Hydrocarbon Degradation by Halophilic Bacteria

Undergraduate Honors Thesis

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Abstract

Halophilic microorganisms are a viable option for the bioremediation of oil spills in saline regions worldwide. Halophiles can be found among all three branches in the tree of life, including bacteria. A mixed culture capable of degrading BTEX (a 1:1:1:1 mixture of benzene, toluene, ethylbenzene, and xylene) under conditions of high salinity (2.5 M NaCl) was enriched from a crude oil-contaminated sediment from Kuwait. The enrichment's metagenome revealed that the culture was dominated by organisms belonging to the genus *Arhodomonas* (>99% abundance). Functional analysis revealed the presence of numerous genes that code for aromatic ring hydroxylating and ring cleaving enzymes, and most of the downstream genes needed for complete mineralization dearomatized intermediates. A pure culture of bacteria that degrades BTEX at high salinity was isolated from produced water collected from the Wilcox oil production facility in Payne County, OK. Amplification of 16S rRNA-gene of the isolate showed >99% sequence similarity to *Modicisalibacter tunisiensis*, a species previously isolated from fracking water in Tunisia. The genome of strain Wilcox was sequenced and its biodegradation potential of petroleum compounds was assessed *in silico.* Laboratory-scale microcosms containing produced water amended BTEX as representative hydrocarbons were set up to test the bioremediation capacity of both Kuwait enrichment and *Modicisalibacter* sp. strain Wilcox. Results showed that both cultures efficiently degrade BTEX and other hydrocarbons at high salinity. Strain Wilcox appears to have the capacity to degrade ethylbenzene as the sole carbon source under nitrate-respiring conditions. These observations are supported by the presence of nitrate reductase encoding genes in the genome.

Introduction

Hypersaline environments are present across the globe. Examples include deep sea brine, salt lakes and salt flats, coastal lagoons, and man-made salterns. The organisms that populate them and require high salinities to live, are known as halophiles, literally “salt-lovers.” Halotolerant organisms, on the other hand, do not require hypersaline conditions but can still grow in them. Halotolerant microbes accumulate compatible solute compounds when present in conditions of high salinity, to create an osmotic balance with the saline external environment. In conditions of lower salinity, these microbes secrete the accumulated compatible solutes. Halophilic microbes employ a different strategy to combat the effects of salinity. Their strategy, known as “salting in,” is to intake K+ salt ions from the environment to counterbalance Na+ extracellular ion concentrations, and therefore must have adaptations such as enzymes that can withstand the salinity and mechanisms of protecting DNA from it. These organisms will lyse if removed from high salinity environments.

Previous studies have been conducted on the potential for the usage of halophiles in hydrocarbon degradation, as well as in various industries. Halophiles produce a variety of secondary metabolites, compounds which have uses from dermatological applications (borinic acid) and potential medicines (aziridine) to plastic production (styrene) and wine flavoring (ethyl acetate) (Selvarajan 2017). Outside of industrial applications, halophiles have been considered for their potential in bioremediation.

Hydrocarbon contaminated water is a byproduct of oil extraction. These compounds are toxic, mutagenic and carcinogenic. Ecosystems such as salt marshes and coastlines could benefit from halophilic microbial bioremediation in the case of oil spills or pipeline leaks. The use of halophiles to degrade hydrocarbons in hypersaline environments has the benefit that there is no need for added steps to remove or dilute the salt before bioremediation can occur. Many studies have been done characterizing the degradation of various hydrocarbons by diverse microorganisms under saline conditions (see Table 1).

With an increase in salinity, dissolved hydrocarbons experience a decrease in solubility. To combat this effect and be more suitable for bioremediation efforts, microbes may need to produce biosurfactants. Surfactants are compounds which reduce surface tension. They allow for easier uptake of hydrocarbons for metabolism by the bacteria. Members of multiple genera, including *Halomonas*, *Marinobacter*, *Brevibacterium*, and *Idiomarina*, were shown to have 50% or more emulsion for both diesel and kerosene (Gomes et al, 2016).

A species of particular interest to the Fathepure lab is *Modicisalibacter* sp. strain Wilcox. This strain was recently isolated from saline produced water by a graduate student in the lab, William Marsh. This organism is phylogenetically (> 99% 16S rRNA-gene sequence similarity) related to the type strain, *Modicisalibacter tunisiencis* isolated from fracking water in Tunisia (Gomes et al, 2016).

