days, 30°C	100	(Villatoro-Monzón et al., 2008)
Bacteria consortium	Soil	Benzene	104		60 days, 20°C		
		Toluene	103		35 days, 20°C		(Dou et al., 2008)
		Ethylbenzene	103	Nitrate	48 days, 20°C	100	

Microorganism	Compartment	Contaminant	Initial Concentration (µM)	Electron Acceptor	Conditions: time (days), Temperature (°C)	Total Degradation (%)	Reference
		m-xylene	98		62 days, 20°C		
		o-xylene	101	Sulfate	69 days, 20°C		
		p-xylene	97		67 days, 20°C		
Bacteria	Groundwater	Benzene	25		230 days, 20°C	100	(Botton and Parsons,
consortium		Toluene	100		160 days, 20°C		2006)
		o-xylene	60		600* days, 20°C		
		p-xylene	60		600* days, 20°C		
				Iron			

*Average values

Table 3. Genes and enzymes in BTEX biodegradation tests under anaerobic conditions *(permission obtained from Chemosphere)*

Microorganism	Contaminant	Gene	Enzyme Encoded	Reference
Bacteria consortium	Toluene	K07540	benzylsuccinate synthase	(Jiang et al., 2023)
		K01075	4-hydroxybenzoyl-CoA	
		K01560	Thioesterase-2-haloacid dehalogenase	
Bacteria consortium	Benzene	K07540	benzylsuccinate synthase	(Wu et al., 2022)
	Toluene	bssA/bamA	6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase	
	Ethylbenzene			
	p-xylene			
	m-xylene			
	o-xylene			
Bacteria consortium	Toluene	K07540	benzylsuccinate synthase	(Huang et al., 2021)
		K07543+K07544	benzylsuccinate CoA-transferase (R)-benzylsuccinyl-CoA dehydro genase	
		K07545	E-phenylitaconyl-CoA hydratase (2S)-[(R)-hydroxy(phenyl)methyl]-succinyl-CoA	
		K07546	dehydrogenase benzoylsuccinyl-CoA thiolase	
		K07547+K07548	benzoyl-CoA reductase	
		K07549, K07550	cyclohexa-1,5-dienecarbonyl-CoA hydratase	

Microorganism	Contaminant	Gene	Enzyme Encoded	Reference
		K04112, K04113, K04114,	6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase	
		K04115	6-oxo-cyclohex-1-ene-carbonyl-CoA hydrolase	
		K07537	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase cyclohex-1-ene-1-carboxylCoA	
		K07538	hydratase	
		K07539	cyclohexanecarboxyl-CoA dehydrogenase cyclohexanecarboxylate-CoA ligase	
		K07535	2-ketocyclohexanecarboxyl-CoA hydrolase	
		K07534		
		K04117		
		K04116		
		K07536		
Bacteria consortium N3	Benzene	K04114	benzoyl-CoA reductase	(Keller et al., 2018)
Bacteria consortium N4				
Aromatoleum aromaticum EbN1	Toluene	bamA	Ring opening hydrolase	(Dorer et al., 2016)
	Ethylbenzene	Bss	Benzylsuccinate synthase	
Georgfuchsia toluolica G5G6				
Bacteria consortium DD-Anox1	Toluene	bamA	Ring opening hydrolase	
	Ethylbenzene	Bss	Benzylsuccinate synthase	

Microorganism	Contaminant	Gene	Enzyme Encoded	Reference
Bacteria consortium	Benzene & Toluene	K25932 K04110	phenylphosphate carboxylase	(Laban et al., 2010)
		UbiD/UbiX	benzoate-CoA ligase	
		UbiD/UbiX	3-octaprenyl4-hydroxybenzoate carboxy-lyase benzene carboxylase	

Aerobic degradation exhibits remarkable versatility in addressing contamination, whether in proximity to or distant from the source zone, as well as in both saturated and unsaturated zones. In heavily contaminated sites (saturated), especially with a higher concentration of hydrocarbons, the aerobic zone may transition rapidly into an anaerobic state due to oxygen depletion. Despite its reliance on oxygen, aerobic degradation offers several alternatives to augment oxygen levels, thereby enhancing its efficiency. These alternatives can be used in both areas with high saturation and those with lower saturation and include using substances that release oxygen, injecting ozone, or oxygen, or using a combination of techniques like air sparging, bioventing or pump and treat. For aerobic biodegradation in soil, the process can be combined with vapor extraction or biopiling (US EPA, 2014). Moreover, biostimulation and bioaugmentation are the most important alternatives for boosting the aerobic biodegradation of petroleum hydrocarbons, both of which significantly contribute to enhancing versatility. Biostimulation is a strategy involving the supplementation of nutrients, electron acceptors (such as oxygen), or electron donors (co-metabolism) to boost the microbial activity and growth of aerobic degraders (Gieg and Toth, 2017; Haleyur et al., 2019). This approach not only fosters the growth of aerobic degraders but can also stimulate the production of enzymes and induce specific degradation pathways in the microbial community at the site.

Similarly, bioaugmentation is an effective strategy for accelerating biodegradation rates. It entails the injection of cultivated degraders into the contaminated environment. Overall, the application of aerobic biodegradation for hydrocarbons has reached the implementation stage with various alternatives that offer significant advantages and adaptability to specific biodegradation goals. The technology readiness level (TRLs) for technologies compatible

with and capable of enhancing aerobic degradation typically ranges between 7 and 8 for soil and between 7 and 9 for water, according to the European Space Agency's (ESA) scale. This indicates a high level of maturity and feasibility for these technologies in real-world applications. Technologies that include excavation, thermal treatment, landfill disposal, pump and treat and bioventing are mature enough to make aerobic processes highly reliable. However, aerobic degradation is not suitable for lightweight hydrocarbons such as BTEX due to their volatilization.

In contrast, anaerobic degradation offers a better option for the removal of such AHs taking into account its environmental fate. Compounds such as BTEX in the saturated zone tend to volatilize to the atmosphere; however, in the unsaturated zone, they can be transported and infiltrated into soil and groundwater (Khalid et al., 2021). BTEX compounds can be easily absorbed in clay, minerals and organic matter in the soil due to their high soil partition coefficient (Koc). In another type of soil, BTEX can infiltrate faster and contaminate the water of aquifers where oxygen is usually scarce and organic matter derivates, metals and minerals are abundant.

The presence of multiple alternative electron acceptors in environments, such as nitrate, methane, sulfate, manganese dioxide, iron oxide, humic substances, and heavy metals provides the anaerobic processes a significant versatility. Despite being acknowledged as a lengthier process, attributed to the comparatively slow metabolic activity of anaerobic microorganisms, it remains a dependable method for the removal of BTEX in groundwater across diverse geological and geographical locations.

Conversely, the strategies available to expedite hydrocarbon removal from aquifers under anaerobic conditions are fewer compared to aerobic processes. Primarily, only surfactants

and bioaugmentation have been explored as technologies to enhance biodegradation rates. Bioaugmentation holds the potential to significantly increase biodegradation rates by several orders of magnitude beyond naturally occurring, non-stimulated rates. However, the application of biosurfactants is more pertinent and specific to the biodegradation of alkanes. (Crisafi et al., 2016; Nikolopoulou and Kalogerakis, 2008; Roy et al., 2018)

As of now, these enhancement technologies have not progressed to the application stage for BTEX-contaminated subsurface, including groundwater and subsoil. Their technology readiness levels (TRLs) are still fluctuating between 4 and 5, having been validated in lab-scale but only preliminarily tested in relevant environments. However, since anaerobic biodegradation processes pose an important interest in future research such as scale-up studies, its TRL levels are expected to increase in the next decades. Moreover, the inadequate understanding of anaerobic biodegradation mechanisms, including pathways, intermediates, and enzymes, is impeding the integration of these technologies. In contrast, aerobic biodegradation understanding is more advanced. For example, aerobic degradation of BTEX is more mature with a clearer picture of the central catabolic routes involved such as the addition of hydroxyl groups and the intermediates such as catechol derivates found in the biodegradation pathways (Lueders 2017).

Additionally, the feasibility of applying aerobic and anaerobic biodegradation for BTEX involves significant economic, logistic, and regulatory implications, depending on the site conditions. Additionally, the feasibility of aerobic and anaerobic biodegradation is assessed based on energy requirements, and in terms of economic feasibility, both degradation processes show high viability in sensitive and urban areas where strict compliance with policies is essential, and sustainable options are preferred.

In cases where BTEX contamination is located in surface areas and highly saturated zones, aerobic degradation emerges as a cost-effective solution due to the rapid oxidation of compounds and the ready availability of atmospheric oxygen. Nevertheless, if BTEX pollution is concentrated in low-permeability soils like clay, aerobic degradation becomes ineffective as it becomes inaccessible, and oxygen is quickly depleted. In contrast, anaerobic biodegradation of BTEX proves to be a better alternative for challenging contaminated sites, particularly when they are buried underground and distant from operational centers in larger cities. The degradation of BTEX in remote and cold sites, such as the northern tundra of European Siberia or Canada, may require the provision of electricity, transportation, logistical operations, and the supply of chemicals, personal protective equipment, and working tools. Meeting these operational requirements can be cost-prohibitive, contradicting the goal of biodegradation to provide cost savings while offering an environmentally friendly and practical solution for addressing BTEX contamination.

Anaerobic biodegradation offers a more practical approach in these cold and remote areas that are often part of conservation areas also, and where biodiversity and water resources protection are prioritized (Dias et al., 2012). Moreover, anaerobic is an approach that requires low maintenance and monitoring in areas of limited access. Once the microbial culture is inoculated into the site it can be monitored on a monthly or yearly basis as it progresses slower. In contrast, aerobic degradation requires more maintenance as the chain of reactions occurs faster and the oxidation of BTEX compounds may also imply a sequence of various redox zones is established moving in the downstream direction from the location of the contaminant. In terms of monitoring, aerobic biodegradation has the advantage of the contaminated site being open access. Moreover, monitoring has been well established by the

United States (US) - EPA, with several regulated field methodologies, parameters determined and analytical methods. For example, methods such as EPA SW-846: 8260B which is widely used along with field Gas chromatography (GC) and Gas Chromatography Mass Spectrometry (GC-MS) to determine organic volatile compounds such as BTEX. Likewise, since the mechanism of aerobic biodegradation is well understood so is the formation of intermediate compounds after the breaking down of BTEX, which could offer insights into the monitoring of the removal progress. Although not at the same level of understanding of aerobic, anaerobic biodegradation also has intermediates like benzylsuccinates/E-phenylitaconates that can be employed as in situ indicators of the degradation progress. More intermediates have also been reported as indicators such as benzoates derivates; however, employing benzylsuccinates proves to be a more dependable and distinctive indicator of anaerobic conditions due to their absence from commercial and industrial sources. Despite the existence of anaerobic intermediates, a major disadvantage for the monitoring of anaerobic biodegradation lies in the scarce metagenomic studies and the underdeveloped molecular tools such as proteomic analysis and stable isotope probing which limit its understanding (Boll et al., 2018b; Ladino-Orjuela et al., 2016).

Finally, the environmental and social impact are critical aspects to differentiate the best alternative between aerobic and anaerobic. For example, aerobic biodegradation possesses the advantage of having less formation of toxic and harmful intermediates. Aerobic conditions are thus safer, considering that BTEX compounds can likely achieve complete mineralization with carbon dioxide and water as end products. Despite the rapid mineralization of BTEX and other petroleum hydrocarbons, aerobic biodegradation is not contaminant-specific and can disrupt the ecological balance and biogeochemical cycles. On

the contrary, anaerobic degradation relies often on the natural attenuation which is slow due to the anaerobe metabolism (Reitzel, 2005). This slow transformation of BTEX compounds could lead to higher levels of intermediates. However, there are no studies suggesting that any of these intermediates might be having a negative impact on aquifers and other sub-surface ecosystems. Overall, anaerobic biodegradation can be considered a more sustainable alternative compared to aerobic when it comes to the large-scale biodegradation of petroleum hydrocarbons such as BTEX. Anaerobic is less disruptive as it provides sufficient time for microbial ecosystems to prepare for the biodegradation process. As a result of this stimulation, natural cycles such as nitrification, ammonification and methanogenesis are stimulating, while enriching the soil and protecting biodiversity. Likewise, anaerobic has fewer energy demands without the use of fuel-operated technologies such as pumps, and therefore, a significant reduction in its carbon footprint and climate change impact. In addition, both types of biodegradation alternatives correspond to a process in which nature repairs itself and thus, they are safe to protect water bodies, groundwater and soil during a BTEX cleanup process.

1.2.2 BenzylCoA Reduction

Benzoyl-CoA, a central metabolite found after the breakdown of AHs like BTEX, is present in several alternative pathways. During the breaking down of BTEX, the biochemical energy demands, terminal electron acceptor preference and the degradation pathway depend on the microorganism. However, all the biodegradation pathways seem to converge on the benzoyl-CoA pathway.

In this pathway, the benzoyl-CoA ring is anaerobically reduced to form a non-aromatic cyclohexene carboxyl CoA, specifically the intermediate cyclohex-1,5-diene-1-carboxyl-CoA (I), by benzoyl-CoA reductase (BCR) (Durante-Rodríguez et al., 2018). Figure 1 displays the benzoyl-CoA reduction pathway, showing that it can be activated either via ATP-dependent enzyme (Class I BCR) or ATP-independent reductase (Class II BCR). During the activation, Class I BCR is responsible for both the de-aromatization of benzoyl-CoA and the hydrolysis of two ATP molecules (Ghattas et al., 2017). Two types of Class I BCR have been identified and characterized with their subunits in species from the *Tahuera*, *magnetospirillum*, *azoarcus*, and *aromatoleum* genus; Bcr-type (BadDEFG) and Bzd-type (BzdNOPQ), whereas for Class II BCR, eight subunits had been reported (BamBCDEFGHI) with its respective active site (BamB) (Boll et al., 2014; Tremblay and Zhang, 2020).

Different authors suggest that class I BCR and class II BCR catalyze the de-aromatization depending on the microorganism. Class I BCR is often found in facultative anaerobic bacteria that utilize terminal electron acceptors with high energy yields, such as denitrifying bacteria (e.g. *Tahuera, Azoarcus, Aromatoleum*), while Class II BCR is commonly found in obligate anaerobes, including some iron (e.g *Geobacter metallireducens*) and sulfate reducers (e.g *Desulfococcus*). (Boll et al., 2018b; Tiedt et al., 2018).

Additionally, Figure 1 illustrates the subsequently modified beta-oxidation of cyclohex-1,5-diene-1-carboxyl-CoA (I) that involves the addition of a water molecule by a cyclohexadienoyl-CoA hydratase (Carmona et al., 2009). The resulting 6-hydroxycyclohex-1ene-carboxyl-CoA (II) is oxidized and hydrated, producing 3-hydroxy-pimelyl-CoA (IV) by hydroxyenoyl-CoA dehydrogenase and a ring-opening oxoenoyl-CoA hydrolase, respectively. Glutaryl-CoA (V), crotonyl-CoA (VI), 3-hydroxy-butanoyl-CoA (VII) and

acetoacyl-CoA (VIII) are further intermediates formed after the steps of beta-oxidation, hydration, decarboxylation, and coenzyme A addition. The enzymes responsible for these transformations include acetoacyl-CoA reductase, glutaconyl-CoA decarboxylase, 3-hydroxy-pimeyl-CoA dehydrogenase, the b-ketopimeyl-CoA thiolase, and glutaryl-CoA dehydrogenase.

Acetoacyl-CoA (VIII) cleavage produces two acetyl-CoA molecules that, along with a molecule of acetyl-CoA produced after the hydrolytic ring fission step, enter the Krebs cycle, which ultimately produces carbon dioxide, NADH, FAHD2, and guanosine triphosphate (GTP). Although the benzoyl-CoA degradation pathway has no major differences for different strains or terminal electron acceptors, two independent enzymes were identified as responsible for the decarboxylation in the last step under sulfate-reducing conditions whereas one single enzyme-catalyzed this reaction under iron-reducing conditions.

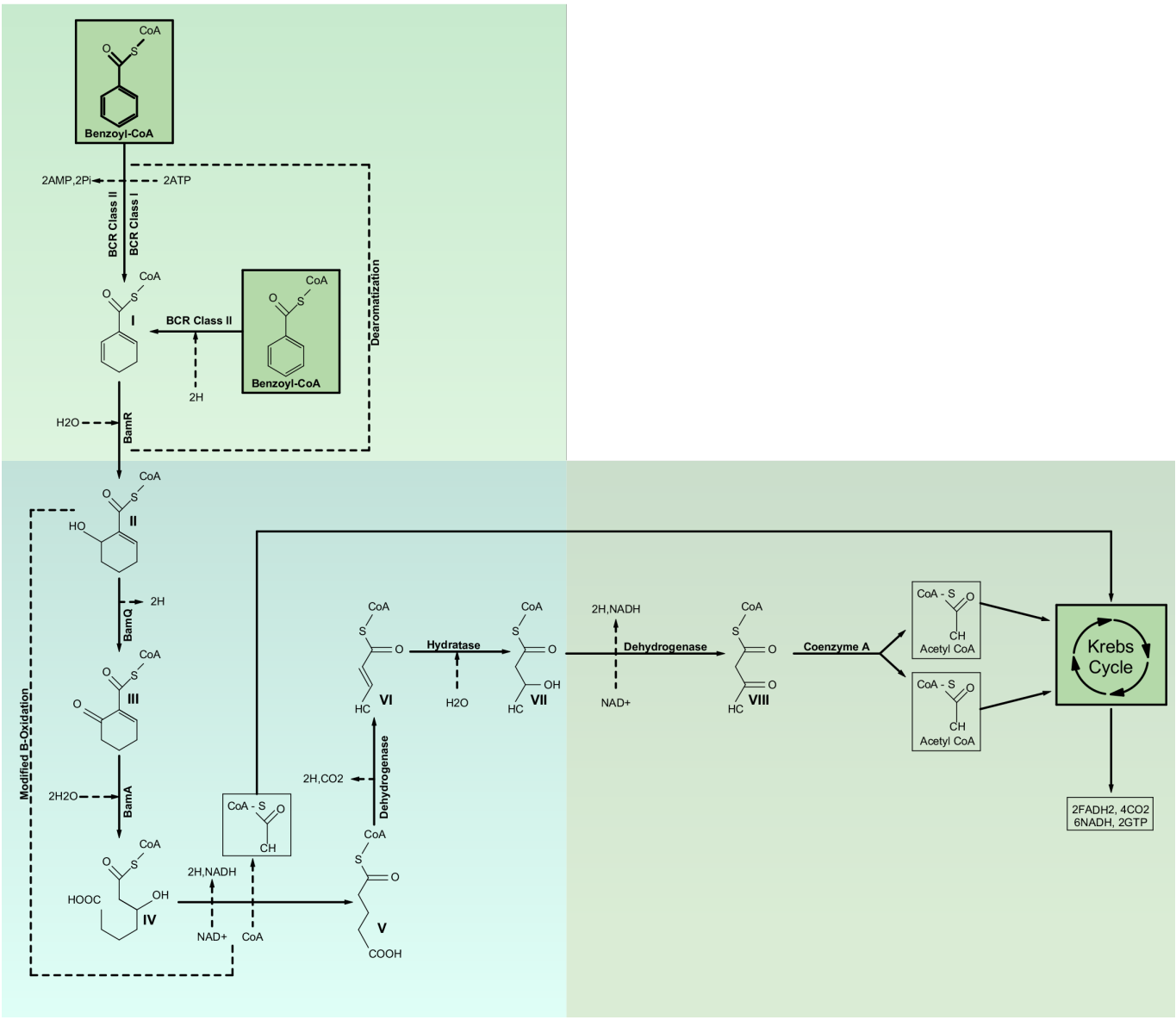


Figure 1. Metabolic pathway from benzoyl-CoA to acetyl CoA. All the metabolite intermediates in the benzoyl CoA degradation are shown including the Krebs cycle final products: I. cyclohex-1,5-diene-1-carboxyl-CoA II. 6-hydroxycyclohex-1ene-1-carboxyl-CoA III. Oxoenoyl-CoA IV. 3-hydroxy-pimeloyl-CoA V. glutaryl-CoA VI. crotonyl-CoA VII. 3-hydroxy-butanoyl-CoA VIII. Acetoacyl-CoA *(permission obtained from Chemosphere).*

1.2.3 Benzene biodegradation pathway

Even though the understanding of benzene biodegradation is still in an intermediate stage in the absence of oxygen, three pathways for benzene anaerobic degradation are proposed: (1) hydroxylation to phenol, (2) carboxylation to benzoate, and (3) methylation to toluene (Castro et al., 2022; Edwards et al., 2021; Gieg and Toth, 2017).

Figure 2 shows that during the hydroxylation of benzene (1), the hydroxyl group from water is introduced into a carbon-hydrogen bond, and a subsequent series of reactions to produce benzoyl-CoA follows this. Initially, benzene is hydroxylated to phenol by a benzene hydroxylase. Then, the resulting phenol is converted into phenyl phosphate (I) by an ATP-dependent phenyl phosphate synthase (PpsABC) with subunits (α, β, γ). Subsequently, phenyl phosphate is converted into 4-hydroxybenzoate (II) through carboxylation mediated by the phenylphosphate carboxylase (PpcBD) comprised of the subunits (d, α, β, γ). Following this, a 4-hydroxybenzoate-CoA ligase (PpcBD) transforms 4-hydroxybenzoate into 4-hydroxybenzoyl-CoA (III). The final step involves the conversion of 4-hydroxybenzoyl-CoA into the central intermediate, benzoyl-CoA, through the activity of 4-hydroxybenzoyl-CoA reductase (PcmRST). An enzyme comprised of three subunits referred to as (α2β2γ2) (Netzer et al., 2020).

On the other hand, benzene biodegradation via carboxylation to benzoate (2) is likewise an important pathway considered the primary mechanism of the benzene activation pathway (Gieg and Toth, 2017).

Carboxylation serves as an activation mechanism for benzene, and it results in the transformation of benzoate catalyzed by benzene carboxylase (UbiD-like) in the presence of

carbon dioxide. Subsequently, benzoate is transformed to benzoyl-CoA by a proposed benzoate-CoA ligase (Bcl1) that is composed of a single protein in the presence of a Coenzyme-A and adenosine triphosphate (ATP) (Tremblay and Zhang, 2020). Moreover, multiple authors have reported this activation step for the biodegradation of benzene under different anaerobic conditions. For example, Ghattas et al., (2017) suggested that the carboxylation of benzene to form benzoate likely occurred under methanogenic, iron, and sulfate-reducing conditions. However, nitrate-reducing microbial consortia have also shown a potential degradation via benzene carboxylation. For instance, (Atashgahi et al., 2018) observed gene transcripts for key enzymes on the biodegradation pathway such as benzoate-CoA ligase and UbiD/UbiX-related carboxylases suggesting the benzoate formation and highlighting the carboxylation to benzoate step as the main activation reaction for the benzene biodegradation pathway in denitrifying enrichment cultures. Similarly, other authors such as (Dong et al., 2017) reported benzoate presence using a sulfate-reducing member of the genus *Pelotomaculum* that converts benzene to carbon dioxide. Nevertheless, certain aspects such as the high energy dissociation of the C-H bond in benzene (473 kJ/mol), the difficulty of benzene to diffuse into cells, and the lack of benzoate-CoA ligase can make it more difficult for direct carboxylation with other enrichment cultures (Meckenstock et al., 2016). Thus, further research is required to clarify and demonstrate whether benzene carboxylation may occur in other cultures and under reducing conditions and tackle these challenges.

Alternatively, benzene methylation to toluene (3) is another proposed metabolic pathway as a potential activation mechanism that remains unknown in certain aspects. Biologically, benzene methylation to toluene was first observed in human bone marrow incubated with S-

adenosylmethionine (Flesher and Myers, 1991). Later, evidence found in benzene biodegradation studies suggested that benzene methylation can be carried out using nitrate, sulfate, and methanogenic enrichment cultures (Chakraborty and Coates, 2005; Ghattas et al., 2017; Ulrich et al., 2005; Vogt et al., 2011). However, authors such as (Atashgahi et al., 2018) stated that further research is required to prove benzene methylation to toluene as the activation step of this anaerobic biodegradation pathway after conducting a meta transcriptomic analysis in different benzene nitrate-reducing enrichment culture. For example, this study could not find transcripts such as bssA genes that encode benzyl succinate synthase, a key enzyme for the metabolism of toluene (Acosta-González et al., 2013). Moreover, these studies explained that no proteins involved in benzene methylation have been found in proteomic studies, still, some benzene degrading consortia had not been able to degrade toluene. A better understanding of the enzymes present is essential to suggest whether this can be an alternative pathway in the anaerobic degradation of benzene considering that benzene is converted to toluene by a methyltransferase whose identity is not yet clear (Wartell et al., 2021). Other studies indicated that the benzene methylation to toluene and benzene hydroxylation to phenol pathways were activated simultaneously, thereby further research is required to better understand this possibility (Chen et al., 2023; Wartell et al., 2021).

Overall, certain metabolic steps in the anaerobic pathways of benzene have not been well characterized and more research is required. Likewise, the mechanism of action and identity of certain enzymes and genes participating in the benzene hydroxylation to phenol is still unclear. For instance, information about benzene hydroxylase and 4-hydroxybenzoate-CoA ligase, enzymes that catalyze the formation of phenol and 4-hydroxybenzoyl-CoA, need

more research (Meckenstock et al., 2016; Tremblay and Zhang, 2020). Further research is also required to explore benzene carboxylation and methylation with SO_4^{-2}, NO_3^-, HCO_3^{-3}, Mn^{+4}, and Fe^{+3} as the electron acceptors in newly found benzene enrichment cultures. These benzene enrichment cultures were isolated from natural ecosystems contaminated with benzene such as the benzene enrichment culture N3 (*Sulfuritalea* and Desulfosporosinus abundant) and N4 (*Thiobacillus abundant*), and microbiota Nitrate supplemented (NS) (*Pseudomonas, Petrimonas, Achromobacter, Intrasporangium, Comamonas*), WE (Without exogenous electron acceptors) and the microbiota Ferric Iron supplemented (FS) (*Sedimentibacter, Pseudomonas, Annwoodia, Mycetohabitans, Desulfoprunum, Ornatilinea, Methanobacterium, Desulfobutbus, Acetobacterium*) that were able to interact and form intricate networks in a metabolic cooperation and therefore is it necessary to discard the possibility of them to use multiple electron acceptors to overcome energetic barriers (Keller et al., 2018; Wu et al., 2022).