Degrader	Hydrocarbon	Salinity (%w/v)	Ref
Alipathic Hydrocarbons			
Actinopolyspora sp.	Pentadecane ($C_{15}H_{32}$) Eicosane ($C_{20}H_{42}$)	25	Al-Mueini et al. (2007)
Actinopolyspora sp. DPD1	Pentacosane ($C_{25}H_{52}$)	25	Al-Mueini et al. (2007)
Alcanivorax sp. Qtet3	Pristane ($C_{19}H_{20}$) Eicosane ($C_{20}H_{42}$) Tetracosane ($C_{24}H_{50}$)	0-15	Dastgheib et al. (2011a,b)
Bacillus sp. DHT	Decane ($C_{10}H_{22}$) Hexadecane ($C_{15}H_{34}$)	10	Kumar et al. (2007)
Bacillus sp. DS1	Hexadecane ($C_{15}H_{34}$)	12-20	Sass et al. (2008)
Haloarcula sp.	Heptadecane ($C_{17}H_{36}$)	>22	Tapilatu et al. (2010)

	Eicosane ($C_{20}H_{42}$)	>22	Tapilatu et al. (2010)
Haloarcula vallismortis EH4	Tetradecane ($C_{14}H_{30}$) Hexadecane (C15H34) Pristane (C19H20) Eicosane (C20H42) Heneicosane (C21H44)	>20	Bertrand et al.(1990)
Halobacterium sp.	Octadecane ($C_{18}H_{38}$)	>26	Al-Mailem et al. (2010)
Halococcus sp.	Octadecane ($C_{18}H_{38}$)	>26	Al-Mailem et al. (2010)
Haloferax sp.	Octadecane ($C_{18}H_{38}$) Heptadecane ($C_{17}H_{36}$) Eicosane ($C_{20}H_{42}$)	>26 >22 >22	Al-Mailem et al. (2010) Tapilatu et al. (2010) Tapilatu et al. (2010)
Halomonas sp. C2SS100	Hexadecane ($C_{15}H_{34}$)	19	Mnif et al. (2009)
Halomonas xianhensis SUR308	Octane (C_8H_{18})	20	Biswas et al. (2015)
Halorientalis sp.	Hexadecane (C15H34)	21	Zhao et al. (2017)
Marinobacter aquaeolei	Pentadecane ($C_{15}H_{32}$) Hexadecane ($C_{15}H_{34}$) Pristane ($C_{19}H_{20}$)	0-20	Huu et al. (1990)
Marinobacter hydrocarbonoclasticus	Hexadecane ($C_{15}H_{34}$) Pristane ($C_{19}H_{20}$) Eicosane ($C_{20}H_{42}$) Heneicosane ($C_{21}H_{44}$)	4.6-20 4.6-20 1-14 4.6-20	Gauthier et al.(1992) Gauthier et al.(1992) Fernandez-Linares et al. (1996) Gauthier et al.(1992)
Psuedomonas sp C450R	Hexadecane ($C_{15}H_{34}$)	10	Mnif et al. (2009)
Polycyclic Aromatic Hydrocarbons			
Actinopolyspora sp. DPD1	Fluorene	5-20	Al-Mueini et al. (2007)
Bacillus sp strain DHT	Naphthalene	10	Kumar et al. (2007)
	Pyrene	10	Kumar et al. (2007)
Chromohalobacter	Anthracene Biphenol Naphthalene Phenanthrene	8.8	Al-Mailem et al. (2017)
Haloarcula hispanica	Naphthalene	20	Erdogmuş et al. (2013)