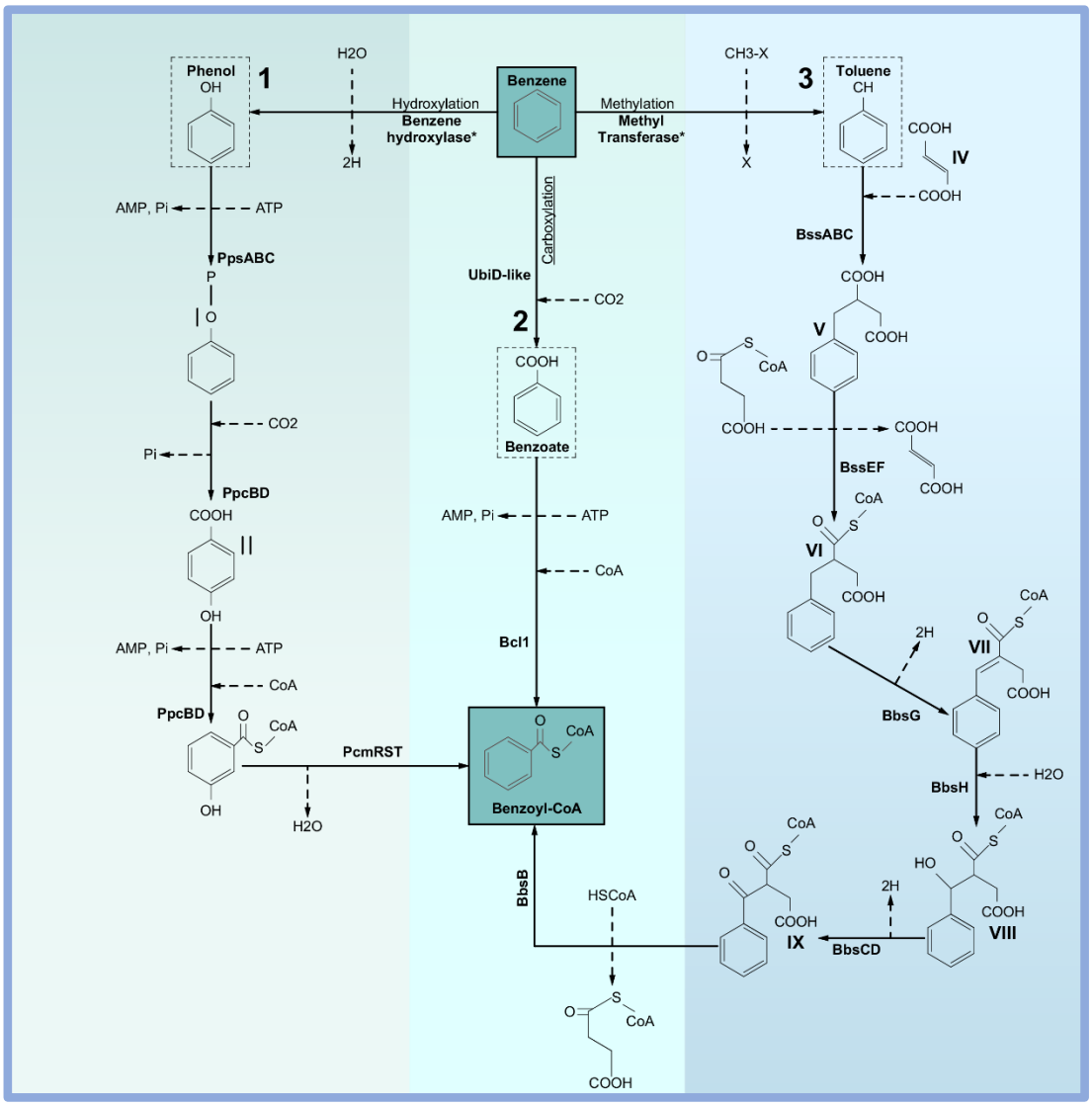


Figure 2. Proposed pathway of benzene degradation via hydroxylation to phenol, methylation to toluene, and carboxylation to benzoate.). PpsABC, ATP-dependent phenylphosphate synthase; PpcBD, phenylphosphate carboxylase; PcmRST, 4-hydroxybenzoyl-CoA reductase; UbiD-like Benzene carboxylase; Bcl1, Benzoate-CoA ligase; BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA (E)-benzylsuccinate CoA transferase; Bbs (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsB, benzoylsuccinyl-CoA

thiolase. Intermediates according to the number shown in the figure in Roman numerals: I. Phenylphosphate II. (4)-hydroxybenzoate III. 4-hydroxybenzoyl-CoA IV. Fumarate V. (R)-benzylsuccinat VI. (R)- benzylsuccinyl-CoA VII. (E)-phenyl-itaconyl-CoA VIII. 2-[hydroxy(phenyl)-methyl]-succinyl-CoA IX. Benzoyl-succinyl-CoA *(permission obtained from Chemosphere).*

1.2.4 Toluene biodegradation pathway

Several studies have revealed that the toluene degradation pathway is primarily activated through fumarate addition (1), specifically the benzyl succinate synthase pathway. This mechanism has been widely observed in the anaerobic biodegradation of toluene by different bacteria with the ability to degrade toluene in the presence of various terminal electron acceptors (TEAs), including nitrate, sulfate, iron, and methane (Blázquez et al., 2018; Gieg and Toth, 2017; Johnson et al., 2023; Tremblay and Zhang, 2020). Figure 3 illustrates the transformation steps that toluene undergoes following the addition of fumarate through a glycyl-radical-driven reaction that targets the central bond of fumarate (VI). Initially, benzylsuccinate (I) is formed by the enzyme benzylsuccinate synthase (bssABC) (Gieg and Toth, 2017; Tremblay and Zhang, 2020). Subsequently, benzylsuccinate CoA transferase (BbsEF) and benzylsuccinyl-CoA dehydrogenase (bbsG) catalyze the formation of benzylsuccinyl-CoA (II) and (E)-(phenyl)itaconyl-CoA (III), respectively (Tremblay and Zhang, 2020). Next, (E)-phenylitaconyl-CoA (III) undergoes hydration and transformation into 2-[hydroxy(phenyl)-methyl]-succinyl-CoA (IV) catalyzed by a phenylitaconyl-CoA hydratase (bbsH) in the presence of a water molecule (Blázquez et al., 2018; Lueders, 2017). The resulting product is then oxidized by a 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase (bbsCD), resulting in the formation of benzoylsuccinyl-CoA (V).

Subsequently, a reaction catalyzed by a benzoylsuccinyl-CoA thiolase (bbsB) replaces the succinyl-CoA moiety of benzoylsuccinyl-CoA with CoA, leading to the formation of benzoyl-CoA (Heider et al., 2016; Lueders, 2017; Tremblay and Zhang, 2020).

Additionally, as it is shown in figure 3, toluene conversion can be activated via two alternative proposed pathways: hydroxylation to benzyl alcohol (2) and para-hydroxylation to cresol (3). In the hydroxylation pathway, toluene metabolism is initiated by the hydroxylation of its methyl group, catalyzed by toluene methyl hydroxylase, resulting in the formation of benzyl alcohol (VII). After that, the transformation of benzyl alcohol (VII) to benzaldehyde (VIII) by the action of a benzyl alcohol dehydrogenase, and after that, the benzaldehyde (VIII) is converted to benzoate (IX) by a benzaldehyde dehydrogenase. Lastly, the synthesis of central aromatic intermediate benzoyl CoA is facilitated by a benzoyl-CoA synthetase.

Likewise, figure 3 illustrates the metabolic pathway of the toluene hydroxylation to p-cresol (3), where toluene is activated by the hydroxylation of the toluene ring at the p-position, with the hydroxyl group derived from water. Subsequently, the oxidation of the methyl group of p-cresol (X) is catalyzed by p-cresol methyl hydroxylase, yielding p-hydroxybenzyl alcohol (XI). Similarly, the resulting p-hydroxybenzyl alcohol is converted to p-hydroxybenzaldehyde (XII) by the same enzyme through a well-characterized reaction in anaerobic microorganisms (Peters et al., 2007; Rabus and Wilkes, 2020). Other proposed subsequent metabolites in this pathway prior to benzoyl CoA are p-hydroxybenzoic acid (XIII) and p-hydroxybenzoyl-CoA (XIV) catalyzed by a p-Hydroxybenzaldehyde dehydrogenase. Nonetheless, the transformation of p-hydroxybenzoic acid (XIII) to benzoate

(IX), another possible intermediate before benzoyl-CoA, may also occur in an alternative pathway (3) (Bonting and Fuchs, 1996).

Although the mentioned pathways have been reviewed in the literature and some intermediate steps have been proposed, further research is required to improve the pathways knowledge. This improvement can be achieved, for example, by the study of the enzymes involved and their characterization. For example, while the subunits of enzymes involved in the fumarate addition (1) pathway, such as benzylsuccinate-CoA transferase, have been identified to be arranged in an α2-β2 structure, but other enzymes like benzylsuccinate synthase (bssABC) have been only partially characterized. Studies have indicated that the ultimate enzyme is structured with three subunits (αβγ)2, wherein the catalytic α-subunit houses a glycyl radical enzyme adjacent to both the glycyl radical cofactor and the active site. However, the functions of the β and γ subunits are not well elucidated. Despite these research gaps, the evidence supporting the existence of a fumarate addition pathway in iron-reducing microorganisms, such as *G. metallireducens*, is strong due to the crucial role of enzymes in the pathway. For example, the importance of benzylsuccinyl-CoA dehydrogenase has been demonstrated by the inability of this strain to transform toluene when the gene bbsG was intentionally downregulated (Chaurasia et al., 2015).

Despite that the alternative pathways 2 and 3 have not been completely proven, enzyme activity in cell extracts has been detected and reported, supporting the existence of the metabolic steps and intermediates discussed earlier. For instance, toluene is degraded via methyl hydroxylation to benzyl alcohol with the denitrifying strain *Thauera aromatica*. However, more research is needed to demonstrate the toluene methyl hydroxylase activity (Durante-Rodríguez et al., 2018).

Regarding evidence of toluene hydroxylation to p-cresol, the presence of small amounts of p-cresol has been detected under methanogenic conditions. Additionally, several studies support the fact that toluene can be degraded via hydroxylation to p-cresol under denitrifying and sulfate-reducing conditions having p-hydroxybenzoic acid as an intermediate.

Further research is required on the three proposed pathways, as none of them can be ruled out. More aspects, such as the slower toluene conversion to p-cresol compared to the rapid toluene breakdown of toluene into benzylsuccinate via fumarate addition is worth to investigate. Similarly, research gaps involving the enzymes and the identification of intermediate compounds need to be addressed.

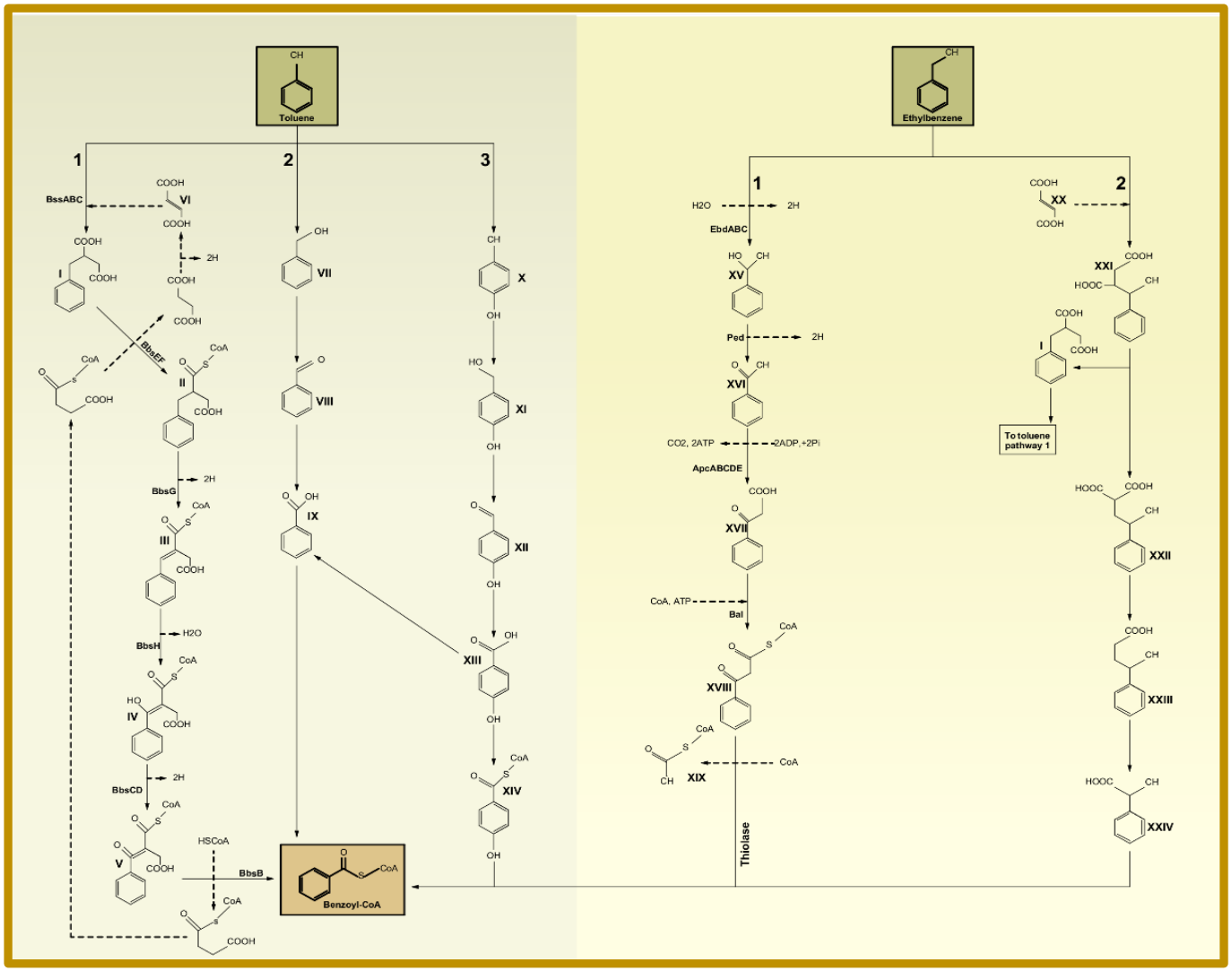

Toluene
Ethylbenzene
1
2
3
BssABC
VI
I
II
BbsEF
BbsG
III
BbsH
IV
BbsCD
V
HSCoA
BbsB
Benzoyl-CoA
VII
VIII
IX
X
XI
XII
XIII
XIV
H2O
2H
EbdABC
XV
Ped
XVI
CO2, 2ATP
2ADP,+2Pi
ApcABCDE
XVII
CoA, ATP
Bal
XVIII
XIX
CoA
Thiolase
XX
XXI
XXII
XXIII
XXIV
To toluene pathway 1

Figure 3. Proposed pathway of toluene degradation via fumarate addition and ethylbenzene degradation via hydroxylation. The intermediates are numbered in Roman numbers: I. Benzylsuccinate II. Benzylsuccinyl-CoA III. (E)-phenylitaconyl-CoA IV. 2-[Hydroxy(phenyl)-methyl]-succinyl-CoA V. Benzoylsuccinyl-CoA VI. Fumarate VII. benzyl alcohol VIII. benzaldehyde IX. benzoate X. p-cresol XI. p-hydroxybenzyl alcohol XII. p-hydroxybenzaldehyde XIII. p-hydroxybenzoic acid XIV. p-hydroxybenzoyl-CoA XV. 1-phenylethanol XVI. Acetophenone XVII. benzoylacetate XVIII. benzoyl acetate-CoA XIX. Acetyl-CoA XX. Fumarate XXI. (1-phenylethyl) succinate; XXII. (2-phenylpropyl)malonate XXIII. 4-phenylpentanoate XXIV. 2-phenylpropanoate (permission obtained from Chemosphere).

1.2.5 Ethylbenzene biodegradation pathway

Although the anaerobic biodegradation pathways of ethylbenzene are still poorly characterized compared to those of toluene, the pathway activated via hydroxylation (1) has been extensively described (Heider et al., 2016; Tremblay and Zhang, 2020). This pathway begins with the hydroxylation of ethylbenzene, which is catalyzed by ethylbenzene dehydrogenase. This enzyme is situated between the outer and the plasma membrane and is characterized as a heterotrimeric molybdenum and heme enzyme (Ghattas et al., 2017; Said et al., 2021). Moreover, the subunits of the ethylbenzene dehydrogenase involved in the direct hydroxylation of ethylbenzene have been identified as (EbdA), (EbdB), and (EbdC) in strains EB1 and EbN1 (Rabus et al., 2016; Weelink et al., 2010).

Figure 3 illustrates the different steps of the hydroxylation reaction of ethylbenzene, providing details such as the hydroxyl group donor (water), and the oxidation of carbon

number one in the methyl group of the side chain, which is facilitated by ethylbenzene dehydrogenase (EbdABC). This reaction produces (S)-1-phenylethanol (XV) as a metabolite (Gieg and Toth, 2017; Wartell et al., 2021). The alcohol group in 1-phenylethanol (XV) is further converted into acetophenone (XVI) by (S)-1-phenylethanol dehydrogenase (Ped) (Höffken et al., 2006). This enzyme is well-documented and categorized within the NAD-dependent alcohol dehydrogenase family, specifically identified as a member of the short-chain alcohol dehydrogenase/aldehyde reductase family. The subsequent metabolism of acetophenone (XVI) is mediated by a complex called acetophenone carboxylase, which consists of five subunits (ApcABCDE). This complex catalyzes an ATP-dependent carboxylation reaction, resulting in the formation of benzoyl acetate (XVII) (Jobst et al., 2010; Tremblay and Zhang, 2020). The presence of acetophenone carboxylase in this pathway was indirectly confirmed by demonstrating the dependence of ethylbenzene and acetophenone degradation on carbon dioxide (CO2). Further biodegradation of benzoylacetate is depicted in Figure 3, where the subsequent reactions leading to the formation of benzoylacetate-CoA (XVIII) are shown. This transformation is facilitated by a type of benzoylacetate-CoA ligase known as (Bal). Lastly, the benzoyl acetate-CoA is cleaved by thiolysis to the central metabolic intermediates acetyl-CoA (XIX) and benozoyl-CoA (Weidenweber et al., 2017).

Alternatively, the metabolic pathway of ethylbenzene activated via fumarate addition (2) has been unsuccessfully described compared to the previously discussed oxygen-independent hydroxylation pathway (Wartell et al., 2021). The fumarate addition pathway in ethylbenzene was initially documented in the strain EbS7 under sulfate-reducing conditions. This strain activated its ethylbenzene metabolism by introducing fumarate to its second carbon, forming

1-phenylethyl-succinate (XXI). The subsequent catabolism of 1-phenylethyl-succinate is depicted in Figure 3, where the carbon skeleton rearrangement and decarboxylation reactions leading to the formation of the intermediates; (2-phenyl propyl) malonate (XXII), 4-phenyl pentanoate (XXIII) and 2-phenylpropionate (XXIV), are illustrated (Heider and Schühle, 2013; Weelink et al., 2010; Yoshikawa et al., 2017).

Furthermore, authors such as Gieg and Toth, (2017) have postulated that, after the creation of 1-phenylethyl-succinate (XXI), the metabolic pathway resembles that of n-alkanes, deviating from the conventional toluene pathway. However, evidence suggests that the addition of fumarate as the initial reaction in the ethylbenzene biodegradation is analogous to the anaerobic catabolic pathways of toluene and xylenes. For example, (R)-benzyl succinate has been detected as an intermediate in both m-xylene and ethylbenzene biodegradation, suggesting the presence of the benzyl succinate synthase (bss) and a possible connection to the fumarate addition pathway of toluene (Boll et al., 2018a).

More studies are indeed required to identify the different types of benzyl succinate synthases isoenzymes that may catalyze intermediate steps and to understand their substrate recognition abilities (Heider et al., 2016). Additionally, although it is generally believed that the preference for either hydroxylation (in denitrifying bacteria) or fumarate addition (in sulfate-reducing bacteria) in ethylbenzene biodegradation is determined by the physiology of the bacterial strain, where more research efforts are important to shed light and clarify the factors that determined which metabolic pathway is followed. Another remaining question in ethylbenzene metabolism is whether fumarate addition occurs exclusively under unfavorable bioenergetic conditions, especially when sulfate reducer bacteria are present, as the redox potential of the first intermediate, phenylethanol, becomes highly positively charged to

enable efficient electron transfer to the electron acceptor. This aspect requires further investigation to determine the specific conditions and the factors that influence the preference for fumarate addition in ethylbenzene metabolism. In addition, more studies are necessary to confirm whether the central intermediate (benzoyl-CoA) from ethylbenzene is also part of ethylbenzene metabolism under anaerobic conditions via fumarate addition with sulfate as an electron acceptor. These studies would involve investigating the intermediate reactions, identifying the enzymes involved, and elucidating the genes responsible for encoding these enzymes. Addressing these research gaps can bring more understanding of the biochemical processes involved in ethylbenzene metabolism and the factors that govern the choice of metabolic pathways.

1.2.6 Xylene biodegradation pathway

Several authors have pointed out that the anaerobic biodegradation pathways of xylenes are activated through fumarate addition, sharing similar reactions and enzymes with toluene anaerobic catabolism (Tremblay and Zhang, 2020; Yoshikawa et al., 2017). For example, the presence of benzyl succinate synthase (bss) has been identified in various xylene-degrading cultures using bssA-targeted primers, suggesting that this enzyme catalyzes the fumarate addition to the methyl group of xylenes. Evidence supporting the similarity between xylene and toluene biodegradation pathways includes the detection of methyl benzyl succinate as an intermediate in o-xylene-degrading cultures and the strain UKTLT, indicating that o-xylene is converted via a mechanism related to toluene biodegradation (Kunapuli et al., 2010). Additionally, different authors have reported the presence of closely related fumarate addition metabolites such as 4-methyl benzyl succinic acid, and 4-methyl phenyl itaconic acid, using diverse toluene-degrading bacteria including *Thauera aromatica, Azoarcus sp*.

and *Georgfuchsia sp*, which support an analogous biodegradation pathway (Rotaru et al., 2010; Sperfeld et al., 2018). Likewise, Godin et al., (2020) reported enzymes closely related to benzylsuccinate synthase (bss isoenzymes) that are involved in the initial fumarate-dependent activation of m-xylene biodegradation. In addition, the presence of this type of bss isoenzymes facilitates the conversion of xylenes to methyl benzyl succinic acids and the formation of intermediates such as m-, o-, or p-Toluic acids (Godin et al., 2020; Heider and Schühle, 2013). These findings suggest a common mechanism of activation for xylenes and toluene anaerobic biodegradation pathways through fumarate addition, although further research is needed to fully understand the specific enzymes involved and their genetic regulation in xylenes metabolism.

Figure 4 shows the fumarate-dependent activation step in the biodegradation pathways of xylene isomers and the different downstream metabolites such as benzylsuccinic acids, phenylitaconic acids and toluic acids that ended up in the central aromatic intermediate benzoyl-CoA.

The additional reaction of fumarate in the m-xylene pathway forms 3-methyl benzyl succinic acid (I) where degradation is initiated by a type of benzyl succinate dehydrogenase yielding 3-methyl phenyl itaconic acid (II). The resulting 3-methyl phenyl itaconic acid (II) can be transformed into either 3-methyl benzoyl CoA (XXVI) or 3-methyl benzoyl succinic acid (III). The intermediate 3-methyl-benzoyl-CoA (XXVI) can be formed after the hypothesized CoA-thioesterification of methylphenylitaconic acid (II) and catalyzed by a benzyl succinate CoA transferase isoenzyme. Moreover, the remaining 3-methyl-benzoyl-CoA (XXVI) activation is carried out by an ATP-dependent 3-methyl benzoyl-CoA reductase, and then it breaks down until its further mineralization to carbon dioxide.

The methylphenylitaconic acid (II) is converted into 3-methylbenzoylsuccinic acid (III), and subsequently transformed into m-toluic acid (IV). Nonetheless, it may be possible for methylphenylitaconic acid (II) to be directly metabolized into m-toluic acid (IV). Afterward, m-toluic acid (IV) may follow two different pathways of transformation to form the central aromatic metabolite benzoyl-CoA. One of the two possible pathways consists of the conversion of m-toluic acid (IV) to methyl-salicylic acid (V) prior to benzoylCoA. Another alternative is the formation of 3-carboxy benzyl succinic acid (VI), isophthalic acid (VII) and benzoic acid (VIII) after its utilization.

In addition, direct toluate conversion into benzoic acid (VIII) is also considered a significant step in toluate conversion into either benzoic acid (VIII) since it is a widely reported intermediate for the anaerobic alkylbenzene metabolism. Figure 4 illustrates the synthesis of 2-methyl benzyl succinic acid (IX) following fumarate addition, a process mediated by a benzyl succinate synthase-like enzyme, particularly in the context of o-xylene. Subsequently, a benzyl succinate dehydrogenase isoenzyme catalyzes the conversion of 2-methyl benzyl succinic acid (IX) to 2-methyl phenyl itaconic acid (X), where the succinyl group is likely replaced by succinyl-CoA. The metabolization of 2-methylphenylitaconic acid (X) leads to two possible downstream intermediates: 2-methyl-benzoyl-CoA (XXV) and 2-methylbenzoylsuccinic acid (XI). For example, the 2-methyl-benzoyl-CoA (XXV) metabolite may be either converted to benzoyl-CoA or undergo a series of intermediary steps such as direct aromatic ring reduction, hydration-dehydrogenation, ring cleavage and β-oxidation until carbon dioxide. Moreover, the formation of 3-o-toluyl propionic acid (XII) might be possible due to the decarboxylation of 2-methyl benzoyl succinic acid (XI), and this is followed by its transformation into o-toluic acid (XIII). However, it can be hypothesized

that the formation of the central intermediate o-toluic acid (XIII), which is commonly detected at high concentrations in contaminated sites, may be possible from the direct metabolization of either (XI) or (XII). Thereafter, the toluic acid biodegradation might follow three alternative pathways: dehydration to methyl-salicylic acid (XV), the formation of phthalic acid (XIV) and its transformation to benzoate (XVI). It must be clarified that it has also been reported a direct metabolization of toluates to benzoate prior to benzoyl-CoA is a possibility.

The benzyl succinic and phenylitaconic acids derivates, such as 4-methyl benzyl succinic acid (XVII), 4-methylphenylitaconic acid (XVIII), and 4-methyl benzyol succinic acid (XIX), are formed after the activation of p-xylene through fumarate addition. Figure 4 illustrates the downstream products found in the p-xylene biodegradation pathway, starting from p-toluic acid (XX) and progressing to 4-methyl-benzoyl-CoA (XXIV) until further degradation to benzoyl-CoA. Similarly, an analogous pathway is depicted for o-xylene and p-xylene, including B-oxidation steps corresponding to their methylbenzoyl-CoA intermediates (XXIV and XXV) before ring reduction. It should be noted that many of these intermediates are common for all three xylene isomers and may be dead-end products for certain microorganisms, while may be utilized as substrates for other strains. An example of this is the methyl benzyl succinic acids which are thought to be co-metabolic with xylenes and may contribute to their relative resistance to biodegradation under specific field conditions and in the presence of indigenous bacteria species. Therefore, further research on this matter would be important to better understand the fate of these intermediates.

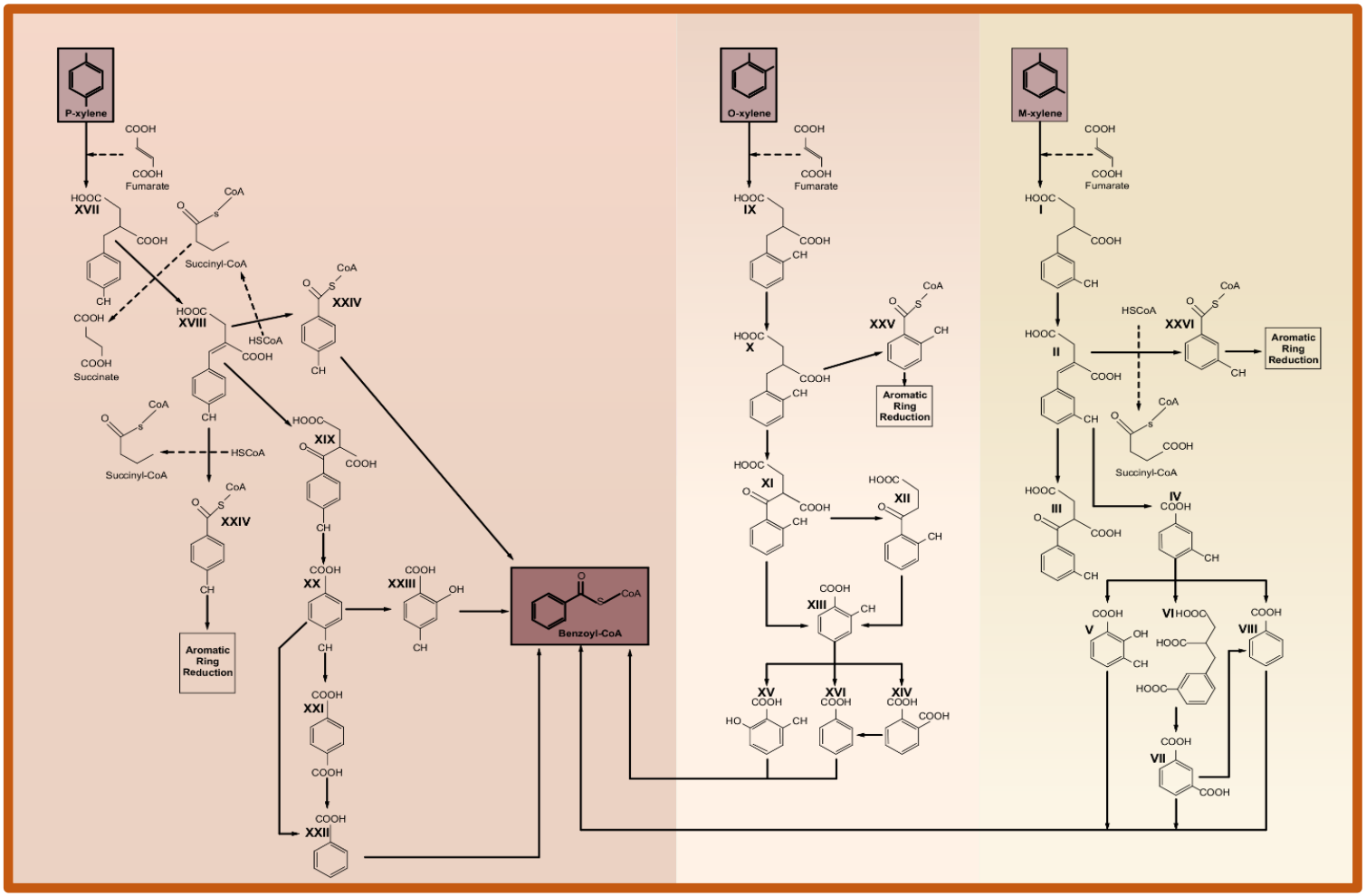

P-xylene
O-xylene
M-xylene
Fumarate
Succinyl-CoA
Succinate
HSCoA
Aromatic Ring Reduction
Benzoyl-CoA
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
IX
X
XI
XII
XIII
XIV
XV
XVI
XXV
I
II
III
IV
V
VI
VII
VIII
XXVI

Figure 4. Proposed pathway for anaerobic biodegradation of xylene isomers (o-xylene, m-xylene, and o-xylene). The intermediates are numbered in Roman numbers: I. 3-methylbenzylsuccinic acid II. 3-methylphenylitaconicacid III. 3-methylbenzoylsuccinic acid IV. m-toluic acid V. methyl-salicylic acid VI. 3-carboxybenzylsuccinic acid VII. isophthalic acid VIII. benzoic acid IX. 2-methylbenzylsuccinic acid X. 2-methylphenylitaconic acid XI. 2-methylbenzylsuccinic acid XII. 3-o-toluyl propionic acid XIII. o-toluic acid XIV. Phthalic acid XV. methyl-salicylic acid XVI. benzoic acid XVII. 4-methylbenzylsuccinic acid XVIII. 4-methylphenylitaconic acid XIX. benzyolsuccinic acid XX. p-toluic acid XXI. terephthalic acid XXII. benzoic acid XXIII. salicylic acid. XXIV 4-Methyl-Benzoyl-CoA. XXV 2-Methyl-Benzoyl-CoA XXVI 3-Methyl-Benzoyl-CoA (*permission obtained from Chemosphere*).

1.3 Proposed Research

1.3.1 Research Gaps

Biological approaches like bioremediation offer an alternative less energy-intensive, and less costly. Likewise, bioremediation offers more specificity towards the contaminant, biodiversity protection and reliability during the removal of BTEX. Several studies have explored the use of single bacteria to target BTEX compounds, converting them into safer substances like carbon dioxide, pyruvate or acetyl CoA (Wongbunmak et al., 2020). Particularly, strains from the *Pseudomonas*, *Streptomyces*, *Microbacterium*, *Bacillus*, *Acinetobacter, Serratia* and *Rhodococcus* genres have been proven as successful BTEX degraders (Chicca et al., 2020; Hocinat et al., 2020; Wongbunmak et al., 2020). For instance, recent studies have reported the potential of *Microbacterium esteraromaticum* to degrade up

to 72 % of BTEX (Kaur et al., 2023). Likewise, Serratia sp. is also capable of breaking down BTEX up to 86% in 21 days (Yavas & Icgen, 2018). Despite the capability of single bacteria such as *Serratia sp*. and *Microbacterium sp*. degrades BTEX, its use presents challenges:

- Limited production and activity of BTEX-degrading enzymes.
- Low tolerance towards environmental changes (temperature, pH, oxygen).
- The performance of a single microorganism for BTEX degradation can be strongly compromised above the average BTEX EPLs in heavily polluted aquifers (>200 mg of BTEX/L).
- Time is an important constraint when it comes to bioremediation using single bacteria, as it can take several days to degrade stable compounds like benzene.
- On-site degradation in polluted sites with low and high oxygen levels as single bacteria is limited to either aerobic or anaerobic degradation.

A potential alternative to address these challenges during BTEX degradation, is the use of a coculture-based biodegradation process. *Using bacterial cocultures for specific compound degradation can provide a more flexible alternative in bioremediation*. Coculturing a facultative anaerobe such as S. *fonticola* with an aerobic such as M. *esteraromaticum*, can be an example of this flexibility. Moreover, coculture offers a more robust tolerance and enzymatic capacity, ultimately enhancing the degradation rates and reducing its duration. This is the first study of a coculture trying to enhance BTEX degradation and offering a competitive removal of BTEX.

1.3.2 Objectives

To screen and understand the potential of a coculture between two known BTEX-degrading bacteria, specific objectives have been identified as follows:

- **Objective I**. Study the tolerance and degradation ability of a bacterial coculture between *S. fonticola* and *M. esteraromaticum* towards BTEX.

  Hypothesis 1: The coculture displays increased tolerance toward BTEX due to the activation of complementary oxidative stress response mechanisms.

  Hypothesis 2: The combined metabolism of the coculture allows for more efficient utilization of BTEX in single and multi degradation compound.

- **Objective II**. Screen the effect of benzyl alcohol in the activity and production of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase using the same bacterial coculture.

  Hypothesis 1: Benzyl alcohol upregulates specific transcription factors involved in the metabolism of AHs in both strains, enhancing the activity of competent BTEX-degrading enzymes such as catechol 1,2-dioxygenase and catechol 2,3-dioxygenase.

1.3.3 Novelty

This project introduces a unique microbial coculture between *Serratia fonticola* and *Microbacterium esteraromaticum* as a solution for the slow and ineffective biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) in groundwater. Unlike traditional single-strain or physicochemical approaches, this coculture's ability to degrade BTEX compounds more efficiently is particularly suitable for sensitive environments where pollutant degradation must balance speed with ecological safety.

One novel aspect is the potential for enhanced co-metabolism, where the interaction between the two strains produces degradation intermediates that activate additional metabolic pathways. This effect is particularly significant in toluene and xylene degradation, facilitated by the coculture's increased synthesis of BTEX-degrading enzymes, such as dioxygenases. Furthermore, the coculture's tolerance to oxidative stress induced by BTEX toxicity and hydrogen peroxide (H_2O_2) suggests a potential application in environments with fluctuating contaminant levels and diverse hydrocarbon pollutants.

By advancing the understanding of microbial tolerance levels and co-metabolic interactions in BTEX degradation, this project opens *new approaches for more effective and eco-friendly bioremediation techniques, providing a critical advantage in the polishing step of the treatment of BTEX-contaminated water bodies*.

Chapter 2: Materials and Methods

2.1 Microorganism, preservation, and inoculum preparation

S. fonticola and *M. esteraromaticum* were isolated from the crude oil-contaminated site in Toronto, Canada dedicated to the generation and supply of electricity from geothermal energy. Both strains were preserved in Petri plates containing tryptic soy agar (TSA) (casein peptone 15 g/L soy peptone 5 g/L; sodium chloride 5 g/L; agar 15 g/L). Subsequently, the strains were grown in tryptic soy broth (TSB) after pre-culturing for three generations at 1% v/v as part of the inoculum preparation in a shaker incubator (Multitron Standard, Switzerland) at 180 rpm and 30±1°C for 12 hours. Individual inoculums were adjusted equally for each strain and the final inoculum was set up by combining the volumes of the same optical density of individual strains to achieve a consistent initial cell density (OD_{600nm} of 0.5) in a 1:1 ratio.

2.2 Flask biodegradation test

To study the biodegradation ability of the coculture between *S. fonticola* and *M. esteraromaticum*, batch treatments were set in 100 ml serum bottles containing 15 ml of TSB media supplemented individually with 50 mg/L of benzene, xylene, toluene and ethylbenzene or with a total 200 mg/L of all the BTEX compounds (1:1:1:1 ratio). Each serum bottle was inoculated with 0.45 ml (M. *esteraromaticum*) and 0.50 ml (S. *fonticola*) to achieve an initial OD_{600nm} of 0.1. The degradation test was performed at 30°C and 180 rpm for 42 hours. The coculture and the strains were grown individually without BTEX addition and considered positive controls. To determine the volatilization grade and culture media effect on BTEX

degradation, serum bottles with just culture media and culture media combined with BTEX (abiotic controls) were utilized as negative controls. All treatments were performed in duplicates.

2.3 Enzyme Production

To study the effect of benzyl alcohol as an inducer of the catabolism of the different individual BTEX compounds, the enzyme activity of C1,2O and C2,3O in coculture was measured. C1,2O and C2,3O activity were indicators of stress-induced BTEX co-metabolism in the coculture after supplementing with different concentrations of benzyl alcohol (0.25, 1 and 3 mM). Batch treatments were set in 100 ml serum bottles containing benzyl alcohol, and 15 ml of TSB media supplemented with 200 and 500 mg/L of benzene, xylene, toluene and ethylbenzene. All the treatments were conducted in duplicates.

2.3 Enzyme Extraction

Cells were harvested by centrifugation at 4500rpm for 15 min. The pellet was then washed with 50 mM phosphate buffer (pH 7.5) and resuspended on 2 ml of the same buffer solution (Lee et al., 2022). Then, cells were disrupted by sonication treatment at 25 KHz with 30-s intervals of sonication (3 min) and 30-s intervals of interruption for a total of 6 min in an ice-cooled bath using a vibra-cell ultrasonic cell processor (Karigar et al., 2006; Manohari and yogalakshmi., 2016). Pellets were removed by centrifugation at 10000rpm for 20 min at 4°C and the supernatant obtained was used as the cell-free extract for subsequent enzyme assays.

2.4 Enzyme assays: Catechol 1,2-dioxygenase and Catechol 2,3-dioxygenase

Bacterial cells were harvested by centrifugation at 5000× g, 4° for 10 min, and then washed twice with 20 mM phosphate buffer (pH 7.0) and resuspended in the same buffer. This suspension was used to assay C12O and C23O. Cells were disrupted using a sonifier at 30kHz for 5 min. The cellular lysates were centrifuged at 12000rpm for 5min, and the supernatant containing the cellular lysate was used for the enzyme assays. The reaction mixture (total 3.0 mL) contained 2.0 mL phosphate buffer (7.5), 0.6 mL 1 mM catechol, 0.2 mL deionized water and 0.2 mL cellular lysates. The reaction was allowed to proceed at 22 °C for 1 min after preincubation in water bath and the addition of the cell lysate. C12O and C23O activities were determined by measuring the production of cis-cis muconic acid at 260 nm and 3-methylcatechol at 388 nm, respectively. Protein concentration was determined according to Bradford method (Li et al., 2014; Fujisawa., 1970).

One unit of dioxygenase activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min (Rodriguez-Salazar et al., 2020). All experiments were carried out in duplicate. Specific activity was defined as the amount of activity required to convert 1nmol of substrate per min per mg of protein.

A control sample contained 0.1 ml of the buffer used for enzyme preparations, instead of enzyme.

2.5 Analytical Methods

2.5.1 Microbial growth and substrate consumption

The microbial growth was monitored by measuring optical density at 600_{nm} using a spectrophotometer Genesys 50 (Thermo Fisher Scientific, United States). Bacterial cells were harvested by centrifugation (2 min at 12,000 r.p.m., room temperature), and the liquid supernatant was used as blank. The cell pellet was resuspended before OD600nm was measured. This procedure was repeated for each sample. Likewise, the microbial biomass was determined gravimetrically by dry cell weight (DCW). Briefly, samples were centrifuged at 11,260 *x* g for 2 minutes at room temperature. Subsequently, the supernatant was stored (4°C) for glucose analysis at 60±1°C, until constant weight.

2.5.2 BTEX degradation

BTEX concentrations were determined with an Agilent 7820A GC system equipped with a GC-MS/Clarus-500 headspace (Perkin-Elmer, United States) and an Agilent-J&W/CP-Sil5CB column (30 m x 0.25 mm x 1.00 µm). The headspace system was maintained at 90±1°C and 120±1°C for the needle and transfer line, respectively. The sample was heated for 10 minutes at 80±1°C before injection. To prepare the samples for BTEX analysis, 20 ml GC-headspace vials were used containing 100 µL of the sample, 5 ml of distilled water and 30 µL of fluorobenzene-D5 (4 ppm, diluted in methanol) as internal standard. All compounds were identified and quantified by comparing them with a Combined Purgeable Internal Surrogate Mixture (Canadian Life Science, Canada).

2.5.3 Protein Determination

The protein content in the crude enzyme was determined by the method of Bradford using bovine serum albumin (BSA) as the standard and reading the absorbance at 595 nm using a spectrophotometer ThermoFisher (Bradford, 1976).

Chapter 3: Study of the biodegradation ability and tolerance of a coculture between S. *fonticola* and M. *esteraromaticum* for the single-compound and multi-compound removal of benzene, ethylbenzene, xylene and toluene.

(Submitted to Science of the Total Environment).

3.1 Introduction

The ever-expanding and dynamic economy has led to a consistent rise in oil-derivates production such as benzene-toluene-ethylbenzene-xylene (BTEX). BTEX compounds, commonly found in storage tanks, pipelines, and gas stations, raise environmental concerns due to their ability to leak and potentially contaminate soil and groundwater. While these compounds can evaporate, some persist in the topsoil and may migrate downwards, impacting subsoil and groundwater (Haiying et al., 2021). Groundwater, comprising only 2% of Earth's water resources, provides around 30% of the world's DW. Likewise, millions of people rely on it for domestic, industrial, and agricultural needs. Concerns arise from the potential spread of BTEX-contaminated groundwater through natural water cycles, reaching groundwater wells and fresh surface water bodies, such as lakes and rivers. Humans and wildlife can be easily exposed and negatively impacted when DW containing BTEX which has been classified as a serious hazardous chemical. BTEX has been the subject of studies during the last few years due to its significant health risks including reproductive disorders, anemia, neurotoxicity, and cancer (Latif et al., 2019). Multiple methods exist to address BTEX contamination in aquifers, including thermal (incineration), physical (pump-and-treat)

and chemical (AOPs) approaches (Hernández-Ospina et al., 2024). However, these methods have disadvantages such as being energy-intensive, incurring high cost and maintenance, lack of specificity, and additional costs related to the transfer, treatment, and disposal of contaminants. Consequently, attention has turned to more responsible nature-based solutions such as bioremediation. This method employs bacteria to target BTEX compounds, converting them into safer substances like carbon dioxide, pyruvate or acetyl CoA (Wongbunmak et al., 2020). Particularly, certain strains from the *Pseudomonas, Streptomyces, Microbacterium, Bacillus, Acinetobacter, Serratia* and *Rhodococcus* genres have been proven as successful BTEX degraders (Chicca et al., 2020; Hocinat et al., 2020; Wongbunmak et al., 2020). Recent studies have reported the potential of *M. esteraromaticum* to degrade up to 72 % of BTEX (Kaur et al., 2023). Likewise, *Serratia* sp. is also capable of breaking down BTEX by up to 86% after 21 days (Yavas & Icgen, 2018). Despite the capability of single bacteria to degrade BTEX, its use presents important challenges such as limited activity and production of competent enzymes, and low tolerance towards environmental changes (temperature, pH, and oxygen concentration). Likewise, the growth of a single strain in degradation can be restricted to the concentration of BTEX below actual EPLs in heavily polluted aquifers (>200 mg L^{-1}). Moreover, time is an important constraint when using single bacteria during the bioremediation process, as it can take several days (up to 21 days) to degrade stable compounds such as benzene in groundwater. A potential alternative to the challenges during BTEX degradation is the use of a coculture-based degradation bioprocess. Using bacterial cocultures for specific compound degradation provides a more robust tolerance to the contaminant and enhances enzymatic activity, ultimately enhancing the degradation and reducing the overall process time. This approach can enhance the removal through co-metabolism, where the degradation intermediates

produced in the process help stimulate further breakdown of BTEX compounds. Likewise, this alternative may offer greater flexibility for on-site degradation due to the oxygen availability as single bacteria is limited to either aerobic or anaerobic degradation (Nagarajan and Loh, 2015).

Studies for coculture *M. esteraromaticum* and *S. fonticola* have shown the great potential that these two microbes must degrade AHs. These strains can complement each other in coculture, as they can be specialized for BTEX compounds. For example, *M. esteraromaticum* has an important tolerance and degradation ability towards ethylbenzene and toluene, whereas *Serratia sp.* has shown a preference towards benzene (Kaur et al., 2023; Avanzi et al., 2015). Likewise, a coculture between the two strains can offer a wider range of applications for different BTEX concentrations. *Serratia* sp. has been able to tolerate and degrade high concentrations of monoaromatic hydrocarbons (up to 500 mg/L); however, *Microbacterium* sp. seems to perform better in lower concentrations (up to 200 mg/L) (Singh et al., 2017; Kaur et al., 2023). Both microorganisms can produce diverse BTEX-degrading oxidases. For example, *Microbacterium* sp. produced enzymes for BTEX degradation like toluene 4-monooxygenase, toluene dioxygenase, ethylbenzene dioxygenase and *Serratia* sp. produced catechol 1,2 dioxygenase and catechol 2,3-dioxygenase.

Finally, based on the type of respiration a coculture between *M. esteraromaticum* (aerobic) and *S. fonticola* (facultative anaerobic) can show potential for batch injection in polluted sites with low and high oxygen levels, and in heavy and non-heavy contamination levels. This study aims to explore the BTEX degradation using a bacterial coculture of *Microbacterium esteraromaticum* and *Serratia fonticola*.

3.2 Results and Discussion

3.2.1 Coculture Performance

3.2.1.1 Energy Consumption

The glucose present in the culture media was consumed in all the treatments by *M. esteraromaticum*, *S. fonticola* and the coculture within the first 12 hours of fermentation except for toluene and ethylbenzene which took up to 18 hours to be consumed by the three BTEX degraders. Within the first 6 hours of cultivation, the maximum glucose consumption (95%) was achieved by the coculture in the BTEX mixture treatment. A rapid decrease in glucose concentration was also observed within the same cultivation time (6 hours) in the benzene and xylene treatments for coculture (76% and 90%, respectively). In the treatment with toluene, coculture and M. *esteraromaticum* had the highest glucose consumption (70% and 74%, respectively) which was higher compared to S. *fonticola* with only 8% of glucose consumed within the first 6 hours.

Likewise, in the toluene treatment, *S. fonticola* showed an increase in glucose consumption, rising from 8% to 96% between 6 to 12 hours of cultivation. A similar pattern was observed for the ethylbenzene treatment in *M. esteraromaticum*, *S. fonticola* and the coculture, with 81% glucose consumption within the first 12 hours. However, after 12 hours, the consumption rate dropped, with only 14% of the glucose being used between 12 and 18 hours of cultivation.

The low glucose consumption (6%) observed in the treatment with toluene for coculture within the first 6 hours, and the time for *M. esteraromaticum* and coculture to finish the

glucose consumption in the treatment with toluene and ethylbenzene can be due to a disruption of the Krebs cycle (Cindy A et al., 2024). The formation of intermediates from the degradation of toluene and ethylbenzene can negatively impact the function or inhibit enzymes from the Krebs cycle, slowing down cellular respiration and ATP production which can be reflected in longer consumption times (Wang et al., 2019). Contrary to the treatments with toluene and ethylbenzene, the treatments with xylene and BTEX mixture had the same glucose consumption rate as that of the control with no alteration in the glucose consumption patterns.

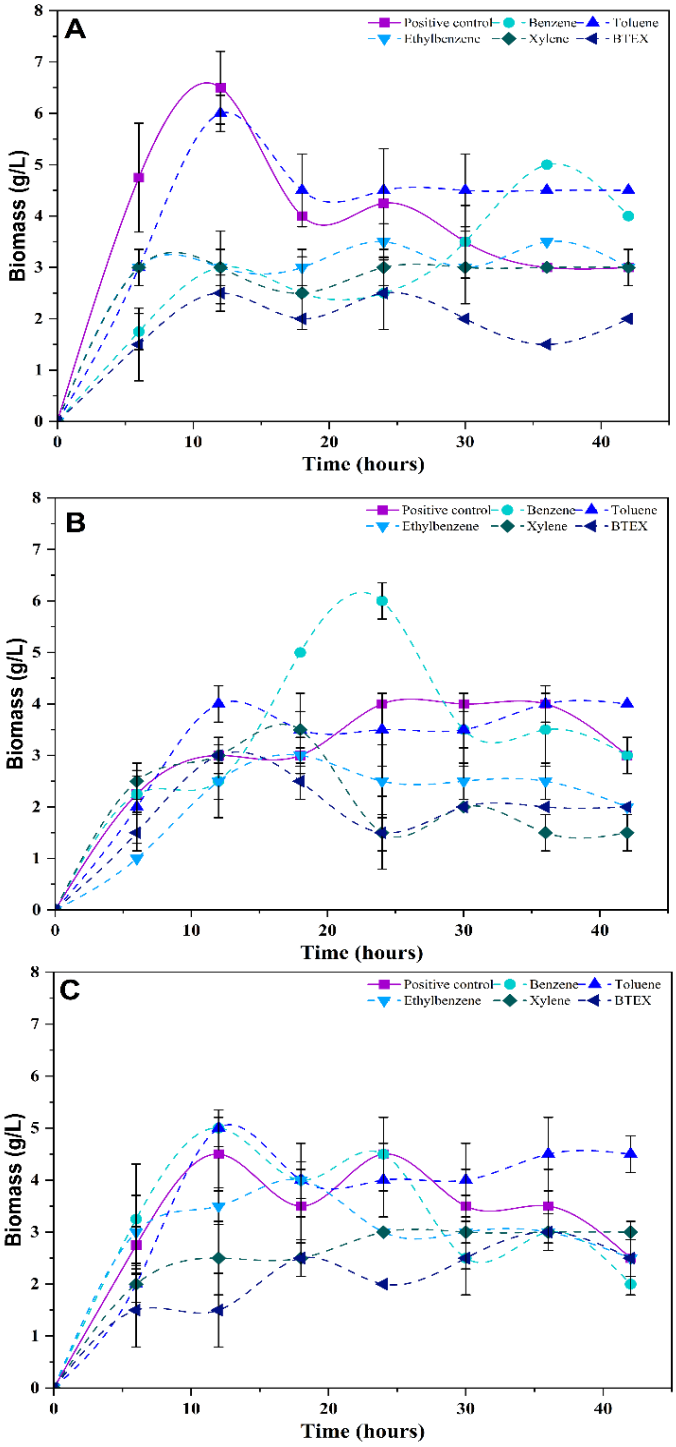


Figure 5. Biomass cell production during BTEX degradation compounds by A) Coculture, B) *S. fonticola,* and C) *M. esteraromaticum*.

3.2.1.2 Biomass growth

Figure 5 shows the microbial growth during BTEX degradation by *M. esteraromaticum*, *Serratia fonticola* and the coculture. As shown in Figure 5A, the coculture exhibited the highest biomass production in the treatment with toluene and benzene coculture (6 ± 0.35 g L^{-1}) and (5 ± 0.35 g L^{-1}) at 12 and 36 hours, respectively. In contrast, treatments with ethylbenzene, xylene and the BTEX mixture resulted in similar biomass levels than individual strains, averaging (3.5±0.70 g L^{-1}; 3±0.70 g L^{-1} and 2.5±0.70 g L^{-1}) without significant changes after 24 hours.

In Figure 5B, *S. fonticola* achieved the highest biomass in the treatment with benzene (6±0.41 g/L) outperforming the positive control and the treatments with toluene, ethylbenzene, xylene and BTEX. Although not as high as the growth performance in the treatment with benzene, *S. fonticola* growth in the treatment with toluene (4±0.35 g L^{-1}) was the most similar to the positive control (4.25±0.21 g L^{-1}). The biomass production for *S. fonticola* remained lower for the treatments with ethylbenzene, xylene and BTEX mixture (3±0.7 g L^{-1}, 3.5±0.7 g L^{-1} and 3±0 g L^{-1}, respectively), stabilizing after 18 hours of cultivation.

Figure 5C shows the growth performance of *M. esteraromaticum* with biomass production in benzene of 5±1.4 g L^{-1} and 5±0.35 g L^{-1} in toluene, respectively at 12 hours, both exceeding the positive control (4.5±0.7 g L^{-1}). While the treatment with ethylbenzene yielded lower biomass (4 ± 0.70 g/L), it still surpassed the treatments with xylene (3 ± 0.7 g L^{-1}) and BTEX mixture (3 ± 0 g L^{-1}).

When compared to other BTEX-degrading strains, the coculture used in this study demonstrated a stronger growth performance than *Arthrobacter ramosus* S2TR-06, a strain

previously reported by Miri et al., (2023). For instance, the coculture achieved 24% and 8% higher microbial growth than *A. ramosus* S2TR-06 in treatments with toluene and benzene, respectively, at similar concentrations (50 mg L^{-1}). However, in the xylene treatment, *A. ramosus* S2TR-06 showed 50% higher biomass production than the coculture.