	Phenanthrene	20	Erdogmuş et al. (2013)
	Pyrene	20	Erdogmuş et al. (2013)
Haloarcula vallismortis (EH4)	Acenaphthene	>20	Bertrand et al.(1990)
	Anthracene	>20	Bertrand et al.(1990)
	Phenanthrene	>20	Bertrand et al.(1990)
Halobacterium salinarum	Naphthalene	20	Erdogmuş et al. (2013)
	Phenanthrene	20	Erdogmuş et al. (2013)
	Pyrene	20	Erdogmuş et al. (2013)
Halobacterium sp.	Biphenol	>26	Al-Mailem et al. (2010)
Halococcus sp.	Biphenol	>26	Al-Mailem et al. (2010)
Haloferax sp.	Anthracene Biphenol Naphthalene Pyrene	20 >26 20 20	Bonfá et al. (2011) Al-Mailem et al. (2010) Bonfá et al. (2011) Bonfá et al. (2011)
Halomonas shengliensis MCAT 10	Phenanthrene Pyrene	9	Gomes et al. (2016)
Halomonas sp.	Phenanthrene	20	Al-Mailem et al. (2017)
Halomonas	Benzopyrene Naphtelene	0-10	Govarthanan et al. (2020)
Idiomarina sp. MOD 32J	Benzopyrene	9	Gomes et al. (2016)
Idiomarina sp. R2A 23.10	Phenanthrene Pyrene	9	Gomes et al. (2016)
Marinobacter flavimaris R2A 36J	Benzopyrene Naphthalene Phenanthrene Pyrene	9	Gomes et al. (2016)
Marinobacter nanhaiticus	Anthracene Naphthalene Phenanthrene	0.5-15	Gao et al. (2013)
Marinobacter sp.	Biphenol	8.8	Al-Mailem et al. (2017)

Modicisalibacter tunisiensis MOD 31J	Benzopyrene	9	Gomes et al. (2016)
Nitratireductor SP. MOD 22.8	Naphthalene	9	Gomes et al. (2016)
BTEX (Benzene, Toluene, Ethylene, Xylene)			
Alcanivorax sp. HA03	Benzene Toluene	3-15	Hassan et al. (2012)
Arhodomonas sp. Strain Rozel	Benzene Toluene	3-23	Azetsu et al. (2009)
Chromohalobacter sp.	Benzene	8.8	Al-Mailem et al. (2017)
Halobacterium sp.	Benzene Toluene	>26	Al-Mailem et al. (2010)
Halococcus sp.	Benzene Toluene	>26	Al-Mailem et al. (2010)
Haloferax sp.	Benzene Toluene	>26	Al-Mailem et al. (2010)
Planococcus sp. Strain ZD22	Benzene Toluene Ethylbenzene Xylene	5-20	Li et al. (2006)
Marinobacter vinifirmus	Benzene Toluene Ethylbenzene Xylene	3-15	Berlendis et al. (2010)
Marinobacter hydrocarbonoclasticuz	Benzene Toluene Ethylbenzene Xylene	3-15	Berlendis et al. (2010)
Phenolics and Benzoates			
Arhodomonas aquaeolei	Phenol	10	Bonfá et al. (2013)
Candida tropicals	Phenol	15	Bastos et al. (2000)

Bacillus flexus MOD08	Phenol	9	Gomes et al. (2016)
Bacillus sp. Strain DHT	Salicyte	10	Kumar et al. (2007)
Chromohalobacter israelensis	p-Coumaric acid	10	Garcia et al. (2005b)
Chromohalobacter sp. Strain HS-2	Benzoate 4-Hydroxy-benzoate	10	Kim et al. (2008)
Haloarcula sp.	4-Hydroxy-benzoate	20	Erdogmuş et al. (2013)
Halobacterium sp.	4-Hydroxy-benzoate	20	Erdogmuş et al. (2013)
Haloferax sp.	Benzoate 4-Hydroxy-benzoate Salicyte	20 20 20	Bonfá et al. (2011) Erdogmuş et al. (2013) Bonfá et al. (2011)
Haloferax sp. D1227	Benzoate Cinnamic acid Phenyl propionic acid	15 5-30 5-29	Emerson et al. (1994) Emerson et al. (1994); Fu et Oriel (1999) Emerson et al. (1994)
Halomonas elongate	Benzoate 4-Hydroxy-benzoate Cinnamic acid Ferulic acid Phenyl propionic acid	10	Garcia et al. (2005b)
Halomonas organivorans	Benzoate 4-Hydroxy-benzoate Cinnamic acid Ferulic acid Phenol Phenyl propionic acid p-Courmaric acid Salicylate	10 1.5-30 1.5-30 1.5-30 1.5-30 10 1.5-30 1.5-30	Garcia et al. (2005b) Garcia et al. (2004) Garcia et al. (2004) Garcia et al. (2004) Garcia et al. (2004, 2005b) Garcia et al. (2005b) Garcia et al. (2004) Garcia et al. (2004)
Marinobacter lipolyticus	Benzoate	10	Garcia et al. (2005b)
Modicisalibacter tunisiensis	Phenol	10	Bonfá et al. (2011)
Thelassobacillus devorans	Phenol	7.5-10	Garcia et al. (2005a)

Table 1. Aerobic halophilic microorganisms reported to be capable of hydrocarbon degradation.