These differences in microbial growth can be also attributed to differences in metabolic activity and due to less formation of toxic intermediates derived from the action of BTEX degrading enzymes (e.g. oxygenases). A. *ramosus* S2TR-06 has less formation of toxic metabolic intermediates in the treatment with xylene, and coculture in the treatments with benzene and toluene.

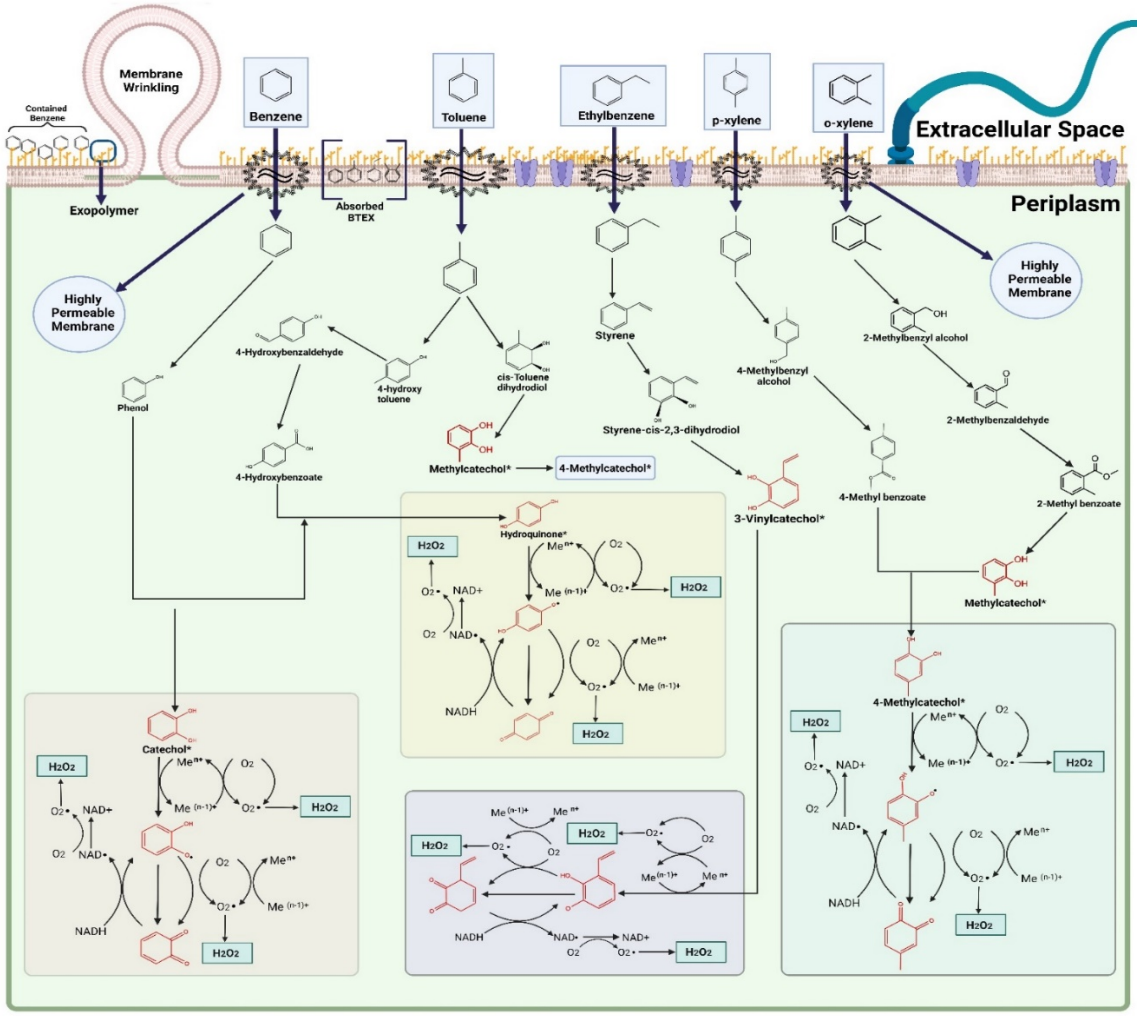

Membrane Wrinkling
Contained Benzene
Exopolymer
Benzene
Toluene
Ethylbenzene
p-xylene
o-xylene
Extracellular Space
Absorbed BTEX
Periplasm
Highly Permeable Membrane
Highly Permeable Membrane
4-Hydroxybenzaldehyde
4-hydroxy toluene
cis-Toluene dihydrodiol
Styrene
4-Methylbenzyl alcohol
2-Methylbenzyl alcohol
Phenol
2-Methylbenzaldehyde
Styrene-cis-2,3-dihydrodiol
4-Hydroxybenzoate
Methylcatechol*
4-Methylcatechol*
3-Vinylcatechol*
4-Methyl benzoate
2-Methyl benzoate
Hydroquinone*
Methylcatechol*
Catechol*
4-Methylcatechol*
H2O2
NAD+
NADH
O2
Me n+
Me (n-1)+

Figure 6. Proposed pathway of the benzene, toluene, ethylbenzene, and xylene biodegradation by *S. fonticola* and *M. esteraromaticum*.

Despite the detrimental effects of BTEX metabolic intermediates, in this study, the microbial coculture shows tolerance to all four compounds. Figure 6 shows a proposed pathway of intermediate formation in *S. fonticola* and *M. esteraromaticum* after the BTEX uptake. The presence of toxic intermediates (catechol and benzoate) has previously been reported to cause growth inhibition in microorganisms such as the algae *Parachlorella kessleri* in BTEX degradation tests even at low concentrations (100 μg L^{-1}) (Takáčová et al., 2015). In addition, catechol and benzoate-derivates can generate active oxygen species (AOS) like H_2O_2 on a small scale and at controlled levels within the cell. The H_2O_2 accumulation can result in the oxidation/carbonylation of amino acids (histidine, cysteine, arginine) in essential large ribosomal subunit protein complexes containing uL10 and bL12 proteins). UL10 proteins are responsible for recruiting G proteins to the ribosome, which act as molecular switches for the translation of proteins. Likewise, bL12 protein is part of a structural protein complex (L7/12 stalk) in the ribosome that plays a crucial role in the activation of key translation factors needed for the initiation, elongation and release phases of mRNA translation into proteins. Moreover, bL12 protein plays an important role in the energy supply by ensuring that GTPase works optimally in the hydrolysis of GTP. GTPase facilitates the energy necessary for the elongation of the protein chain, also facilitating the transport of amino acids and the movement of the ribosome along the mRNA strand (Fasnacht and Polacek, 2021).

Tolerance towards BTEX in bacteria can be observed in the growth performance and can be developed based on the ability of the microorganism to induce physiological and metabolic functions such as protein or RNA reparation in the presence of toxic compounds. For

example, tolerance towards benzene-derived superoxide-generating compounds in *M. esteraromaticum* can be attributed to the activation of transcription factors such as the PerP regulatory protein, common in Gram-positive bacteria, and responsible for the activation of the synthesis of BTEX-degrading enzymes that ameliorate the effect of oxidative species (Imlay, 2015);. In the case of *Serratia fonticola*, it has been reported that H_2O_2-induced transcription factors promoted by the OxyR and SoxR regulatory proteins, take part in the detection, modification and excretion of oxidative compounds. Likewise, OxyR and SoxR proteins are important in Gram-negative bacteria to induce the rRNA reparation rRNA involved in the synthesis of enzymes that contribute to the lesser detrimental effects of superoxide compounds in the bacterial cells and enhance its tolerance towards BTEX. In this sense, the low coculture biomass production observed (< 3.5g L^{-1}) in the treatments with ethylbenzene, xylene and BTEX can be due to either an abundant presence of AOS or to less efficient oxidative stress response systems in the protection of ribosomal proteins (e.g. transcription factors PerP, OxyR and SoxR).

The robust benzene and toluene tolerance found in coculture and *S. fonticola*, and the ethylbenzene tolerance found in *M. esteraromaticum* can be attributed to these oxidative stress response systems which allows for rapid growth rates compared to algae and fungi. For example, recent studies reporting the growth performance of microalgae *Chlorella vulgaris* in the presence of low concentrations of BTEX (0.5 mg L^{-1}) achieved a maximum cell production (9.6e3 cells L^{-1}) only after 3-6 days, whereas for coculture and the individual strains, it only took 1 day. This time gap represents a difference in the BTEX tolerance of *C. vulgaris* and coculture and can be due to a disruption to the photosynthesis process of the BTEX compounds. Particularly, Duan et al., (2017) showed that toxic intermediates from the

degradation of BTEX can interfere with the formation of the chlorophyll-protein complex (LHC) that captures light for energy conversion and disrupts the thylakoidal membrane as well as the biomass production of microalgae. Coculture growth in the presence of BTEX is significantly faster compared to that of fungi. For instance, Usman et al., (2020) reported the growth of *Gliocladium sp*. in minimal saline media (MSM) supplemented with BTEX (1% v v^{-1}) and achieving a maximum spore production (6.3e4 spores L^{-1}) after 15 days. The *Gliocladium sp*. similar to other fungi species such as *Aspergillus terreus* have a long exponential phase which is 10 or 12 times longer than that of the bacterial coculture (Usman et al., 2020). Although *Gliocladium sp*. was grown in extremely high concentrations of BTEX (>1000 mg L^{-1}) and MSM, filamentous fungi present typically lower biomass production and growth rates. The fungal cell wall comprises glucan, chitin and glycoproteins in both algae and fungi that provide extra protection and tolerance to high BTEX concentrations (Cindy A. et al., 2024). Likewise, the cellular wall in microalgae acts as a barrier preventing or reducing BTEX toxicity and alleviating oxidative stress. In both fungi and microalgae, the cell wall delays the interaction between BTEX compounds reducing internal toxicity.

Overall, a better understanding of the different levels of tolerance BTEX-degrading strains in the biodegradation process of toxic emerging contaminants such as BTEX and the effect of the oxidative stress induced by degradation intermediates in the growth performance can bring new insights into bioremediation technologies.

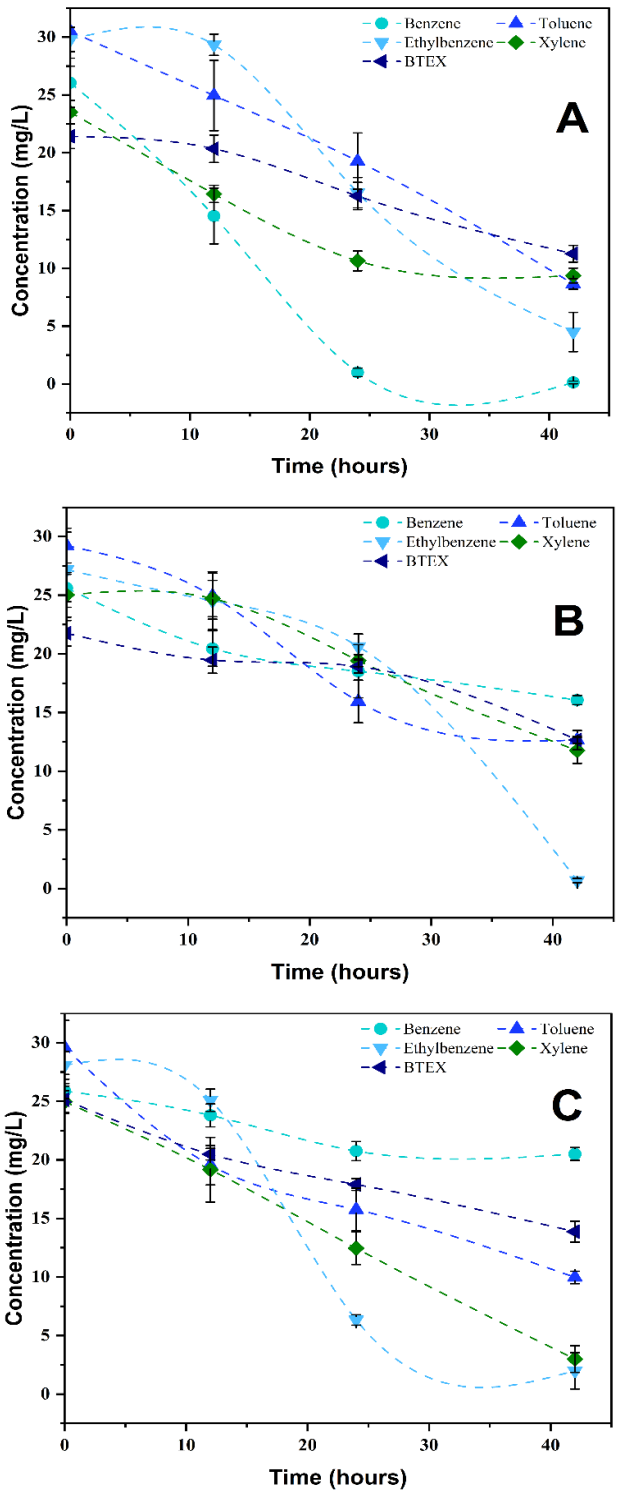


Figure 7. BTEX degradation by A) Coculture B) *S. fonticola* C) *Microbacterium esteraromaticum*.

3.2.2 BTEX degradation

3.2.2.1 Multiple Compound Degradation

BTEX compounds are often found together due to their similar physicochemical properties and use in the formulation of fuels. Figure 7 shows the BTEX degradation profile by *M. esteraromaticum*, *Serratia fonticola* and its coculture. A total BTEX degradation of 47.2% was achieved by the coculture after 42 hours. In the case of *S. fonticola* and *M. esteraromaticum* (Figure 7B and Figure 7C), a total degradation of 44.6% (9.1 mg L^{-1}) and 41.74% (7.84 mg L^{-1}) was achieved, respectively. For *S. fonticola* more than 50% of the degradation occurred only after 24 hours, whereas for M. *esteraromaticum* occurred within the first 12 hours. In the treatments in which the monocultures were used, the BTEX multi-compound degradation was only 77% of the achieved in the treatment in which coculture was used for the same BTEX multi-compound degradation.

Studies in which BTEX multi-compound degradation test has been performed are not as common as studies performing BTEX single compound degradation. Moreover, these studies are mainly focused on genomic and metagenomic analysis (Banerjee et al., 2022; Wu et al., 2023; Eze, 2021). The coculture BTEX degradation performance from this study can be compared to the BTEX multi-compound degradation reported by Wongbunmak et al., (2020). Coculture achieved a higher degradation (47%) compared with the results reported by (Wongbunmak et al., 2020) where only 38% of BTEX total degradation was achieved in two days using *Bacillus amyloliquefaciens* W1 growing in culture media supplemented with a BTEX concentration three times smaller (60 mg L^{-1}). Likewise, coculture outperforms

Bacillus amyloliquefaciens W1, achieving a higher depletion of 22, 17, 20 and 7 (mg L^{-1}) for benzene, toluene, ethylbenzene and xylene in the BTEX mixture, respectively.

It is not common for bacteria to degrade all four BTEX compounds as there are challenges to be addressed such the BTEX toxicity, enzyme activity and inhibition. Moreover, bacteria often tend to specialize. For example, *M. esteraromaticum* showed a preference for xylene (88% removal) and *S. fonticola* for ethylbenzene (97% removal), which is evident in their outstanding degradation performance. This compound preference for degradation can be explained by the ability to produce oxygenase enzymes which are compound-specific (e.g. XMO) (Miri et al., 2021). The enhanced BTEX degradation with 6 mg L^{-1} more depletion in the coculture during the multi-compound degradation test compared to the individual strains, can be explained by the complementary specialization of the strains. Another reason for this enhanced coculture BTEX degradation performance is the occurrence of induced co-metabolism between xylene and toluene, as both compounds share the same degradation intermediate (3-methyl catechol) (Khodaei et al., 2017). Although coculture improved significantly and achieved 47% of the removal of BTEX mixture in only 42 hours, the multi-compound presence of BTEX difficulted complete degradation. This can be due to previously reported inhibition of dioxygenase enzymes when the hydroxymethyl substituent of the benzene ring is positioned in an axial orientation (Bugg, 2003). Moreover, the aromatic structure between the four BTEX compounds can lead to competition for the binding site of BTEX-degrading enzymes.

Interestingly, BTEX multi-compound biodegradation presented a slow degradation within the first 12 hours of fermentation in coculture, and the individual strains S. *fonticola* and M. *esteraromaticum* with only 5%, 11% and 18% of BTEX removal, respectively (Figure 7).

Contrary to what has been observed in single-compound BTEX degradation, BTEX multi-compound degradation presented slow initial degradation rates which is consistent with other studies conducted (Wongbunmak et al., 2020; Bacosa et al., 2021). Finally, coculture enhanced the biodegradation of BTEX and it is a more robust alternative to degrade higher BTEX concentrations (200 mg L^{-1}).

3.2.2.2 Single-compound degradation

3.2.2.2.1 Benzene degradation

Figure 7 illustrates the coculture, *S. fonticola* and *M. esteraromaticum* single compound and BTEX degradation. Figure 7A shows a rapid benzene degradation by coculture, with 96% (25.9 mg L^{-1}) of depletion before 24 hours. Total benzene degradation was observed at the end of the test at 42 hours. Figures 7B and 7C show lower benzene removal, achieving only 37% (9.5 mg L^{-1}) and 24% (5.5 mg L^{-1}) degradation at 42 hours in the treatments using *S. fonticola* and *M. esteraromaticum*, respectively. That said, the biodegradation achieved by coculture in the treatment with benzene was three times and five times higher than the achieved by S. *fonticola* and *M. esteraromaticum*, respectively.

The total benzene degradation achieved by coculture this study, it was 5 hours faster than the reported by Zhou et al., (2016) growing *Acinetobacter baumannii* DD1 and using benzene (60 mg/ L-1) as the only carbon and energy source. Likewise, when compared to other studies that used *Serratia* sp. as a benzene-degrading strain and similar benzene concentrations (30 mg $L^{-1}$and 50 mg L^{-1}), the coculture used in this study was significantly faster (2-3 days) as they achieved 100% benzene removal until 10-14 days (Ortega-González et al., 2013; Avanzi et al., 2015). The difference in results can be attributed to a higher

enzyme production and activity of more specific and competent benzene-degrading enzymes such as C12O and C23O. Kaur et al., (2023) used M. *esteraromaticum* as a strain with the same benzene concentration as this study (50 mg L-1); however, it achieved only 70% of benzene removal in the period (30 hours) in which coculture achieved 100% of benzene removal. This 30% difference in the biodegradation efficiency can be attributed to an enhancement in the activity of catechol dioxygenases and monooxygenases, and microbial-substrate interaction due to the presence of S. *fonticola* in the coculture.

3.2.2.2.2 Ethylbenzene degradation

For the treatment with ethylbenzene, the total degradation achieved by coculture at 42 hours was 85% (25.3 mg/ L^{-1}removed) which is only 12.5% (1.17 mg/ L^{-1}) and 8% (0.7 mg/ L^{-1}) less degradation than the achieved by *S. fonticola* and *M. esteraromaticum,* respectively.

Moreover, the degradation achieved by coculture in the treatment with ethylbenzene is better compared to the studies reported by Wongbunmak et al., (2017) and Wongbunmak et al., (2020) in which *Bacillus amyloliquefaciens* W1 and *M. esteraromaticum* were used as ethylbenzene-degrading strains. The ethylbenzene degradation achieved by the coculture in this study was higher (4.5 mg/ $L^{-1}$and 2.5 mg L^{-1}) compared to *Bacillus amyloliquefaciens* W1 and *M. esteraromaticum*, respectively.

Moreover, the degradation pattern observed in Figure 7A (corresponding to coculture metabolism) from 12 to 24 hours follows the rapid ethylbenzene degradation observed in Figure 3C (corresponding to M. *esteraromaticum*) during the same period. Likewise, the degradation pattern from 24 to 42 hours in coculture follows the ethylbenzene degradation observed in Figure 3B within the same period. This tendency in the degradation patterns

suggests the action of both strains and synergistic effect in the coculture for the single compound degradation of ethylbenzene. The catabolism of ethylbenzene is switched on earlier in *M. esteraromaticum* playing a major role in the first 24 hours, whereas *S. fonticola* showed higher degradation rates in the last 24 hours period of the degradation.

3.2.2.2.3 Xylene degradation

In the single compound degradation of xylene, the treatment using coculture achieved a total removal of 14 mg L^{-1}after 42 hours of cultivation. This xylene removal in the treatment with coculture was 3 mg L^{-1} higher than the achieved in the treatment with *S. fonticola*; however, it was 8.5 mg L^{-1} lower than the xylene removal achieved in the treatment with *M. steraromaticum*. Similar to the treatment with single ethylbenzene, coculture did not contribute to an enhanced xylene degradation, whereas *M. esteraromaticum* achieved 2-fold higher xylene degradation. *M. esteraromaticum* performance in this study was the same as the reported by Kaur et al., (2023), with 85% of xylene degradation. When compared to a study in which (Banerjee et al., 2022) used a xylene degrading enrichment, the coculture achieved 14 mg L^{-1} xylene removal whereas the enrichment of only 5 mg L^{-1} in the same average period (32 hours). This indicates that coculture is a better alternative in bioremediation for the degradation of single compounds such as xylene and that more diverse cultures such as enrichments guarantee a better degradation performance. Overall, the xylene removal achieved by coculture is better than the previously reported microbial degradation studies in which it took several days to achieve the same degradation reported in this study (Avanzi et al., 2015; Ortega-González et al., 2013).

3.2.2.2.4 Toluene degradation

For the treatment with toluene and coculture, a total removal of 22 mg L^{-1} was found after 42 hours of cultivation. These results are higher in comparison with the observed in S. *fonticola* (5.2 mg L-1) and *M. esteraromaticum* (2.1 mg L^{-1}). These results were similar to the reported in other studies using *M. esteraromaticum* strain, achieving 30 mg L^{-1} of toluene degradation within the same period (42 hours) (Kaur et al., 2023). Interestingly, the time for the maximum biomass production (12 hours) was the same as when 50% of toluene was degraded. This suggests that there is a direct relationship between biomass production with the degradation of this specific compound.

The contribution to the degradation of the individual compounds of S. *fonticola* and M. *esteraromaticum* in the coculture can be elucidated based on the degradation patterns and the reported ability of M. *esteraromaticum* and S. *fonticola* to grow and assimilate BTEX. Likewise, Wongbunmak et al., (2017) observed the same tendency, an increase in the microbial growth of *M. esteraromaticum* when toluene and m-xylene were supplemented in high concentrations (>80 g L^{-1}). Finally, when compared to the single compound degradation observed in the treatments with benzene, ethylbenzene and xylene using coculture, the toluene removal was the third highest with more than 70 % proving that the substituent methyl is an alternative route of attack and oxidize the benzene ring in toluene (Bacosa et al., 2021). Moreover, (Bacosa et al., 2021) used *Burkholderia* sp. B5, *Pseudomonas* sp. T1 and *Cupriavidus* sp. X5 to degrade 100 mg/ L^{-1} of toluene in a study of binary mixtures with octane which aimed to determine the ability and preference of the strains between aromatic and aliphatic hydrocarbons. Although the concentration of toluene was half of the

supplemented by Bacosa et al., (2021), the coculture in this study outperformed the three strains which took 80, 70 and 250 hours to remove toluene to the same extent, respectively.

3.3 Conclusions

This study reveals that the BTEX-degrading bacterial coculture had different tolerance levels. The fast glucose consumption and low biomass in xylene and BTEX treatments indicated higher toxicity. Likewise, according to biomass production, coculture displayed a high tolerance for benzene and toluene treatments. The lowest biomass production corresponded to the BTEX mixture, evidencing cumulative toxicity. Coculture enhanced both BTEX multi-compound and single-compound degradation; especially for compounds such as benzene, toluene and ethylbenzene with more than 70% removal. Future studies should explore concentration effects on toxicity, tolerance towards BTEX toxicity, and mitigation of toxicity effects. Hybrid approaches like bioaugmentation, biostimulation and immobilization should be study to enhance coculture survival, activity and BTEX biodegradation efficiency. Likewise, biostimulation strategies involving the supplementation not only of nutrients but also substances that promote the stress-induced catabolism of BTEX such as the expression of BTEX-degrading enzymes and stress-induce co-metabolism among the BTEX compounds should be explored. Further work is necessary to enhance the activity of oxygenases such as catechol dioxygenases as enzymes which are crucial to enhanced BTEX degradation rates and to make the biodegradation process more BTEX-specific with the coculture.

Chapter 4: Benzyl alcohol as an inducer of catechol 2,3 dioxygenase activity in a BTEX-degrading coculture comprising S. *fonticola* and M. *esteraromaticum*

(to be submitted).

4.1 Introduction

The surge in petroleum hydrocarbons exploration has resulted in the contamination of fragile environments and sensitive water bodies which are a supply of DW to the population in several developed and underdeveloped countries. Diverse ecosystems are being contaminated with oil-derived toxic AHs such as BTEX due to their potential to escape from infrastructure and machines using oil and diesel, infiltrating in the subsoil, groundwater and finally in the ocean's subsurface through the submarine groundwater discharge (Huang et al., 2021). Once in the environment, BTEX high toxicity effect and carcinogenic nature can alter the delicate marine ecosystems balance like coral reefs and endangered the public health in coastal areas (Wang et al., 2016; Latif et al., 2019). Likewise, BTEX-associated diseases such as cancer represent trillion of dollars from the global gross domestic product (GDP), thus being considered both as an economic and health burden (Chen et al., 2023).

Currently, alternatives to address BTEX-pollution in sensitive environments rely on thermophysical techniques, like incineration and pump-and-treat systems which are often expensive and energy-demanding (Soleimani, 2014). Similarly, chemical approaches, such

as advanced oxidation processes (AOPs) lack precision as they are not tailored to specific pollutants (Alori at el., 2022). Moreover, as environmental regulations become stricter, the demand for not only effective BTEX remediation solutions but greener alternatives is superlative. Novel approaches such as bioremediation that employs natural occurring microorganisms offer advantages such as contaminant specificity, cost efficiency, reliability, and the preservation of biodiversity and the ecological balance. Likewise, research has focused on utilizing individual bacterial strains to degrade BTEX compounds, breaking them down into non-toxic byproducts such as carbon dioxide, pyruvate, or acetyl CoA (Wongbunmak et al., 2020). Among the most effective BTEX-degrading bacteria are those from genera including *Pseudomonas*, *Microbacterium*, *Bacillus*, *Acinetobacter*, *Serratia*, and *Rhodococcus* (Chicca et al., 2020; Hocinat et al., 2020; Wongbunmak et al., 2020). Particularly, *Microbacterium esteraromaticum* and *Serratia* sp. have shown a promising potential to efficiently degrade BTEX (Kaur et al., 2023; Yavas & Icgen, 2018). However, the use of single bacteria such as *Serratia* sp. and *Microbacterium* sp. faces limitations, including a low production and activity of competent BTEX-degrading enzymes and low microbial growth due to high BTEX concentrations in heavily contaminated aquifers, exceeding 200 mg/L (Hocinat et al., 2020). Thus, using bacterial cocultures for specific compound degradation provides a more robust tolerance and enzymatic capacity (Mukherjee et al., 2012). Another way to ameliorate BTEX degradation, it is to explore the use of enzymes instead of coculture, ultimately improving the degradation rates. Despite the improvements seen in chapter 3, multi-compound BTEX degradation can be further improved using enzymes (Miri et al., 2021).

On the other hand, among the options to enhance biological degradation of BTEX can be found biostimulation, bioaugmentation, immobilization and biosurfactant addition to improve bioavailability and promote the microorganism survival and activity (Rahmati et al., 2022; Eras-Muñoz et al., 2022). Although biostimulation typically promotes the microbial activity of BTEX degraders after the addition of nutrients, it is also possible to supplement substances that can help to induce BTEX catabolism.

Overall, few are the studies of stress-induce metabolic degradation of AHs. For example, salicylate, propane, butanol, carvone, salicylic acid and limonene had been used as inducers based on their structure similarity to degradation intermediates in the biodegradation of polyaromatic hydrocarbons and polychlorinated biphenyls such as phenanthrene, 1,4 dioxane and biphenyl (Young et al., 2006; Hand et al., 2015; Tandlich et al., 2001; Singer et al., 2000). With no studies so far focused on a catabolic inducer compound that is commercially available, affordable, naturally occurring and nontoxic for bioremediation processes, the proposal of supplementation benzyl alcohol in coculture is unique. Benzyl alcohol as the first intermediate in one of the toluene aerobic degradation pathways, can contribute to the enhancement of toluene degradation considering the multiple reported co-metabolism between toluene, benzene and the xylene isomers (Wongbunmak et al., 2020; Miri et al., 2022).

This study aims to explore the benzyl alcohol stress-induced activity of the potent BTEX-degrading enzyme (C23O) activity, and it is the first of its kind to use a natural occurring substance combined with a coculture of two competent BTEX-degrading bacteria. Moreover, it is intended to use the C23O activity as indicator of BTEX degradation, and for

future enzymatic application using the coculture between S. *fonticola* and M. *esteraromaticum*.

4.2 Results and Discussion

4.2.1 Catechol 2,3 Dioxygenase Stress-induced Activity

Figure 8 shows the results of the Catechol 2,3 Dioxygenase (C23O) after monitoring the formation of 3-methyl catechol (388 nm) in the treatments with different concentrations of BTEX (200 – 500 mg L-1) and benzyl alcohol (0.25 – 3 mM). Treatments with not C23O activity or a negligible C23O activity were not included in figure 8. The concentration of BTEX and benzyl alcohol was selected based on their water solubility at 22°C and in alignment with the bioavailability and tolerance reported in previous stress-induced degradation studies (Allen et al., 2008; Beggs et al., 1976; Handayani et al., 2019).

The coculture cell lysate showed the presence of C12O for different benzyl alcohol concentration acting as inducer, as follows: 1mM > 0.25mM > 3mM. The highest C23O activity in coculture was observed in the treatment **(II)** with 1mM benzyl alcohol and 200 mg BTEX L-1 (4.35 U/mg) at 32 hours. This was followed by the treatment **(IX)** with 500 mg BTEX L-1 (4.23 U/mg) and the treatment **(VIII)** with 200 mg BTEX L-1 (3.85 U mg-1), at 32 and 16 hours respectively. Treatments supplemented with a minimum concentration of benzyl alcohol (0.25 mM) showed activities of C23O above 1.3 U mg-1, whereas for the highest concentration of benzyl alcohol (3 mM), the presence of C23O was very less with values in the activity equal or below 0.6 U mg-1.

The presence of C23O suggests the degradation of BTEX as multiple authors reported its high specificity and its role after the substituents of aromatic ring are dissembled by action of monooxygenases for the BTEX compounds (Hassan and Aly, 2018; Wu et al., 2023). Likewise, an early C23O activity in treatments such as the one with 3 mM of benzyl alcohol and 200 mg BTEX L-1 **(I)** is an indicator of BTEX enhance degradation due to that C23O is expressed only after the activation of degradation pathway. On the other hand, inducer dominance was observed in coculture, M. *esteraromaticum* and S. *fonticola*. For instance, S. *fonticola* showed a preference for benzyl alcohol which is put in evidence by an earlier (16 hours) and higher (3.16 U mg-1) maximum C23O activity in the treatment **(VII)** with 0.25 mM of benzyl alcohol compared to the treatment **(VIII)** with 200 mg BTEX L-1 (2.91 U mg-1 at 32 h). Likewise, M. *esteraromaticum* had a higher C23O activity in the treatment **(VII)** supplemented with 0.25 mM of benzyl alcohol (4.47 U mg-1) compared to the treatment **(VIII)** supplemented with 200 mg L-1 (1.67 U mg-1) of BTEX. In contrast, BTEX was the dominant inducer of C23O in coculture as it showed a maximum higher activity in the treatment **(VIII)** with 200 mg BTEX L-1 (3.85 U mg-1) compared to treatments in which only benzyl alcohol was supplemented such the treatment **(VI)** with 1mM Benzyl alcohol (1.55 U mg-1).

This inducer dominance in C23O is in alignment with previous degradation studies in which M. *esteraromaticum* and S. *fonticola* showed a strong affinity for benzyl alcohol, direct intermediate in the toluene degradation pathway. These studies prove the use of Toluene as the only source of carbon and energy by both individual strains. Interestingly, the opposite was observed in coculture indicating an dominance of BTEX as inducer which can be due to

more diverse pathways and intermediates compared to the only and specific pathway known for benzyl alcohol in BTEX-degrading bacteria (Wongbunmak et al., 2020).

Nevertheless, an enhanced activity (4.4 U mg-1) was observed in coculture for the treatment **(II)** with 1 mM of benzyl alcohol and 200 mg of BTEX L-1 which achieved 2-fold higher C23O activity than the treatment **(IV)** with 0.25 mM of benzyl alcohol and its control **(VII)**. This demonstrates the importance of finding the optimal bacteria and contaminant-specific concentration of inducer to stimulate C23O activity and BTEX degradation as demonstrated in other literature findings demonstrating the induction effect in oxygenases activity after the addition of structurally similar compounds (Hand et al., 2015; Tandlich et al., 2001). Moreover, co-metabolism among BTEX compounds is of great importance in the selection of benzyl alcohol as an inducer of BTEX catabolism. Co-metabolism between benzene and o-xylene, and toluene and p-xylene has been widely reported in the enhancement of biodegradation rates as a result of competent enzymes (Khodaei et al., 2017). Likewise, the occurrence of co-metabolism between BTEX compounds helps to explain the enhancement in C23O activity as a direct and known induction route, especially in the treatment supplemented with the optimal concentration of benzyl alcohol (1mM).

Stress-induced enhanced C23O activity can be compared to other studies in which BTEX-degrading leading microorganisms had been highlighted. For example, Miri et al., (2021) reported significant C23O activity (4.07 U mg-1) which is 8% lower than the achieve in this study. This decrease in the enzyme activity can be explain by the absence of inducer and the presence of only one of the xylene isomers (p-xylene) instead of all the BTEX compounds. At the optimal inducer concentration (1 mM), C23O activity was also superior compared to individual strains. For instance, it was 7% higher than the reported by Kaur et al., (2023)

before the stationary phase of M. *esteraromaticum* when grown in media supplemented with 200 mg BTEX L-1 which is comparatively accurate with the treatment with optimal inducer concentration, demonstrating the robustness of coculture and effect of benzyl alcohol.

Finally, the effect of benzyl alcohol showed enhancement in C23O activity and further studies are suggested in order to explore the potential of coculture to degrade other AHs to which degradation is specific to the C23O action. Moreover, it is important to explore the use of more inducers and their effect in the activity of other crucial enzymes such as toluene monooxygenase, xylene monooxygenase, xylene dioxygenase and ethylbenzene dioxygenase.

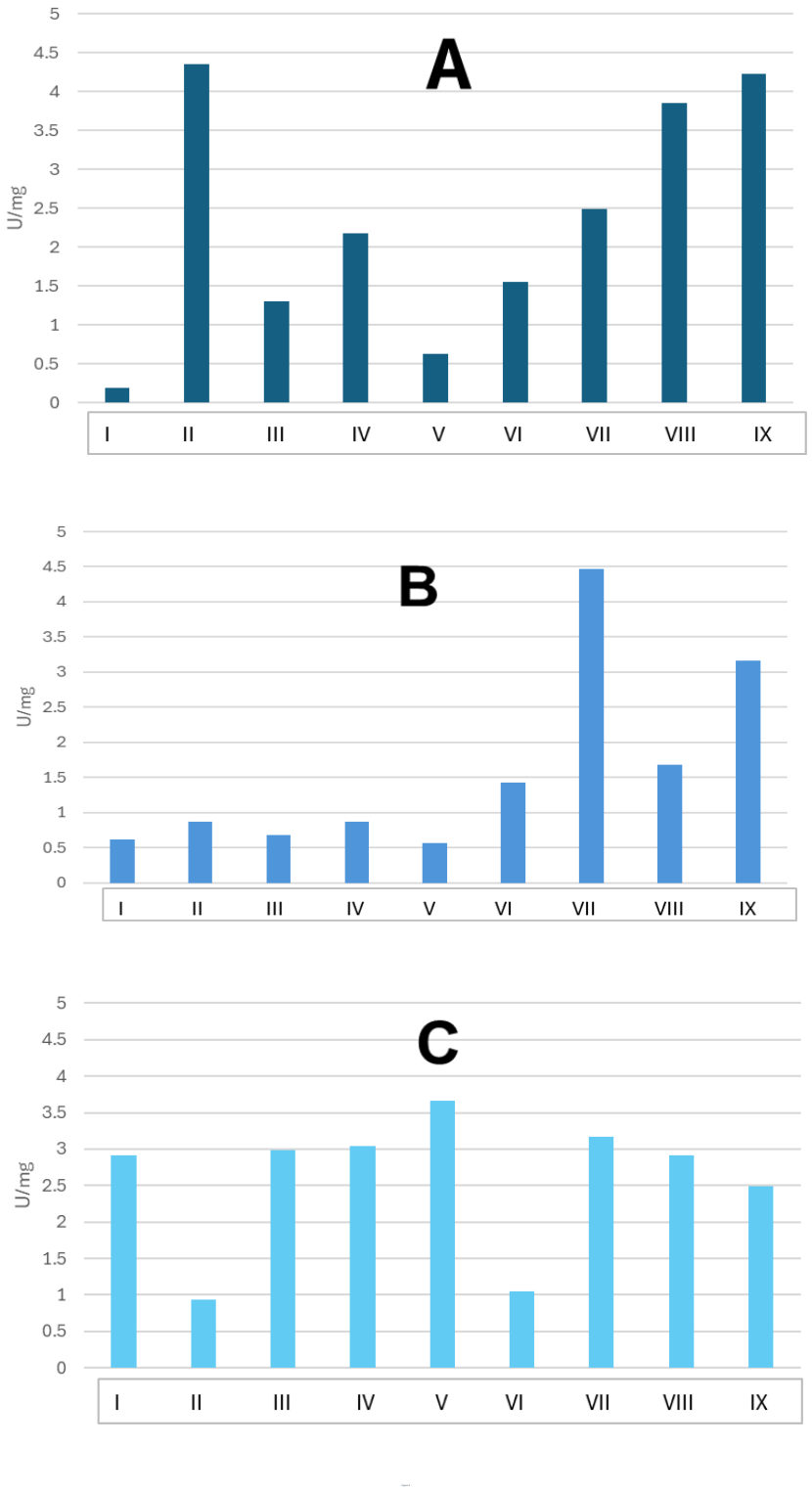


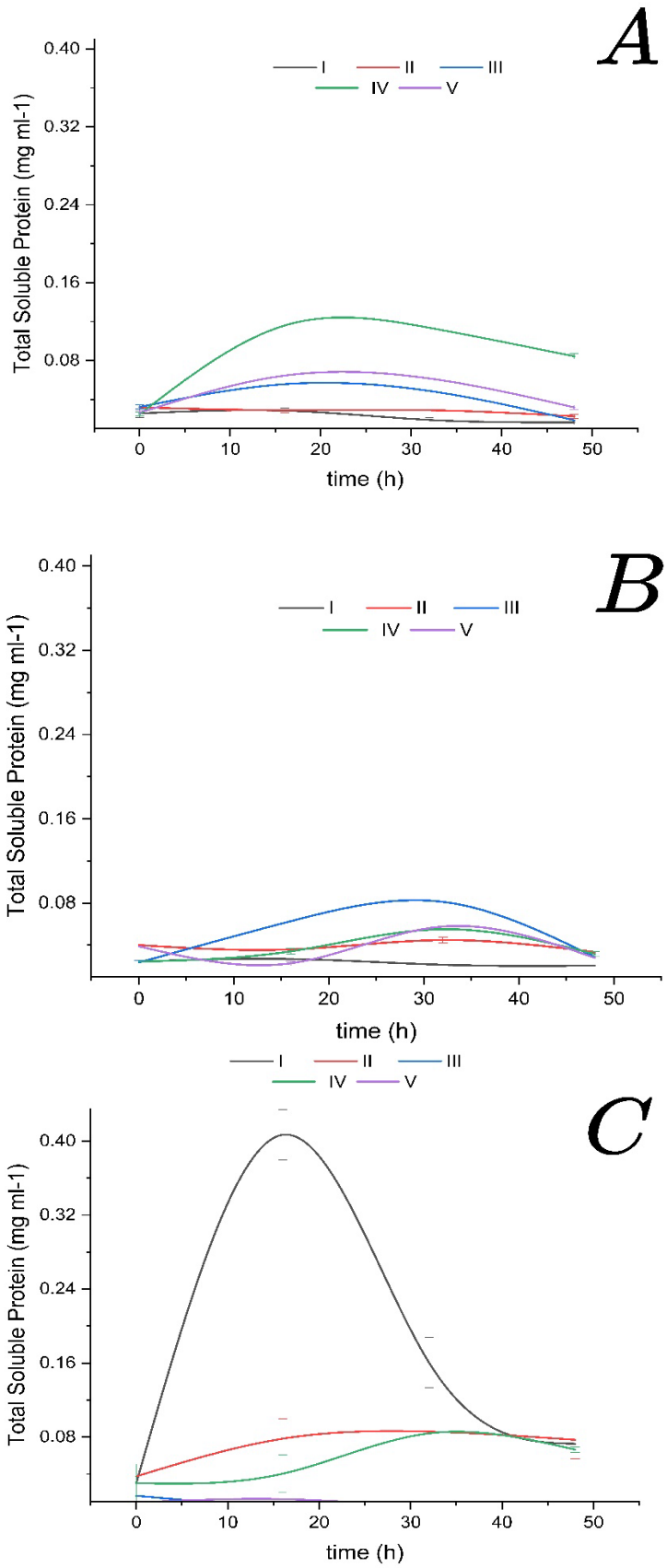
Figure 8. Catechol 2,3 dioxygenase (C23O) determination in the coculture between *M. esteraromaticum* and *S. fonticola,* and the individual strains in the presence of benzyl alcohol

(0, 0.25, 1 and 3 mM) and BTEX mixture (0, 200 and 500 mg L-1). The enzyme production in the different treatments is display for **A)** Coculture **B)** *M. esteraromaticum* **C)** *S. fonticola* as follows: **I).** 3 mM Benzyl Alcohol and 200 mg BTEX L-1 **II).** 1 mM Benzyl Alcohol and 200 mg BTEX L-1 **III).** 0.25 mM Benzyl Alcohol and 500 mg BTEX L-1 **IV).** 0.25 mM Benzyl Alcohol and 200 mg BTEX L-1 **V).** 3 mM Benzyl Alcohol, 0 mg BTEX L-1 **VI).** 1 mM Benzyl Alcohol, 0 mg BTEX L-1 **VII).** 0.25 mM Benzyl Alcohol, 0 mg BTEX L-1 **VIII).** 0 mM Benzyl Alcohol, 200 mg BTEX L-1 **IX).** 0 mM Benzyl Alcohol, 500 mg BTEX L-1.

4.2.2 Total Soluble Protein

Figure 9 displays the Total Soluble Protein (TSP) in coculture, S. *fonticola* and M. *esteraromaticum* growing in the different treatments containing benzyl alcohol (0.25, 1 ad 3 mM) and BTEX (200 and 500 mg L-1). Figure 9A and 9B, show a relatively high TSP in the treatments supplemented with 0.25 mM and 1 mM, which can correspond to optimal inducer concentration for the activity of BTEX-degrading enzymes other than C23O for coculture (0.12 mg ml-1) and M. *esteraromaticum* (0.08 mg ml-1) respectively. Enzymes including but not limited to monooxygenases such as ToMO and XMO which facilitate the incorporation of an oxygen atom to BTEX compounds initially and can occur extracellularly (Sathesh-Prabu et al., 2023). In figure 9C, it can be observed the TSP produced by S. *fonticola* throughout the experiment (48 hours) and illustrating an outstanding TSP production (>0.4 mg ml-1) in the treatment containing the maximum benzyl alcohol (3 mM) and BTEX (500 mg L-1) concentration. This can be explained by the numerous reports of *Serratia* sp. ability to produce a wide group of degradative hydrolytic enzymes (e.g. lipase, nuclease, laccase, esterase) and its variety in secretory systems to transport them extracellularly. Interestingly,

Elistratova et al., (2022) investigated the presence of extracellular protective proteins in *Serratia marcescens* SM6 which were expressed as a stress response to H2O2-derivated oxidative stress. It was particularly reported the presence of a low molecular weight DUF1471-containing protein which is specific to the resistance to the oxidative stress from toxic compounds such as catechol and benzoate, intermediates in the BTEX biodegradation pathways. S. *fonticola* can thus be attributed to produce similar group of these type of proteins extracellularly, explaining its outstanding TSP production in this experiment.


A
I
II
III
IV
V
Total Soluble Protein (mg ml-1)
0.40
0.32
0.24
0.16
0.08
0
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40
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time (h)
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Total Soluble Protein (mg ml-1)
0.40
0.32
0.24
0.16
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Total Soluble Protein (mg ml-1)
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time (h)

Figure 9. Total Soluble Protein in the treatments with a major production in the supernatant for **A)** Coculture **B)** *M. esteraromaticum* **C)** *S. fonticola* after 48h as follows: **I).** 3 mM Benzyl Alcohol and 500 mg BTEX L-1 **II).** 3 mM Benzyl Alcohol and 200 mg BTEX L-1 **III).** 1 mM Benzyl Alcohol and 200 mg BTEX L-1 **IV).** 0.25 mM Benzyl Alcohol and 500 mg BTEX L-1 **V).** 0.25 mM Benzyl Alcohol and 500 mg BTEX L-1.

4.3 Conclusions

This study demonstrated the potential of benzyl alcohol as an effective inducer of C23O activity using a BTEX-degrading coculture of S. *fonticola* and M. *esteraromaticum*. The optimal benzyl alcohol concentration as an inducer of the enhanced C23O activity was 1 mM, which was twice the activity achieved in the controls. Future work should include purification of BTEX-degrading enzymes, and the exploration of alternative affordable and sustainable inducers to maximize other crucial dioxygenases and monooxygenases for the enzyme application using the coculture. Further research efforts should be focused in obtaining a better understanding on how stress-induced oxygenase activity can be employed as an indicator of BTEX degradation and other AHs. Likewise, recommendations include investigating the use of immobilization materials and carriers such as biochar or chitosan beads for enzyme application and its effectiveness when combined with stress-induced BTEX degradation. Finally, it is necessary to explore the potential use of coculture for either the biological or enzymatic degradation of other POPs such as polyaromatic hydrocarbons.

Chapter 5: Conclusions and Future Recommendations

5.1 Conclusions

5.1.1 Treatments with ethylbenzene and toluene showed a low glucose consumption after 12 hours, consuming only 4% and 14% of the glucose from 12 to 18 hours, respectively. The slow glucose consumption observed, especially when compared to other treatments between 12 to 18 hours, indicated a significant disruption in the glucose metabolism in coculture and the single strains. This highlights the metabolic challenges posed by ethylbenzene and toluene, which could guide further optimization of metabolic pathways in future studies.

5.1.2 The coculture achieved an outstanding biomass production in the treatment with toluene (6 ± 0.35 g L^{-1}) and benzene (5 ± 0.35 g L^{-1}) which can be attributed to its tolerance to the compounds and its oxidative stress response systems. These findings underscore the coculture's potential as a robust candidate for biotechnological applications in environments with high toluene and benzene concentrations.

5.1.3 The lowest biomass production corresponded to the treatment with the BTEX mixture (2.5 ± 0.70 g L^{-1}) which is 62 % less production than the observed in toluene, evidencing cumulative toxicity of the compounds. This result emphasizes the need for tailored strategies to mitigate compound-specific and cumulative toxic effects in mixed BTEX environments.

5.1.4 Coculture displayed an outstanding single compound degradation for benzene with 99% removal within 42 hours. Likewise, coculture showed a high ability compared to the individual strains for single toluene (72%) and ethylbenzene (85%) and xylene (62%)

degradation. These results demonstrate the coculture's superior degradation efficiency, reinforcing its applicability for targeted bioremediation of BTEX-contaminated environments.

5.1.5 An enhanced BTEX catabolism was observed in coculture for multi compound degradation, outperforming the individual strains, S. *fonticola* (44.6%) and M. *esteraromaticum* (41.74%) with 47.2% removal. This suggests the synergistic advantage of the formulated combined bacterial metabolism by potentially inducing co-metabolism among the BTEX compounds (e.g., shared intermediate 3-methyl catechol for xylene and toluene degradation) and as a tool for future bioremediation applications

5.1.6 The potential of benzyl alcohol as a natural occurring inducer of C23O activity in the coculture was demonstrated at an optimal supplementation of inducer (1 mM benzyl alcohol) and BTEX (200 mg BTEX L-1), achieving twice the C23O activity (4.35 U/mg) that of the controls. This highlights benzyl alcohol's role as enhancer of enzymatic activity of C23O, paving the way for its use in the production BTEX-degrading enzymes and as part of future bioremediation strategies for BTEX-contaminated environments.

5.2 Future Recommendations

5.2.1 Expand Field Trials: Conduct tests in varied natural settings, including differing salinities, temperatures, and contaminant concentrations, to assess the coculture's real-world applicability and resilience.

5.2.2 **Integrate Soil Studies:** Study the coculture performance in different types of soil through column test and the potential improvements in bioavailability using amendments (e.g., biochar). Likewise, explore how the coculture could be adapted for in situ bioremediation of BTEX-contaminated soils.

5.2.2 Explore Metabolic Pathways: Further research on the metabolic pathways and enzyme systems involved in BTEX degradation will provide insight into optimizing the coculture's effectiveness and targeting specific BTEX compounds.

5.2.3 Develop Improved Bioaugmentation Techniques: Investigate the formulation of the coculture for bioaugmentation, including the use of protective carriers (e.g., biochar) or immobilization techniques, to enhance stability and effectiveness in field applications.

5.2.4 Regulatory and Environmental Impact Studies: Evaluate the coculture's regulatory feasibility and long-term environmental impacts to find market pathways and its implementation in ecologically sensitive and regulated areas.

5.2.5 Investigate Broader Hydrocarbon Applications: Assess the coculture's ability to degrade other persistent organic pollutants (POPs) or hydrocarbon derivatives beyond BTEX, especially in environments prone to mixed-contaminant exposure.