Objective

Exploring the capabilities of hydrocarbon degradation in halophilic bacteria.

Methods

In a previous experiment conducted in the Fathepure lab, the Kuwait consortium (KWTB1ANY culture originating from an oil contaminated soil in Kuwait) was shown to be effective in degrading BTEX (benzene, toluene, ethylbenzene and xylene). This experiment aimed to understand the Kuwait culture's suitability in produced water treatment. Experiments were carried out in 1L bottles containing 300 mL of produced water sourced from Payne County, Oklahoma. Bottles were closed with Teflon-coated septa and aluminum crimp. They were spiked with 12 uL BTEX (mixed in equal proportion). Bottles were inoculated with 12 mL of the Kuwait enrichment. Un-inoculated autoclaved bottles containing BTEX were set up as controls. The bottle's headspace was monitored for the removal of hydrocarbons using gas chromatography (Nicholson and Fathepure 2014). Both Kuwait enrichment and strain Wilcox were actively maintained in mineral salts medium (MSM) containing 2.5 M NaCl with BTEX as the sole sources of carbon in separate bottles and these cultures served as the source inocula for all the experiments conducted in this work.

The second experiment was conducted to determine whether strain Wilcox could degrade benzoate. 250 mL flasks containing 100 mL of MSM (Nicholson and Fathepure 2014) supplemented with 2.5M NaCl were inoculated with 2 mL of strain Wilcox. Three of the flasks were designated active, and were given 1mM of benzoate. The other two were negative controls, containing neither inoculum nor a carbon source.

While the degradation of BTEX under aerobic conditions by strain Wilcox has been proven, it had not yet been shown to occur under anaerobic conditions. An anaerobic medium was prepared, consisting of autoclaved MSM medium, 5 mM $NaNO_3$, and Resazurin. Ultrapure nitrogen gas was bubbled through the medium to remove oxygen via a gas manifold containing sterile filters for 20-30 min. Resazurin is an oxygen indicator, becoming colorless when oxygen is not present. For each 50 mL of medium, 100 uL of 10mg/mL resazurin solution was added. Of the five flasks, three were actives which had strain Wilcox added. Gas chromatography readings were conducted periodically.

The experiment was repeated with ethylbenzene as the sole carbon source, on a larger scale of four actives and three controls.

Results

1.BTEX degradation by Kuwait consortium

For the first experiment, the aerobic degradation of BTEX by the Kuwait consortium, GC readings were taken on days 0, 4, 7, and 8. All four hydrocarbon compounds were completely degraded by day 8. This was as expected. No degradation occurred in control bottles.

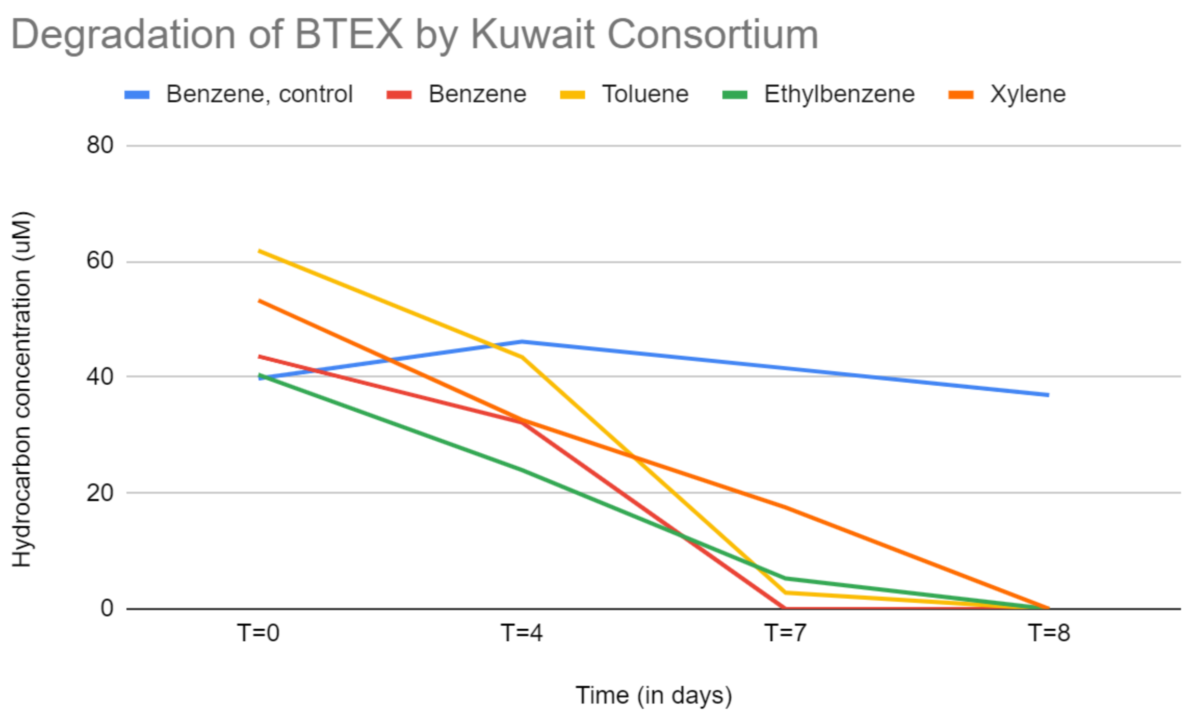

Figure 1 showing the complete degradation of all four BTEX hydrocarbons by the Kuwait consortium, in 8 days.

2. Anaerobic degradation of hydrocarbons by *M.tunisciensis* sp. Wilcox

However, with the strain Wilcox degradation experiments, it was found that only one of the four hydrocarbons was capable of being degraded anaerobically. This was ethylbenzene. In the presence of benzene, toluene, and xylene, ethylbenzene degradation occurred in 7 days. However, when present as the sole carbon source, ethylbenzene was degraded in 4 days. The reason for slower degradation when the organism was provided with BTEX rather than pure ethylbenzene is unknown. This could be due to a toxicity exerted by benzene, toluene and xylene.