Bibliography

Aburto-Medina, A., Ball, A.S., 2015. Microorganisms involved in anaerobic benzene degradation. Ann. Microbiol. 65, 1201–1213. https://doi.org/10.1007/S13213-014-0926-8

Allen, T.D., Kraus, P.F., Lawson, P.A., Drake, G.R., Balkwill, D.L., Tanner, R.S., 2008. Desulfovibrio carbinoliphilus sp. nov., a benzyl alcohol-oxidizing, sulfate-reducing bacterium isolated from a gas condensate-contaminated aquifer. Int J Syst Evol Microbiol 58, 1313–1317. https://doi.org/10.1099/ijs.0.65524-0

Acosta-González, A., Rosselló-Móra, R., Marqués, S., 2013. Diversity of Benzylsuccinate Synthase-Like (bssA) Genes in Hydrocarbon-Polluted Marine Sediments Suggests Substrate-Dependent Clustering. Appl. Environ. Microbiol. 79, 3667–3676. https://doi.org/10.1128/AEM.03934-12

Alori, E.T., 2015. Phytoremediation using microbial communities: II. Phytoremediation Manag. Environ. Contam. 2, 183–190. https://doi.org/10.1007/978-3-319-10969-5_15

Alori, E.T., Gabasawa, A.I., Elenwo, C.E., Agbeyegbe, O.O., 2022. Bioremediation techniques as affected by limiting factors in soil environment. Front. Soil Sci. 2, 937186. https://doi.org/10.3389/fsoil.2022.937186

Atashgahi, S., Hornung, B., Waals, M.J.V.D., Rocha, U.N.D., Hugenholtz, F., Nijsse, B., Molenaar, D., Spanning, R.V., Stams, A.J.M., Gerritse, J., Smidt, H., 2018. A benzene-degrading nitrate-reducing microbial consortium displays aerobic and anaerobic benzene degradation pathways. Sci. Rep. 8, 1–12. https://doi.org/10.1038/s41598-018-22617-x

Avanzi, I. R., Gracioso, L. H., Baltazar, M. dos P. G., Karolski, B., Perpetuo, E. A., & Nascimento, C. A. O. (2015). Aerobic Biodegradation of Gasoline Compounds by Bacteria Isolated from a Hydrocarbon-Contaminated Soil. *Environmental Engineering Science*, *32*(12), 990–997. https://doi.org/10.1089/ees.2015.0122

Bacosa, H. P., Mabuhay-Omar, J. A., Balisco, R. A. T., Omar, D. M., & Inoue, C. (2021). Biodegradation of binary mixtures of octane with benzene, toluene, ethylbenzene or xylene (BTEX): Insights on the potential of Burkholderia, Pseudomonas and Cupriavidus isolates. World Journal of Microbiology and Biotechnology, 37(7), 122. https://doi.org/10.1007/s11274-021-03093-4

Banerjee, S., Bedics, A., Harkai, P., Kriszt, B., Alpula, N., & Táncsics, A. (2022). Evaluating the aerobic xylene-degrading potential of the intrinsic microbial community of a legacy BTEX-contaminated aquifer by enrichment culturing coupled with multi-omics analysis: Uncovering the role of Hydrogenophaga strains in xylene degradation. *Environmental Science and Pollution Research*, *29*(19), 28431–28445. https://doi.org/10.1007/s11356-021-18300-w

Beggs, J.D., Cook, A.M., Fewson, C.A., 1976. Regulation of Growth of Acinetobacter calcoaceticusncib8250 on Benzyl Alcohol in Batch Culture. Microbiology 96, 365–374. https://doi.org/10.1099/00221287-96-2-365

Blázquez, B., Carmona, M., Díaz, E., 2018. Transcriptional regulation of the peripheral pathway for the anaerobic catabolism of toluene and m-Xylene in Azoarcus sp. CIB. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.00506

Boll, M., Estelmann, S., Heider, J., 2018a. Anaerobic Degradation of Hydrocarbons: Mechanisms of Hydrocarbon Activation in the Absence of Oxygen. Anaerob. Util. Hydrocarb. Oils Lipids 1–27. https://doi.org/10.1007/978-3-319-33598-8_2-1

Boll, M., Estelmann, S., Heider, J., 2018b. Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Monocyclic Aromatic Compounds. Anaerob. Util. Hydrocarb. Oils Lipids 1–50. https://doi.org/10.1007/978-3-319-33598-8_6-1

Boll, M., Löffler, C., Morris, B.E.L., Kung, J.W., 2014. Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: Organisms, strategies and key enzymes. Environ. Microbiol. 16, 612–627. https://doi.org/10.1111/1462-2920.12328

Bonting, C.F., Fuchs, G., 1996. Anaerobic metabolism of 2-hydroxybenzoic acid (salicylic acid) by a denitrifying bacterium. Arch. Microbiol. 165, 402–408. https://doi.org/10.1007/s002030050344

Botton, S., Parsons, J.R., 2006. Degradation of BTEX compounds under iron-reducing conditions in contaminated aquifer microcosms. Environ. Toxicol. Chem. 25, 2630–2638. https://doi.org/10.1897/06-004R.1

Bugg, T. D. H. (2003). Dioxygenase enzymes: Catalytic mechanisms and chemical models. *Tetrahedron*, *59*(36), 7075–7101. https://doi.org/10.1016/S0040-4020(03)00944-X

Busca, G., 2021. Production of gasolines and monocyclic aromatic hydrocarbons: From fossil raw materials to green processes. Energies 14, 4061. https://doi.org/10.3390/en14134061

Carmona, M., Zamarro, M.T., Blázquez, B., Durante-Rodríguez, G., Juárez, J.F., Valderrama, J.A., Barragán, M.J.L., García, J.L., Díaz, E., 2009. Anaerobic Catabolism of

Aromatic Compounds: a Genetic and Genomic View. Microbiol. Mol. Biol. Rev. 73, 71–133. https://doi.org/10.1128/mmbr.00021-08

Castro, A.R., Martins, G., Salvador, A.F., Cavaleiro, A.J., 2022. Iron Compounds in Anaerobic Degradation of Petroleum Hydrocarbons: A Review. Microorganisms 10, 2142. https://doi.org/10.3390/microorganisms10112142

Chakraborty, R., Coates, J.D., 2005. Hydroxylation and Carboxylation—Two Crucial Steps of Anaerobic Benzene Degradation by Dechloromonas Strain RCB. Appl. Environ. Microbiol. 71, 5427. https://doi.org/10.1128/AEM.71.9.5427-5432.2005

Chaurasia, A.K., Tremblay, P.L., Holmes, D.E., Zhang, T., 2015. Genetic evidence that the degradation of para-cresol by Geobacter metallireducens is catalyzed by the periplasmic para-cresol methylhydroxylase. FEMS Microbiol. Lett. 362, 145. https://doi.org/10.1093/femsle/fnv145

Chen, C., Zhang, Z., Xu, P., Hu, H., Tang, H., 2023. Anaerobic biodegradation of polycyclic aromatic hydrocarbons. Environ. Res. 223, 115472. https://doi.org/10.1016/j.envres.2023.115472

Chicca, I., Becarelli, S., Dartiahl, C., La China, S., De Kievit, T., Petroni, G., Di Gregorio, S., & Levin, D. B. (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*(29), 36203–36214. https://doi.org/10.1007/s11356-020-09650-y

Cindy A, P. R., Sáenz, S. E., Gordillo, R. M., & Castañón González, J. H. (2024). Effect of aromatic hydrocarbons on Chlorella vulgaris growth. *Pakistan Journal of Botany*, *56*(5). https://doi.org/10.30848/PJB2024-5(17)

Crisafi, F., Genovese, M., Smedile, F., Russo, D., Catalfamo, M., Yakimov, M., Giuliano, L., Denaro, R., 2016. Bioremediation technologies for polluted seawater sampled after an oil-spill in Taranto Gulf (Italy): A comparison of biostimulation, bioaugmentation and use of a washing agent in microcosm studies. Mar. Pollut. Bull. 106, 119–126. https://doi.org/10.1016/j.marpolbul.2016.03.017

Dias, R.L., Ruberto, L., Hernández, E., Vázquez, S.C., Lo Balbo, A., Del Panno, M.T., Mac Cormack, W.P., 2012. Bioremediation of an aged diesel oil-contaminated Antarctic soil: Evaluation of the "*on site*" biostimulation strategy using different nutrient sources. Int. Biodeterior. Biodegrad. 75, 96–103. https://doi.org/10.1016/j.ibiod.2012.07.020

Dong, X., Dröge, J., Toerne, C.V., Marozava, S., McHardy, A.C., Meckenstock, R.U., 2017. Reconstructing metabolic pathways of a member of the genus Pelotomaculum suggesting its potential to oxidize benzene to carbon dioxide with direct reduction of sulfate. FEMS Microbiol. Ecol. 93. https://doi.org/10.1093/femsec/fiw254

Dorer, C., Vogt, C., Neu, T.R., Stryhanyuk, H., Richnow, H.H., 2016. Characterization of toluene and ethylbenzene biodegradation under nitrate-, iron(III)- and manganese(IV)-reducing conditions by compound-specific isotope analysis. Environ. Pollut. Barking Essex 1987 211, 271–281. https://doi.org/10.1016/j.envpol.2015.12.029

Dou, J., Liu, X., Hu, Z., Deng, D., 2008. Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction. J. Hazard. Mater. 151, 720–729. https://doi.org/10.1016/j.jhazmat.2007.06.043

Duan, W., Meng, F., Lin, Y., & Wang, G. (2017). Toxicological effects of phenol on four marine microalgae. *Environmental Toxicology and Pharmacology*, *52*, 170–176. https://doi.org/10.1016/j.etap.2017.04.006

Durante-Rodríguez, G., Gómez-Álvarez, H., Blázquez, B., Fernández-Llamosas, H., Martín-Moldes, Z., Sanz, D., Nogales, J., Carmona, M., Díaz, E., 2018. Chapter 13: Anaerobic Pathways for the Catabolism of Aromatic Compounds. RSC Energy Environ. Ser. 333–390.

Ben8

Elistratova, A.A., Matrosova, L.E., Khilyas, I.V., Shirshikova, T.V., Danilova, I.V., Laikov, A.V., Romanova, Y.D., Sierra-Bakhshi, C.G., Sharipova, M.R., Bogomolnaya, L.M., 2022. Serratia marcescens DUF1471-Containing Protein SrfN Is Needed for Adaptation to Acid and Oxidative Stresses. mSphere 7, e00212-22. https://doi.org/10.1128/msphere.00212-22

Eze, M. O. (2021). Metagenome Analysis of a Hydrocarbon-Degrading Bacterial Consortium Reveals the Specific Roles of BTEX Biodegraders. *Genes*, *12*(1), Article 1. https://doi.org/10.3390/genes12010098

Fasnacht, M., & Polacek, N. (2021). Oxidative Stress in Bacteria and the Central Dogma of Molecular Biology. Frontiers in Molecular Biosciences, 8. https://www.frontiersin.org/articles/10.3389/fmolb.2021.671037

Flesher, J.W., Myers, S.R., 1991. Methyl-substitution of benzene and toluene in preparations of human bone marrow. Life Sci. 48, 843–850. https://doi.org/10.1016/0024-3205(91)90100

Ghattas, A.K., Fischer, F., Wick, A., Ternes, T.A., 2017. Anaerobic biodegradation of (emerging) organic contaminants in the aquatic environment. Water Res. 116, 268–295. https://doi.org/10.1016/j.watres.2017.02.001

Gieg, L.M., Toth, C.R.A., 2017. Signature Metabolite Analysis to Determine In Situ Anaerobic Hydrocarbon Biodegradation, in: Boll, M. (Ed.), Anaerobic Utilization of Hydrocarbons, Oils, and Lipids, Handbook of Hydrocarbon and Lipid Microbiology. Springer International Publishing, Cham, pp. 1–30. https://doi.org/10.1007/978-3-319-33598-8_19-1

Godin, S., Kubica, P., Ranchou-Peyruse, A., Hecho, I.L., Patriarche, D., Caumette, G., Szpunar, J., Lobinski, R., 2020. An LC-MS/MS Method for a Comprehensive Determination of Metabolites of BTEX Anaerobic Degradation in Bacterial Cultures and Groundwater. Water 2020 Vol 12 Page 1869 12, 1869. https://doi.org/10.3390/W12071869

Haiying, H., Jiang, Y., Zhao, J., Li, S., Schulz, S., & Deng, L. (2021). BTEX biodegradation is linked to bacterial community assembly patterns in contaminated groundwater ecosystem. *Journal of Hazardous Materials*, *419*, 126205. https://doi.org/10.1016/j.jhazmat.2021.126205

Haleyur, N., Shahsavari, E., Jain, S.S., Koshlaf, E., Ravindran, V.B., Morrison, P.D., Osborn, A.M., Ball, A.S., 2019. Influence of bioaugmentation and biostimulation on PAH degradation in aged contaminated soils: Response and dynamics of the bacterial community. J. Environ. Manage. 238, 49–58. https://doi.org/10.1016/j.jenvman.2019.02.115

Hand, S., Wang, B., Chu, K.-H., 2015. Biodegradation of 1,4-dioxane: Effects of enzyme inducers and trichloroethylene. Science of The Total Environment 520, 154–159. https://doi.org/10.1016/j.scitotenv.2015.03.031

Handayani, S., Safitri, R., Astika, H., 2019. Biodegradation of BTEX by indigenous microorganisms isolated from UCG project area, South Sumatra - IOPscience [WWW Document]. URL https://iopscience.iop.org/article/10.1088/1755-1315/308/1/012017 (accessed 11.19.24).

Hassan, H.A., Aly, A.A., 2018. Isolation and characterization of three novel catechol 2,3-dioxygenase from three novel haloalkaliphilic BTEX-degrading Pseudomonas strains. International Journal of Biological Macromolecules 106, 1107–1114. https://doi.org/10.1016/j.ijbiomac.2017.08.113

Heider, J., Schühle, K., 2013. Anaerobic biodegradation of hydrocarbons including methane. Prokaryotes Prokaryotic Physiol. Biochem. 605–634. https://doi.org/10.1007/978-3-642-30141-4_80

Heider, J., Szaleniec, M., Sünwoldt, K., Boll, M., 2016. Ethylbenzene Dehydrogenase and Related Molybdenum Enzymes Involved in Oxygen-Independent Alkyl Chain Hydroxylation. J. Mol. Microbiol. Biotechnol. 26, 45–62. https://doi.org/10.1159/000441357

Hernández-Ospina, D. A., Osorio-González, C. S., Miri, S., & Kaur Brar, S. (2024). New perspectives on the anaerobic degradation of BTEX: Mechanisms, pathways, and intermediates. *Chemosphere*, *361*, 142490. https://doi.org/10.1016/j.chemosphere.2024.142490

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H.H., Vogt, C., 2010. Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. Environ. Microbiol. 12, 401–411. https://doi.org/10.1111/j.1462-2920.2009.02077.x

Hidalgo, K.J., Teramoto, E.H., Soriano, A.U., Valoni, E., Baessa, M.P., Richnow, H.H., Vogt, C., Chang, H.K., Valéria, M.O., 2020. Taxonomic and functional diversity of the microbiome in a jet fuel contaminated site as revealed by combined application of in situ microcosms with metagenomic analysis. Sci. Total Environ. 708, 135152. https://doi.org/10.1016/j.scitotenv.2019.135152

Hocinat, A., Boudemagh, A., Ali-Khodja, H., & Medjemadj, M. (2020). Aerobic degradation of BTEX compounds by Streptomyces species isolated from activated sludge and agricultural soils. *Archives of Microbiology*, *202*(9), 2481–2492. https://doi.org/10.1007/s00203-020-01970-4

Höffken, H.W., Duong, M., Friedrich, T., Breuer, M., Hauer, B., Reinhardt, R., Rabus, R., Heider, J., 2006. Crystal Structure and Enzyme Kinetics of the (S)-Specific 1-Phenylethanol Dehydrogenase of the Denitrifying Bacterium Strain EbN1,. Biochemistry 45, 82–93. https://doi.org/10.1021/bi051596b

Huang, H., Jiang, Y., Zhao, J., Li, S., Schulz, S., Deng, L., 2021. BTEX biodegradation is linked to bacterial community assembly patterns in contaminated groundwater ecosystem. J. Hazard. Mater. 419, 126–205. https://doi.org/10.1016/j.jhazmat.2021.126205

Imlay, J. A. (2015). Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. Annual Review of Microbiology, 69(1), 93–108. https://doi.org/10.1146/annurev-micro-091014-104322

Jiang, H., Chen, D., Zheng, D., Xiao, Z., 2023. Anaerobic mineralization of toluene by enriched soil-free consortia with solid-phase humin as a terminal electron acceptor. Environ. Pollut. 317, 120794. https://doi.org/10.1016/j.envpol.2022.120794

Jobst, B., Schühle, K., Linne, U., Heider, J., 2010. ATP-dependent carboxylation of acetophenone by a novel type of carboxylase. J. Bacteriol. 192, 1387–1394. https://doi.org/10.1128/jb.01423-09

Johnson, M.E., Guelph, C., Dunfield, K.E., 2023. Assessing toluene biodegradation potential in groundwater using molecular biological tools. https://hdl.handle.net/10214/27429

Kalantary, R.R., Mohseni-Bandpi, A., Esrafili, A., Nasseri, S., Ashmagh, F.R., Jorfi, S., Jafari, M., 2014. Effectiveness of biostimulation through nutrient content on the bioremediation of phenanthrene contaminated soil. J. Environ. Health Sci. Eng. 12, 1–9. https://doi.org/10.1186/S40201-014-0143-1

Kaur, G., Lecka, J., Krol, M., & Brar, S. K. (2023). Novel BTEX-degrading strains from subsurface soil: Isolation, identification and growth evaluation. Environmental Pollution, 335, 122303. https://doi.org/10.1016/j.envpol.2023.122303

Keller, A.H., Kleinsteuber, S., Vogt, C., 2018. Anaerobic Benzene Mineralization by Nitrate-Reducing and Sulfate-Reducing Microbial Consortia Enriched From the Same Site: Comparison of Community Composition and Degradation Characteristics. Microb. Ecol. 75, 941–953. https://doi.org/10.1007/S00248-017-1100-1

Khalid, F., Hashmi, M.Z., Jamil, N., Qadir, A., Ali, M.I., 2021. Microbial and enzymatic degradation of PCBs from e-waste-contaminated sites: a review. Environ. Sci. Pollut. Res. 28, 10474–10487. https://doi.org/10.1007/s11356-020-11996-2

Khodaei, K., Nassery, H. R., Asadi, M. M., Mohammadzadeh, H., & Mahmoodlu, M. G. (2017). BTEX biodegradation in contaminated groundwater using a novel strain (Pseudomonas sp. BTEX-30). International Biodeterioration & Biodegradation, 116, 234–242. https://doi.org/10.1016/j.ibiod.2016.11.001

Konopásek, I., Strzalka, K., Svobodová, J., 2000. Cold shock in Bacillus subtilis: different effects of benzyl alcohol and ethanol on the membrane organisation and cell adaptation. Biochimica et Biophysica Acta (BBA) - Biomembranes 1464, 18–26. https://doi.org/10.1016/S0005-2736(99)00240-0

Kunapuli, U., Jahn, M.K., Lueders, T., Geyer, R., Heipieper, H.J., Meckenstock, R.U., 2010. Desulfitobacterium aromaticivorans sp. nov. and Geobacter toluenoxydans sp. nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic hydrocarbons. Int. J. Syst. Evol. Microbiol. 60, 686–695. https://doi.org/10.1099/IJS.0.003525-0

Laban, N.A., Selesi, D., Rattei, T., Tischler, P., Meckenstock, R.U., 2010. Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environ. Microbiol. 12, 2783–2796. https://doi.org/10.1111/j.1462-2920.2010.02248.x

Ladino-Orjuela, G., Gomes, E., Silva, R. da, Salt, C., Parsons, J.R., 2016. Metabolic pathways for degradation of aromatic hydrocarbons by bacteria. Rev. Environ. Contam. Toxicol. 237, 105–121. https://doi.org/10.1007/978-3-319-23573-8_5

Latif, M.T., Abd Hamid, H.H., Ahamad, F., Khan, M.F., Mohd Nadzir, M.S., Othman, M., Sahani, M., Abdul Wahab, M.I., Mohamad, N., Uning, R., Poh, S.C., Fadzil, M.F., Sentian, J., Tahir, N.M., 2019. BTEX compositions and its potential health impacts

in Malaysia. Chemosphere 237, 124451. https://doi.org/10.1016/j.chemosphere.2019.124451

Lueders, T., 2017. The ecology of anaerobic degraders of BTEX hydrocarbons in aquifers. FEMS Microbiol. Ecol. 93, 220. https://doi.org/10.1093/femsec/fiw220

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Tarouco, P.C., Weyrauch, P., Dong, X., Himmelberg, A.M., 2016. Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. J. Mol. Microbiol. Biotechnol. 26, 92–118. https://doi.org/10.1159/000441358

Miri, S., Robert, T., Davoodi, S. M., Brar, S. K., Martel, R., Rouissi, T., & Lauzon, J.-M. (2023). Evaluation of scale-up effect on cold-active enzyme production and biodegradation tests using pilot-scale bioreactors and a 3D soil tank. *Journal of Hazardous Materials*, *450*. Scopus. https://doi.org/10.1016/j.jhazmat.2023.131078

Miri, S., Davoodi, S.M., Brar, S.K., Rouissi, T., Sheng, Y., Martel, R., 2021. Psychrozymes as novel tools to biodegrade p-xylene and potential use for contaminated groundwater in the cold climate. Bioresour. Technol. 321, 124464. https://doi.org/10.1016/j.biortech.2020.124464

Miri, S., Naghdi, M., Rouissi, T., Kaur Brar, S., Martel, R., 2019. Recent biotechnological advances in petroleum hydrocarbons degradation under cold climate conditions: A review. Crit. Rev. Environ. Sci. Technol. 49, 553–586. https://doi.org/10.1080/10643389.2018.1552070

Nadaf, G., 2011. Purification and characterization of catechol 1,2-dioxygenase (EC 1.13.11.1; catechol-oxygen 1,2-oxidoreductase; C12O) using the local isolate of phenol-degrading Pseudomonas putida | Folia Microbiologica https://link.springer.com/article/10.1007/s12223-023-01090-8 (accessed 11.19.24).

Nagarajan, K., Loh, K.-C., 2015. Formulation of microbial cocktails for BTEX biodegradation. Biodegradation 26, 51–63. https://doi.org/10.1007/s10532-014-9715-0

Netzer, F. von, Granitsiotis, M.S., Szalay, A.R., Lueders, T., 2020. Next-Generation Sequencing of Functional Marker Genes for Anaerobic Degraders of Petroleum Hydrocarbons in Contaminated Environments. Anaerob. Util. Hydrocarb. Oils Lipids 257–276. https://doi.org/10.1007/978-3-319-50391-2_15

Nikolopoulou, M., Kalogerakis, N., 2008. Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combined with biosurfactants and molasses. Mar. Pollut. Bull. 56, 1855–1861. https://doi.org/10.1016/j.marpolbul.2008.07.021

Olajire, A., Essien, J., 2014. Aerobic degradation of petroleum components by Microbial Consortia. J Pet Env. Biotechnol 5, 195–217.

Olukunle, O.F., 2023. Characterization of polycyclic aromatic hydrocarbon (PAH) degrading bacteria and optimization of physicochemical conditions for the production of catechol 1, 2-dioxygenase. NJPAS 4547–4558. https://doi.org/10.48198/NJPAS/22.B16

Ortega-González, D. K., Zaragoza, D., Aguirre-Garrido, J., Ramírez-Saad, H., Hernández-Rodríguez, C., & Jan-Roblero, J. (2013). Degradation of benzene, toluene, and xylene isomers by a bacterial consortium obtained from rhizosphere soil of Cyperus sp. Grown in a petroleum-contaminated area. *Folia Microbiologica*, *58*(6), 569–577. https://doi.org/10.1007/s12223-013-0248-4

Peters, F., Heintz, D., Johannes, J., van Dorsselaer, A., Boll, M., 2007. Genes, Enzymes, and Regulation of para-Cresol Metabolism in Geobacter metallireducens. J. Bacteriol. 189, 4729–4738. https://doi.org/10.1128/JB.00260-07

Pradhan, N., Ingle, A.O., 2007. Mineralization of phenol by a Serratia plymuthica strain GC isolated from sludge sample. International Biodeterioration & Biodegradation 60, 103–108. https://doi.org/10.1016/j.ibiod.2007.01.001

Rabus, R., Boll, M., Heider, J., Meckenstock, R.U., Buckel, W., Einsle, O., Ermler, U., Golding, B.T., Gunsalus, R.P., Kroneck, P.M.H., Krüger, M., Lueders, T., Martins, B.M., Musat, F., Richnow, H.H., Schink, B., Seifert, J., Szaleniec, M., Treude, T., Ullmann, G.M., Vogt, C., Bergen, M.V., Wilkes, H., 2016. Anaerobic Microbial Degradation of Hydrocarbons: From Enzymatic Reactions to the Environment. Microb. Physiol. 26, 5–28. https://doi.org/10.1159/000443997

Rabus, R., Wilkes, H., 2020. Functional Genomics of Sulfate-Reducing Bacteria Degrading Hydrocarbons, in: Boll, M. (Ed.), Anaerobic Utilization of Hydrocarbons, Oils, and Lipids. Springer International Publishing, Cham, pp. 225–232. https://doi.org/10.1007/978-3-319-50391-2_12

Rahman, W.U., Khan, M.D., Khan, M.Z., Halder, G., 2018. Anaerobic biodegradation of benzene-laden wastewater under mesophilic environment and simultaneous recovery of methane-rich biogas. J. Environ. Chem. Eng. 6, 2957–2964. https://doi.org/10.1016/j.jece.2018.04.038

Reitzel, L., 2005. Quantification of Natural Attenuation using Analytical-Chemical Tools.

Ron, E.Z., Rosenberg, E., 2014. Enhanced bioremediation of oil spills in the sea. Curr. Opin. Biotechnol. 27, 191–194. https://doi.org/10.1016/j.copbio.2014.02.004

Rotaru, A.E., Probian, C., Wilkes, H., Harder, J., 2010. Highly enriched Betaproteobacteria growing anaerobically with p-xylene and nitrate. FEMS Microbiol. Ecol. 71, 460–468. https://doi.org/10.1111/J.1574-6941.2009.00814.X

Roy, A., Dutta, A., Pal, S., Gupta, A., Sarkar, J., Chatterjee, A., Saha, A., Sarkar, P., Sar, P., Kazy, S.K., 2018. Biostimulation and bioaugmentation of native microbial community accelerated bioremediation of oil refinery sludge. Bioresour. Technol. 253, 22–32. https://doi.org/10.1016/j.biortech.2018.01.004

Said, K.A.M., Ismail, A.F., Karim, Z.A., Abdullah, M.S., Hafeez, A., 2021. A review of technologies for the phenolic compounds recovery and phenol removal from wastewater. Process Saf. Environ. Prot. 151, 257–289. https://doi.org/10.1016/j.psep.2021.05.015

Sathesh-Prabu, C., Woo, J., Kim, Y., Kim, S.M., Lee, S.B., Jeon, C.O., Kim, D., Lee, S.K., 2023. Comparative Genomic Analysis and BTEX Degradation Pathways of a Thermotolerant Cupriavidus cauae PHS1. J Microbiol Biotechnol 33, 875–885. https://doi.org/10.4014/jmb.2301.01011

Setlhare, B., Kumar, A., Mokoena, M.P., Olaniran, A.O., 2019. Catechol 1,2-Dioxygenase is an Analogue of Homogentisate 1,2-Dioxygenase in Pseudomonas chlororaphis Strain UFB2. International Journal of Molecular Sciences 20, 61. https://doi.org/10.3390/ijms20010061

Sheng, S., Liu, B., Hou, X., Wu, B., Yao, F., Ding, X., Huang, L., 2018. Aerobic Biodegradation Characteristic of Different Water-Soluble Azo Dyes. Int. J. Environ. Res. Public. Health 15, 35. https://doi.org/10.3390/ijerph15010035

Singh, T., Srivastava, N., Bhatiya, A. K., & Mishra, P. K. (2017). Analytical study of effective biodegradation of p-cresol using Serratia marcescens ABHI001: Application in bioremediation. *3 Biotech*, *7*(6), 384. https://doi.org/10.1007/s13205-017-1006-0

Soleimani, M., 2014. Comparison of Biological and Thermal Remediation Methods in Decontamination of Oil Polluted Soils. J. Bioremediation Biodegrad. 05. https://doi.org/10.4172/2155-6199.1000E145

Soo, P.-C., Horng, Y.-T., Lin, C.-S., Tsai, Y.-H., Wei, J.-R., Lu, C.-C., & Lai, H.-C. (2014). A Serratia marcescens Derived Biosensor for Detection of Environmental Xenobiotic Compounds. *Journal of Biomedical & Laboratory Sciences*, *26*(1), 1–9

Sperfeld, M., Rauschenbach, C., Diekert, G., Studenik, S., 2018. Microbial community of a gasworks aquifer and identification of nitrate-reducing Azoarcus and Georgfuchsia as key players in BTEX degradation. Water Res. 132, 146–157. https://doi.org/10.1016/j.watres.2017.12.040

Tandlich, R., Brežná, B., Dercová, K., 2001. The effect of terpenes on the biodegradation of polychlorinated biphenyls by Pseudomonas stutzeri. Chemosphere 44, 1547–1555. https://doi.org/10.1016/S0045-6535(00)00523-3

Takáčová, A., Smolinská, M., Semerád, M., & Matúš, P. (2015). *DEGRADATION OF BTEX BY MICROALGAE Parachlorella kessleri*.

Tiedt, O., Fuchs, J., Eisenreich, W., Boll, M., 2018. A catalytically versatile benzoyl-CoA reductase, key enzyme in the degradation of methyl- and halobenzoates in denitrifying bacteria. J. Biol. Chem. 293, 10264–10274. https://doi.org/10.1074/jbc.RA118.003329

Tremblay, P.-L., Zhang, T., 2020. Functional Genomics of Metal-Reducing Microbes Degrading Hydrocarbons. Anaerob. Util. Hydrocarb. Oils Lipids 233–253. https://doi.org/10.1007/978-3-319-50391-2_13

Ulrich, A.C., Beller, H.R., Edwards, E.A., 2005. Metabolites detected during biodegradation of 13C 6-benzene in nitrate-reducing and methanogenic enrichment cultures. Environ. Sci. Technol. 39, 6681–6691. https://doi.org/10.1021/ES050294U

Usman, N., Atta, H. I., & Tijjani, M. B. (2020). Biodegradation Studies of Benzene, Toluene, Ethylbenzene and Xylene (BTEX) Compounds by Gliocladium sp. And Aspergillus terreus. *Journal of Applied Sciences and Environmental Management*, *24*(6), Article 6. https://doi.org/10.4314/jasem.v24i6.19

US EPA, O., 2014. How to Evaluate Alternative Cleanup Technologies for Underground Storage Tank Sites: A Guide for Corrective Action Plan Reviewers [WWW Document]. URL https://www.epa.gov/ust/how-evaluate-alternative-cleanup-technologies-underground-storage-tank-sites-guide-corrective (accessed 3.5.24).

Villatoro-Monzón, W.R., Morales-Ibarria, M.G., Velázquez, E.K., Ramírez-Saad, H., Razo-Flores, E., 2008. Benzene biodegradation under anaerobic conditions coupled with metal oxides reduction. Water. Air. Soil Pollut. 192, 165–172. http://dx.doi.org/10.1007/s11270-008-9643-x

Vogt, C., Kleinsteuber, S., Richnow, H.H., 2011. Anaerobic benzene degradation by bacteria. Microb. Biotechnol. 4, 710–724. https://doi.org/10.1111/J.1751-7915.2011.00260.X

Wang, Z., Yang, C., Yang, Z., Brown, C.E., Hollebone, B.P., Stout, S.A., 2016. Petroleum biomarker fingerprinting for oil spill characterization and source identification. Stand. Handb. Oil Spill Environ. Forensics 131–254. https://doi.org/10.1016/B978-0-12-803832-1.00004-0

Wang, Y., Meng, F., Li, H., Zhao, S., Liu, Q., Lin, Y., Wang, G., & Wu, J. (2019). Biodegradation of phenol by Isochrysis galbana screened from eight species of marine microalgae: Growth kinetic models, enzyme analysis and biodegradation

pathway. *Journal of Applied Phycology*, *31*(1), 445–455. https://doi.org/10.1007/s10811-018-1517-z

Wartell, B., Boufadel, M., Rodriguez-Freire, L., 2021. An effort to understand and improve the anaerobic biodegradation of petroleum hydrocarbons: A literature review. Int. Biodeterior. Biodegrad. 157, 105156. https://doi.org/10.1016/J.IBIOD.2020.105156

Weelink, S.A.B., Eekert, M.H.A. van, Stams, A.J.M., 2010. Degradation of BTEX by anaerobic bacteria: physiology and application. Rev. Environ. Sci. Biotechnol. 2010 94 9, 359–385. https://doi.org/10.1007/S11157-010-9219-2

Weidenweber, S., Schühle, K., Demmer, U., Warkentin, E., Ermler, U., Heider, J., 2017. Structure of the acetophenone carboxylase core complex: prototype of a new class of ATP-dependent carboxylases/hydrolases. Sci. Rep. 2017 71 7, 1–10. https://doi.org/10.1038/srep39674

Wongbunmak, A., Khiawjan, S., Suphantharika, M., & Pongtharangkul, T. (2017). BTEX- and naphthalene-degrading bacterium Microbacterium esteraromaticum strain SBS1-7 isolated from estuarine sediment. *Journal of Hazardous Materials*, *339*, 82–90. https://doi.org/10.1016/J.JHAZMAT.2017.06.016

Wongbunmak, A., Khiawjan, S., Suphantharika, M., Pongtharangkul, T., 2020. BTEX biodegradation by Bacillus amyloliquefaciens subsp. plantarum W1 and its proposed BTEX biodegradation pathways. Sci. Rep. 10, 17408. https://doi.org/10.1038/s41598-020-74570-3

Wu, Z., Liu, G., Ji, Y., Li, P., Yu, X., Qiao, W., Wang, B., Shi, K., Liu, W., Liang, B., Wang, D., Yanuka-Golub, K., Freilich, S., Jiang, J., 2022. Electron acceptors determine the BTEX degradation capacity of anaerobic microbiota via regulating the microbial

community. Environ. Res. 215, 114420. https://doi.org/10.1016/J.ENVRES.2022.114420

Wu, H., Du, X., Wu, W., Zheng, J., Song, J., & Xie, J. (2023). Metagenomic analysis reveals specific BTEX degrading microorganisms of a bacterial consortium. *AMB Express*, *13*(1), 48. https://doi.org/10.1186/s13568-023-01541-y

Yavas, A., & Icgen, B. (2018). Aerobic Bacterial Degraders With Their Relative Pathways for Efficient Removal of Individual BTEX Compounds. *CLEAN – Soil, Air, Water*, *46*(11), 1800068. https://doi.org/10.1002/clen.201800068

Yoshikawa, M., Zhang, M., Toyota, K., 2017. Biodegradation of Volatile Organic Compounds and Their Effects on Biodegradability under Co-Existing Conditions. Microbes Environ. 32, 188–200. https://doi.org/10.1264/JSME2.ME16188

Zhou, Y., Huang, H., Shen, D., 2016. Multi-substrate biodegradation interaction of 1, 4-dioxane and BTEX mixtures by Acinetobacter baumannii DD1. Biodegradation 27, 37–46. https://doi.org/10.1007/s10532-015-9753-2

APPENDICES

APPENDIX A: Supplementary information for chapter 3

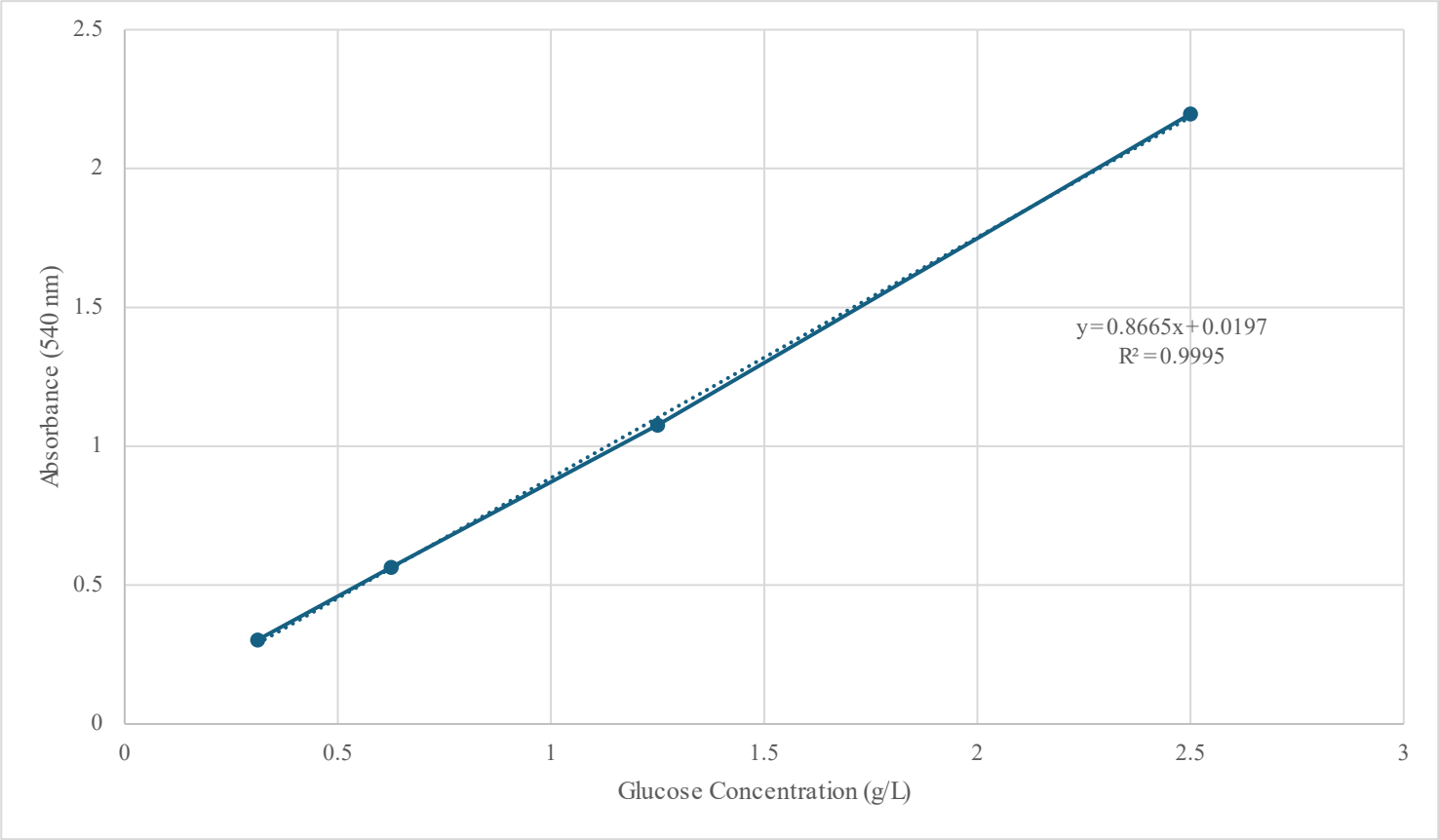


Figure A1. Calibration curve for Glucose consumption using the DNS method

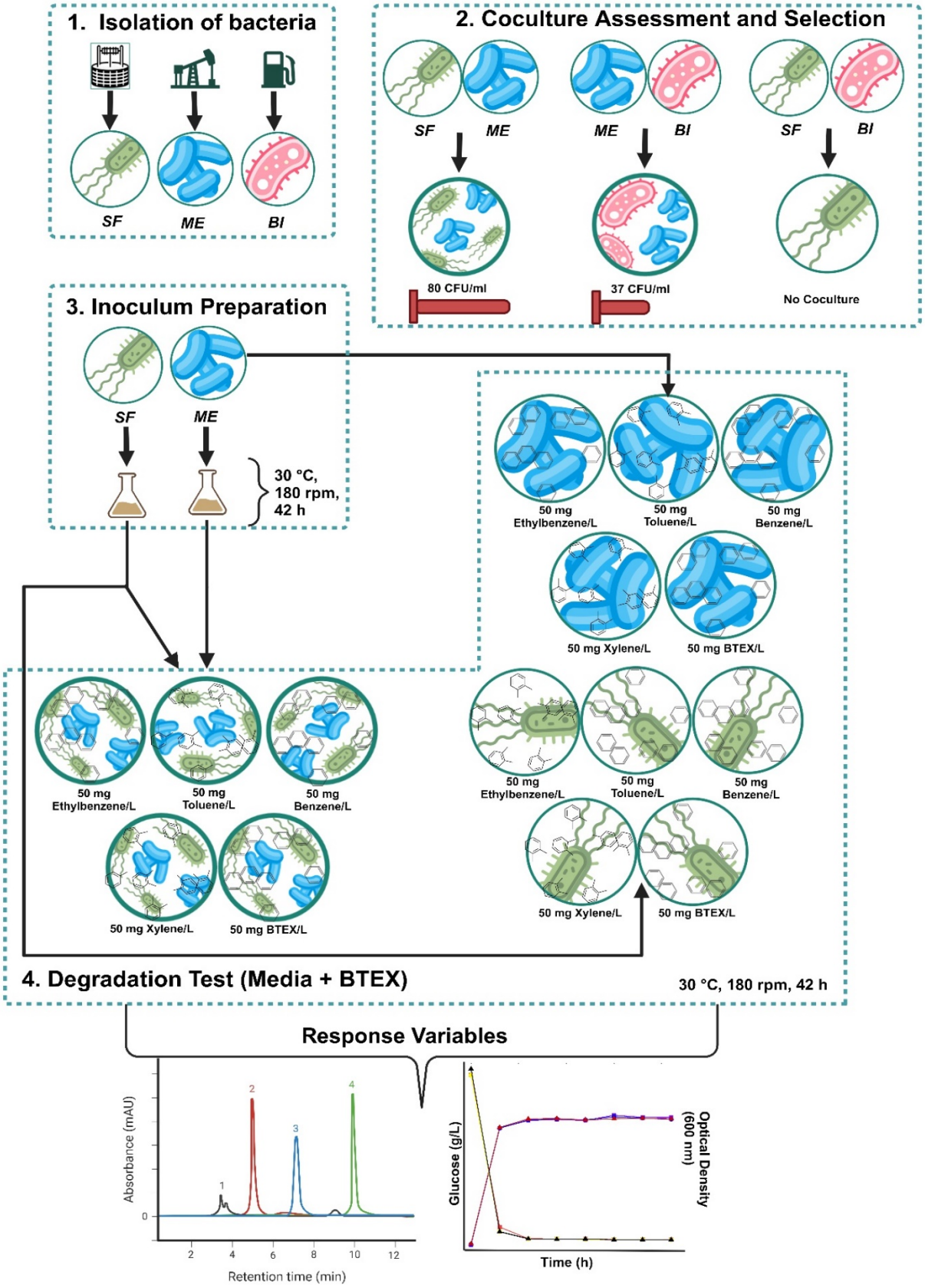
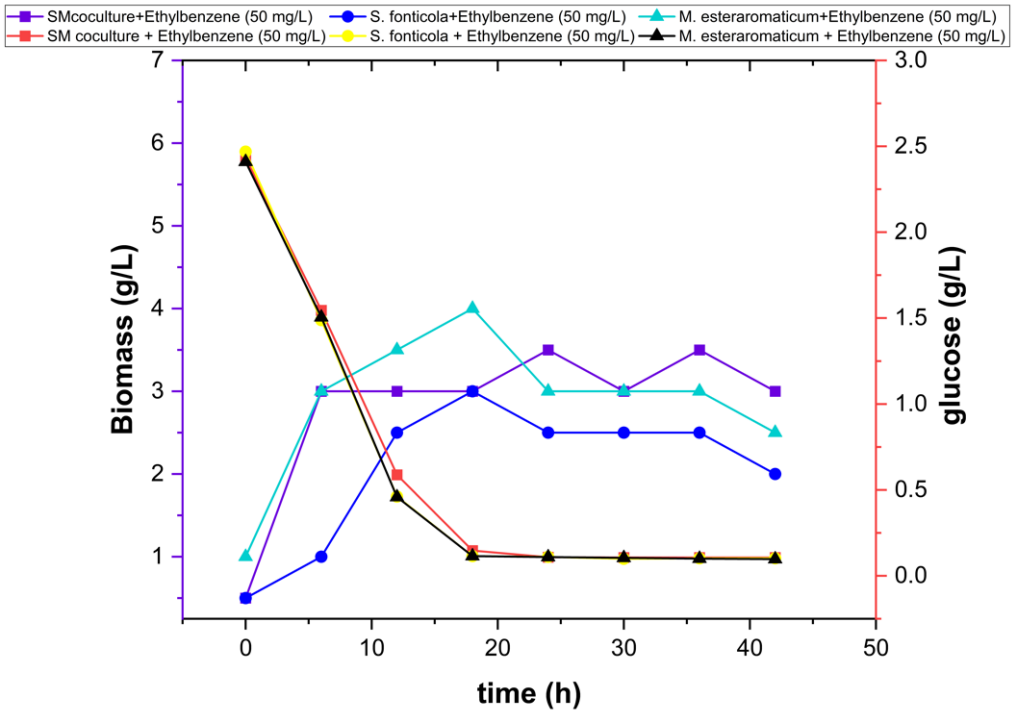
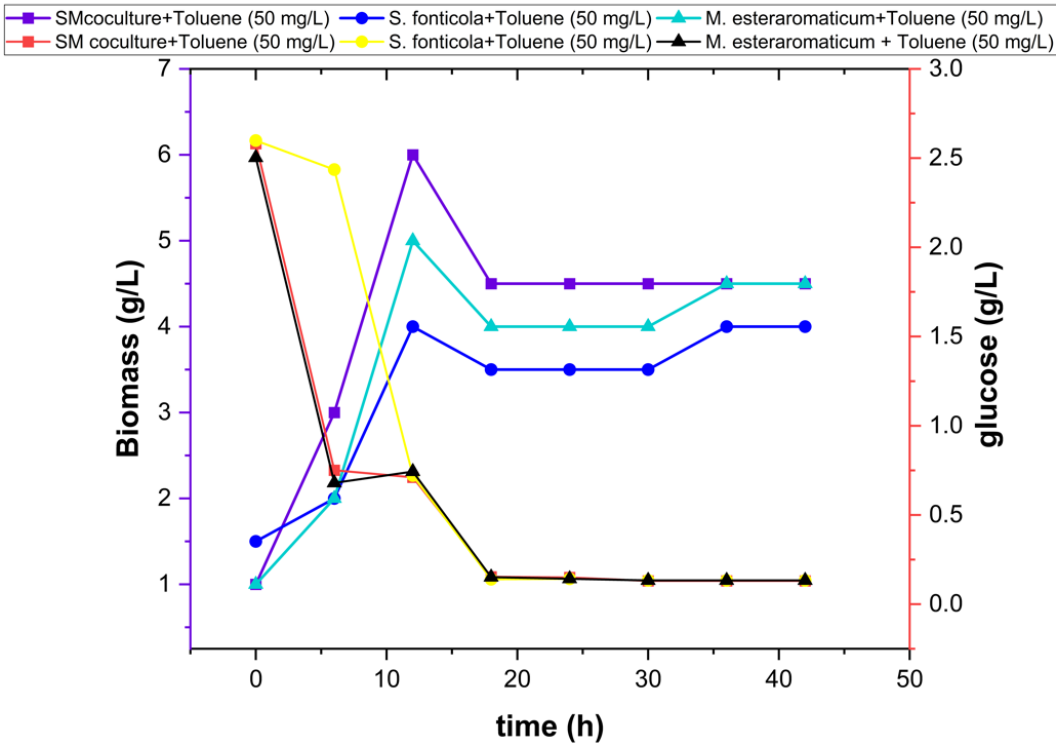
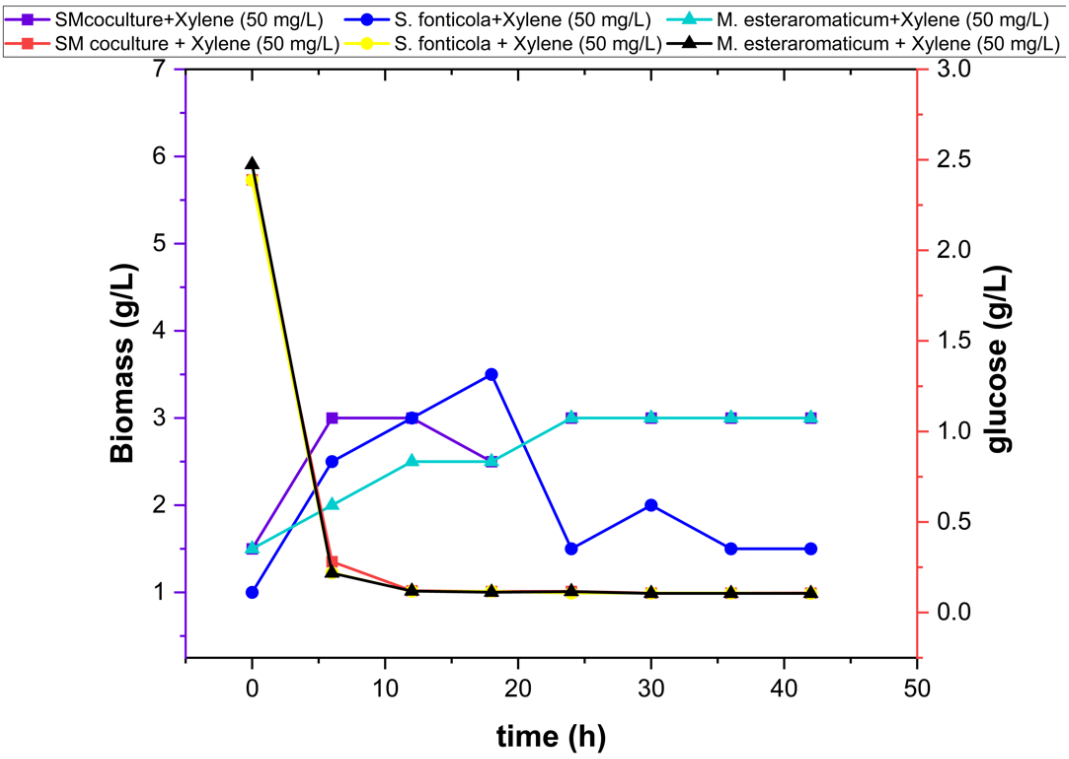
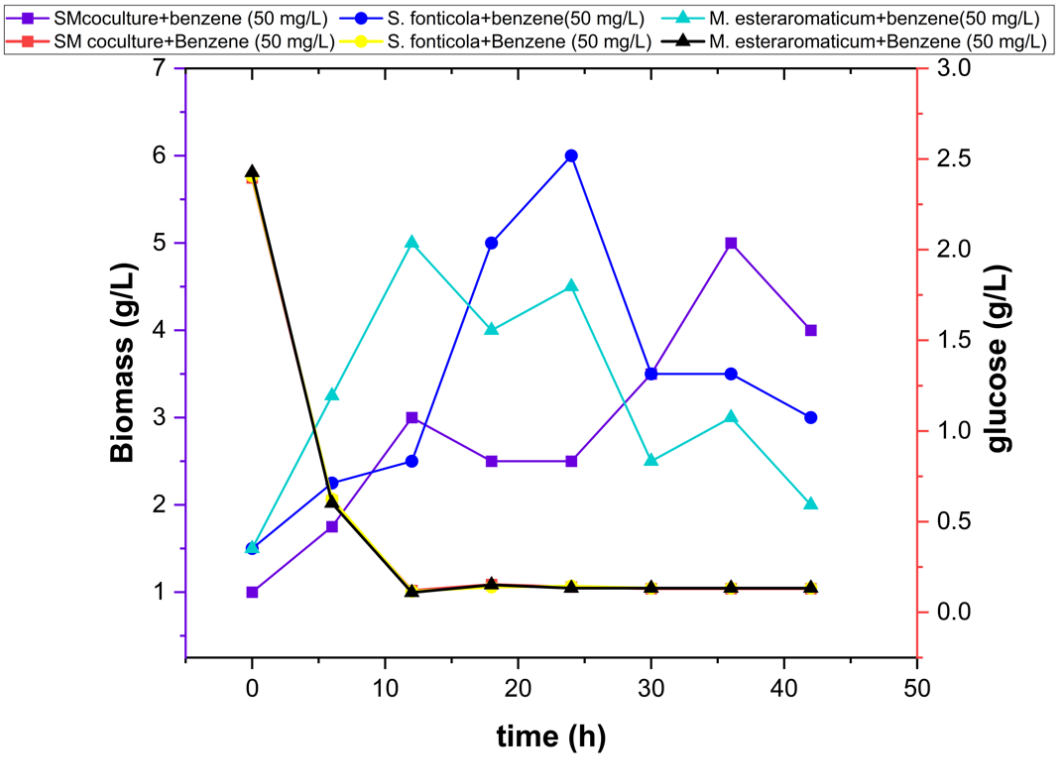


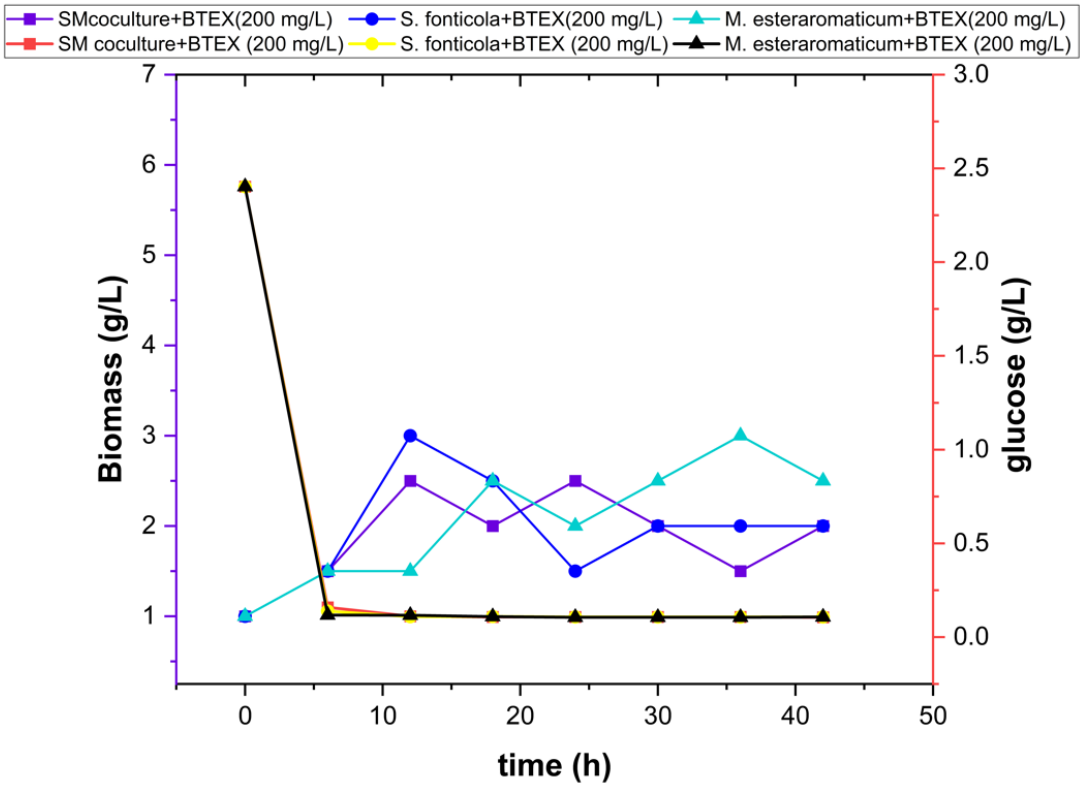
Figure A2. Methodology for the formulation of BTEX-degrading bacterial cocultures


SMcoculture+Ethylbenzene (50 mg/L)
S. fonticola+Ethylbenzene (50 mg/L)
M. esteraromaticum+Ethylbenzene (50 mg/L)
SM coculture + Ethylbenzene (50 mg/L)
S. fonticola + Ethylbenzene (50 mg/L)
M. esteraromaticum + Ethylbenzene (50 mg/L)
Biomass (g/L)
glucose (g/L)
time (h)


SMcoculture+Toluene (50 mg/L)
S. fonticola+Toluene (50 mg/L)
M. esteraromaticum+Toluene (50 mg/L)
SM coculture+Toluene (50 mg/L)
S. fonticola+Toluene (50 mg/L)
M. esteraromaticum + Toluene (50 mg/L)
Biomass (g/L)
glucose (g/L)
time (h)


SMcoculture+Xylene (50 mg/L)
S. fonticola+Xylene (50 mg/L)
M. esteraromaticum+Xylene (50 mg/L)
SM coculture + Xylene (50 mg/L)
S. fonticola + Xylene (50 mg/L)
M. esteraromaticum + Xylene (50 mg/L)
Biomass (g/L)
glucose (g/L)
time (h)


SMcoculture+benzene (50 mg/L)
S. fonticola+benzene(50 mg/L)
M. esteraromaticum+benzene(50 mg/L)
SM coculture+Benzene (50 mg/L)
S. fonticola+Benzene (50 mg/L)
M. esteraromaticum+Benzene (50 mg/L)
Biomass (g/L)
glucose (g/L)
time (h)


SMcoculture+BTEX(200 mg/L)
S. fonticola+BTEX(200 mg/L)
M. esteraromaticum+BTEX(200 mg/L)
SM coculture+BTEX (200 mg/L)
S. fonticola+BTEX (200 mg/L)
M. esteraromaticum+BTEX (200 mg/L)
Biomass (g/L)
glucose (g/L)
time (h)

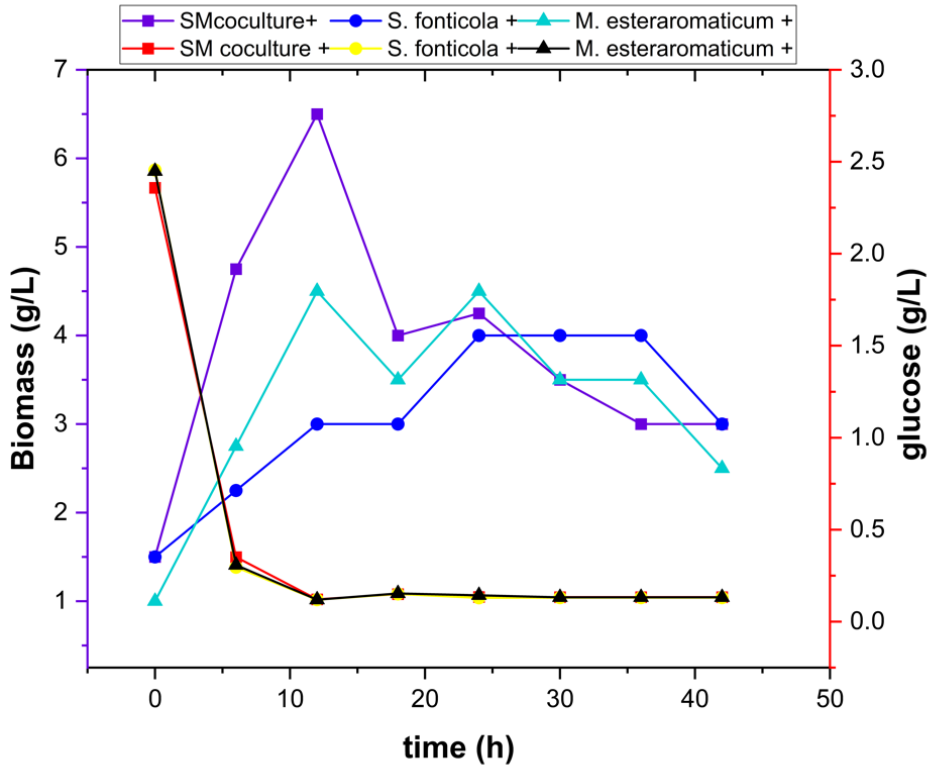

SMcoculture+
S. fonticola +
M. esteraromaticum +
SM coculture +
S. fonticola +
M. esteraromaticum +
Biomass (g/L)
glucose (g/L)
time (h)

Figure A3. Biomass cell production and sugar consumption of S. *fonticola*, M. *esteraromaticum*, and coculture Strains in TSB Media Supplemented with BTEX compounds (50 mg L-1) individually and collectively (200 mg L-1).

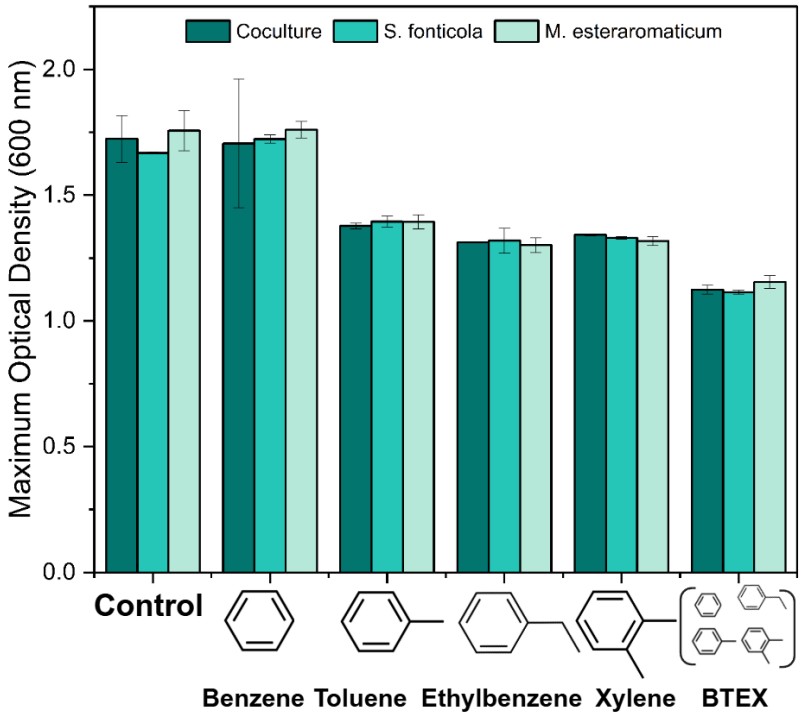


Figure A4. Maximum Microbial Growth in the presence of BTEX (50 mg L-1)

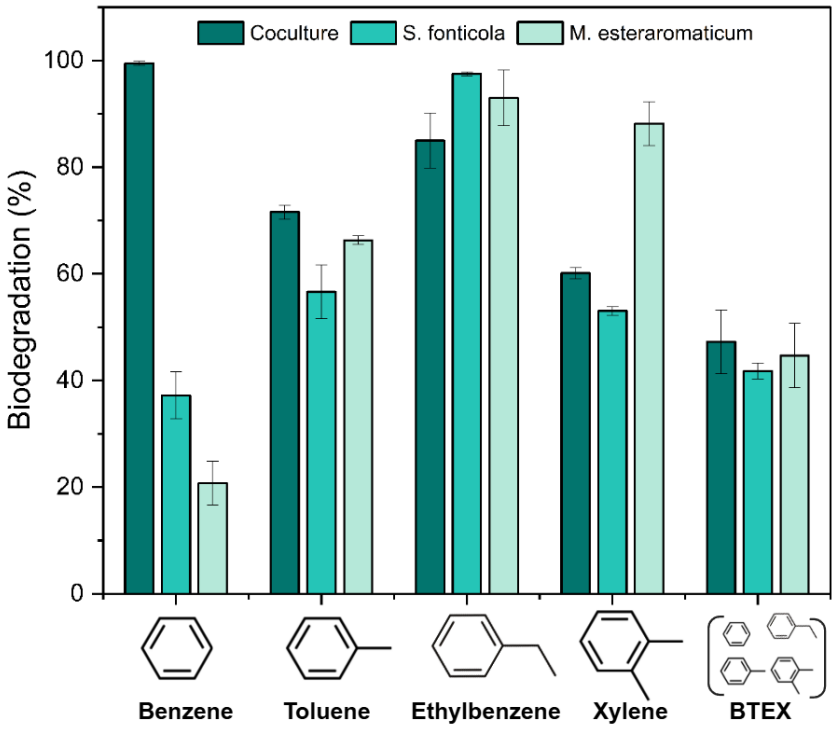


Figure A5. Biodegradation achieved by the single strains and coculture after 42 hours.

APPENDIX B: Supplementary information for chapter 4

Stress-Induced Catechol 1,2 Dioxygenase Activity

The enzyme production for Catechol 1,2 dioxygenase (C12O) was monitored based on the product formation of cis-cis muconic acid (260 nm) in the reaction mixture during the enzyme assay. Likewise, the C12O production in the coculture and the individual strains was monitored based on the activity of C12O at different supplemented concentrations of BTEX mixture (0, 200 and 500 mg L-1) and benzyl alcohol (0, 0.25, 1 and 3 mM), which is displayed in figure B1.

C12O in coculture, *M. esteraromaticum* and *S. fonticola* presented a maximum activity in all the treatments between 16 and 32 hours of fermentation. Moreover, all the treatments supplemented with benzyl alcohol presented a C12O maximum activity in coculture at 16 hours except for the treatments **(I, V))** supplemented with 3 mM of benzyl alcohol, which presented a maximum C12O activity at 32 hours. The highest C12O activity in coculture was observed either in the presence of benzyl alcohol or BTEX, specifically, in the treatments supplemented with 0.25 mM of benzyl alcohol and 200 mg BTEX L-1 (0.026 U mg-1); 0.25 mM of benzyl alcohol with no BTEX (0.027 U mg-1); and 200 mg BTEX L-1 with no benzyl alcohol (0.027 U mg-1). In the other treatments the activity of C12O in coculture was between (0.017 – 0.021 U mg-1), with the lowest activity observed in the treatments supplemented with 3mM of benzyl alcohol.

On the other hand, the C12O in M. *esteraromaticum* and S. *fonticola* achieved a maximum activity at 32 hours except for the treatments in which benzyl alcohol was the only inducer

supplemented (0.25, 1 and 3 mM) and taking about 16 hours to achieve the maximum activity. The maximum C12O activity was achieved faster in the treatments with lower concentrations of benzyl alcohol. Another exception was the treatment with 500 mg BTEX L-1 and no benzyl alcohol (0.019 U mg-1), in which the individual strains achieved the C12O maximum activity before 32 hours.

Moreover, the C12O highest activity was observed to be in the treatment **(VIII)** containing 200 mg BTEX L-1 with no benzyl alcohol (0.0189 U/mg) and treatment **(IV)** 200 mg BTEX L-1 with 0.25 mM of benzyl alcohol (0.023 U mg-1) for M. *esteraromaticum* and S. *fonticola*, respectively. Figure B1,B and B1,C. This represents 53 % and 16% less than the maximum for coculture in the same treatments for M. *esteraromaticum* and S. *fonticola*, respectively.

The role of benzyl alcohol as inducer in the expression of BTEX-degrading enzymes such as C12O is sensitive to the concentration of benzyl alcohol supplemented. That said, a high concentration of benzyl alcohol can negatively impact and result in the C12O expression. For example, the treatments with the highest concentration of benzyl alcohol (3 mM) presented a delay of 16 hours to achieve the maximum C12O activity compared to the treatments with a lower concentration of benzyl alcohol in the coculture (1 and 0.25 mM).

This can be explained by benzyl alcohol having an additional contribution to the BTEX-derived oxidative stress given the accumulation of catechol and benzoate, important intermediates during toluene, benzene, xylene and benzyl alcohol biodegradation pathways (Wongbunmak et al., 2020).

A higher oxidative stress can result in the suppression of C12O enzyme activity by disrupting amino acids such as arginine and cysteine prone to oxidation, which ultimately inhibit its

function and alter the C12O structure (Fasnacht and Polacek, 2021). This can be demonstrated by comparing the treatment **(I)** with 3 mM of benzyl alcohol and 200 mg BTEX L-1, with the treatment **(V)** supplemented only with 3mM of benzyl alcohol, in which the first had 39% less C12O activity in coculture. Likewise, the toxic effect of benzyl alcohol it is evident in the coculture performance parameters such as the microbial growth and the glucose consumption. When compared the treatment with only 200 mg of BTEX L-1 with the treatment containing the same concentration of BTEX and 3mM of benzyl alcohol, the growth is 4e8 cells ml-1 higher. The rapid microbial growth disruption caused by Benzyl alcohol can help explain this phenomenon. Such disruption in the growth can be due to its adverse effect in the fluidisation phenomena in the bacterial membrane structure or membrane anisotropy – A disorder on the membrane fatty acids levels (Konopásek et al., 2000). The glucose consumption also revealed the toxicity of benzyl alcohol at high concentrations (3mM) with only 28% of the glucose consumed in 48 hours, whereas the control consumed 100% in less than 8 hours. As catechol-derivates such as 3-methylcatechol from the degradation of benzyl alcohol can also inhibit enzymes in the Krebs cycle, slowing down cell respiration and C12O production (Wang et al., 2019).

Based on the results from this experiment, it can be stated that either BTEX (200 mg L-1) or benzyl alcohol (0.25 mM) induce C12O activity (0.026 U mg-1) at low concentrations. However, when compared to other studies, the C12O activity induced was significantly low. Studies reported 5-6 times higher C12O activity for *Serratia* sp. (0.134 U mg-1) and *Microbacterium* sp. (0.140U mg-1) in the presence of phenol and naphthalene (Olukunle, 2023; Pradhan and Ingle, 2007). This difference is similar when compared to other BTEX-

degrading strains such as P. *synxantha* with a high C12O (0.12 U mg-1) after 48 hours (Miri et al., 2021).

Likewise, the literature demonstrates that relevant parameters such as pH, temperature should be optimized for a higher activity of C12O. Importantly, the optimal pH values (7-8) as well as temperature (22-30)°C for the maximum C12O activity are contradictory with the conditions for the enzyme assay (Nadaf, 2011; Setlhare et al., 2019). Thus, strain-specific optimization studies in addition to a purification step of the enzyme can be explored to improve C12O activity in the coculture.

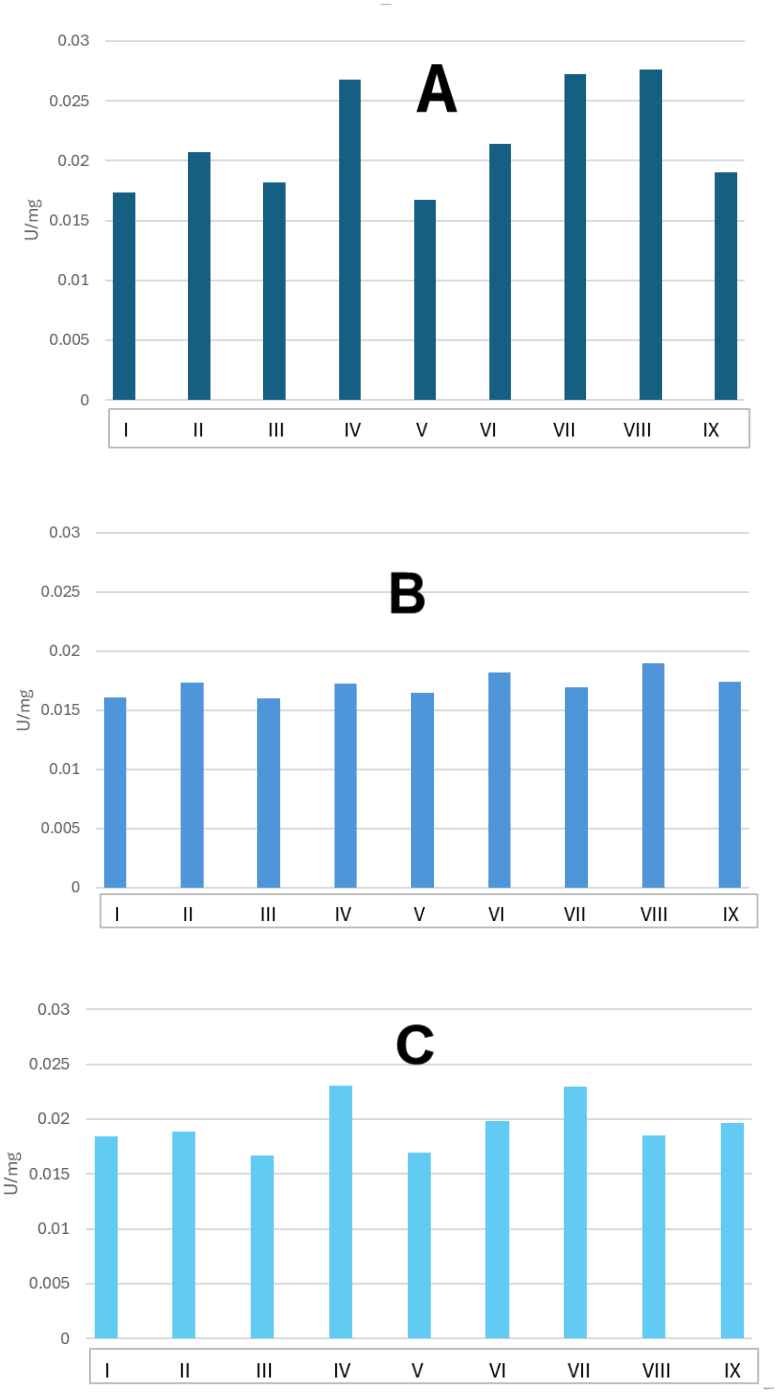


Figure B1. Catechol 1,2 dioxygenase (C12O) determination in the coculture between *M. esteraromaticum* and *S. fonticola,* and the individual strains in the presence of benzyl alcohol (0, 0.25, 1 and 3 mM) and BTEX mixture (0, 200 and 500 mg L-1). The enzyme production in the different treatments is display for **A)** Coculture **B)** *M. esteraromaticum* **C)** *S. fonticola*

as follows: **I).** 3 mM Benzyl Alcohol and 200 mg BTEX L-1 **II).** 1 mM Benzyl Alcohol and 200 mg BTEX L-1 **III).** 0.25 mM Benzyl Alcohol and 500 mg BTEX L-1 **IV).** 0.25 mM Benzyl Alcohol and 200 mg BTEX L-1 **V).** 3 mM Benzyl Alcohol, 0 mg BTEX L-1 **VI).** 1 mM Benzyl Alcohol, 0 mg BTEX L-1 **VII).** 0.25 mM Benzyl Alcohol, 0 mg BTEX L-1 **VIII).** 0 mM Benzyl Alcohol, 200 mg BTEX L-1 **IX).** 0 mM Benzyl Alcohol, 500 mg BTEX L-1.

Table B1. Experimental design for the supplementation of Benzyl alcohol as an inducer of catechol 1,2 dioxygenase (C1,2O) and catechol 2,3 dioxygenase (C2,3O) activity in a BTEX-degrading coculture between S. *fonticola* and M. *esteraromaticum.*

Treatment	Benzyl Alcohol (mM)	BTEX conc. (mg/L)	Response Variables
Co-culture S. *fonticola* M. *esteraromaticum*	5	500	Glucose Consumption (DNS)
	1	350	
	1	500	Biomass Production (Dry Weight)
	1	200	
	3	200	
	5	350	Microbial Growth (Optical Density 600nm)
	5	200	
	3	500	
	3	350	
Negative control (NO bacteria)	0	200	BTEX concentration (GC analysis)
		350	
		500	
Positive control 1 (NO BTEX)	1	0	Total Protein (Bradford)
	3		
	5		
Positive control 2 (NO inducer)	0	200	C1,2O and C2,3O enzyme activity
		350	
		500	
Positive control 3	0	0	

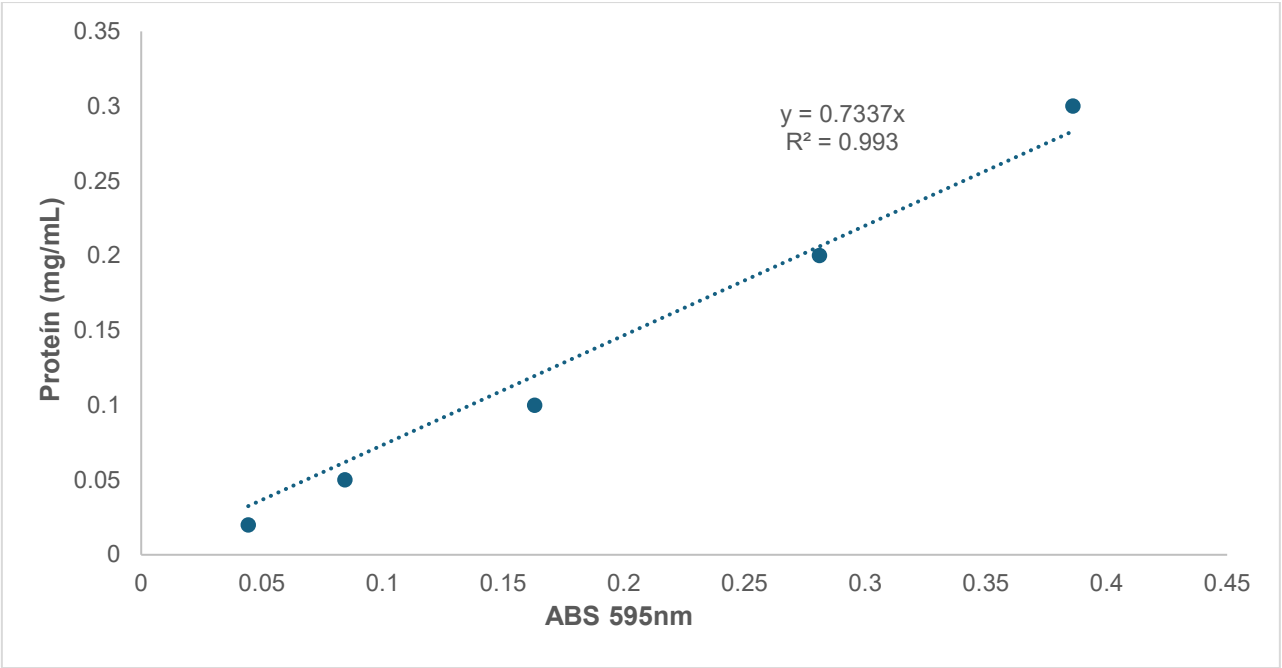


Figure B2. Calibration curve for the Total soluble protein using the Bradford Method

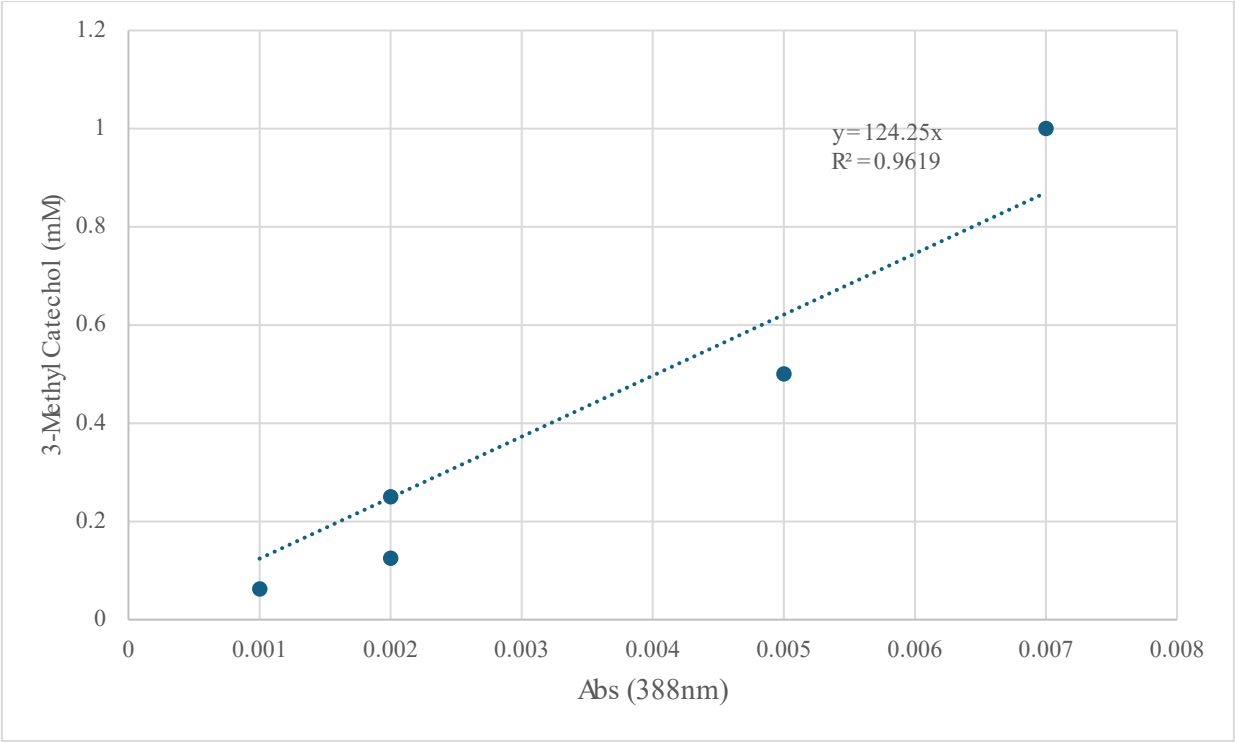


Figure B3. Calibration curve for the C23O assay based on the formation of 3-Methyl catechol

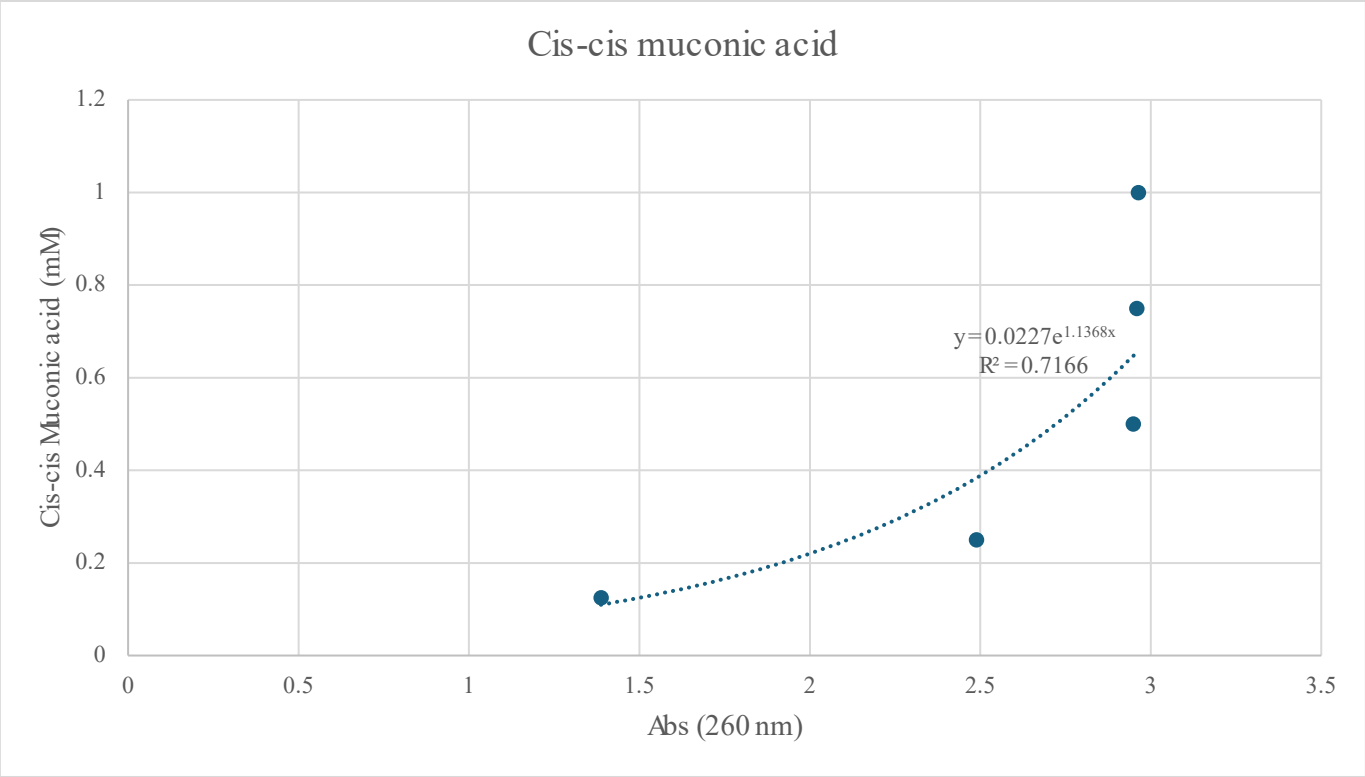


Figure B3. Calibration curve for the C12O assay based on the formation of Cis-cis Muconic acid