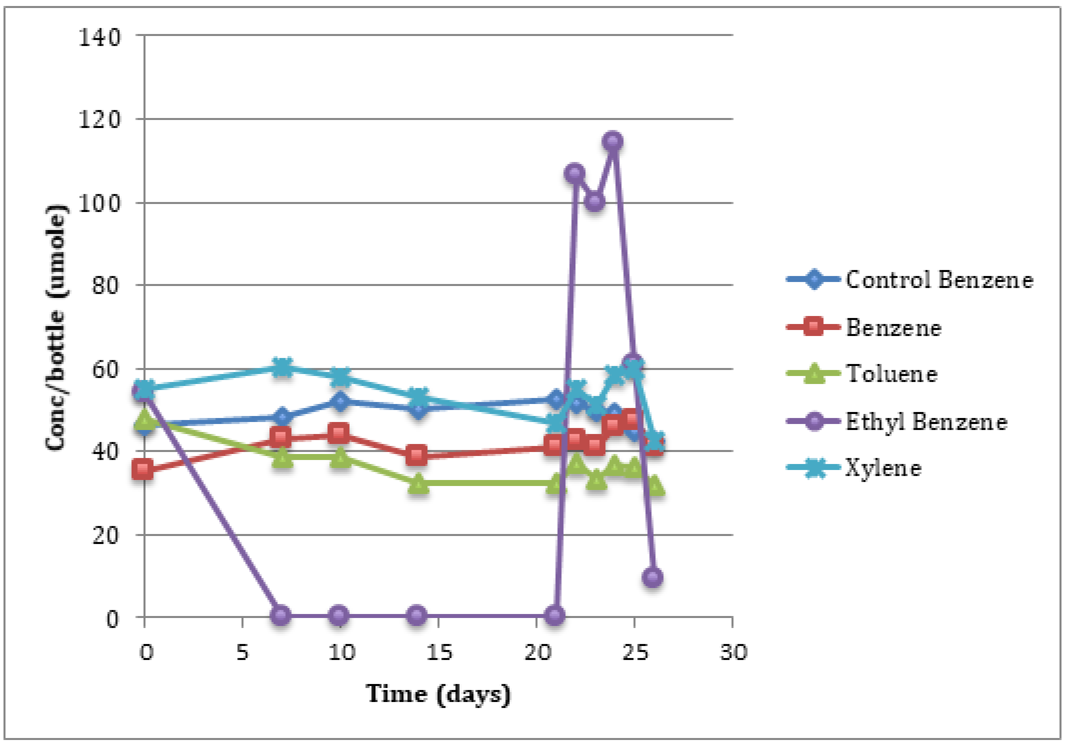

Figure 2 shows the anaerobic degradation of ethyl benzene in 7 days, with ethylbenzene being again added to the culture, and degraded again in 5 days. The effective rate for the instance of ethyl benzene removal is 5.7 uM/day initially and 20 uM/day for the second round.

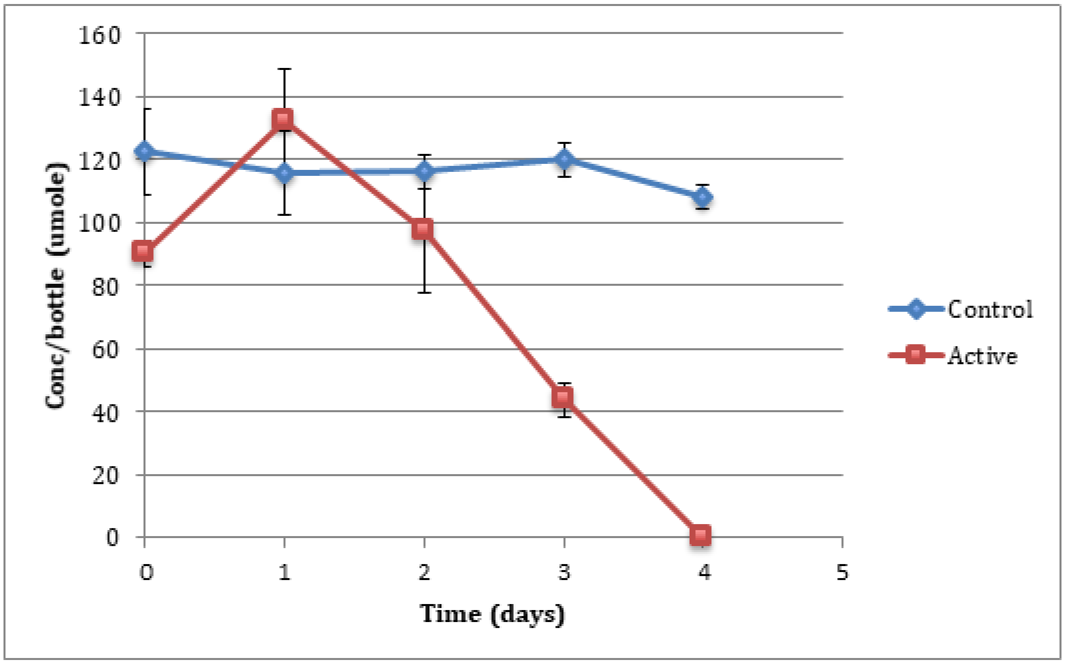

Figure 3 shows the anaerobic degradation of ethylbenzene in a 4 day period. The effective rate of ethyl benzene degradation is 22.5 uM per day.

Discussion

BTEX compounds are of particular interest because they are found in crude oil and produced water. They are also found in the environment near naturally occuring crude oil deposits and may contaminate the environment as humans access the oil. Microbial bioremediation of contaminated environments is an environmentally friendly option compared to the use of chemical cleaning agents. The Kuwait consortium consistantly degrades BTEX in a matter of days suggesting its potential use in the cleanup of saline produced water. This is important because few microorganisms can survive high salinity, and produced water is often saline. However, removal of salt is needed for the beneficial use of produced water. Environments where oil spills occur are also often saline sites, such as the ocean.

Strain Wilcox being capable of anaerobic degradation of hydrocarbons has not been previously studied. It was found that ethylbenzene was the sole hydrocarbon degraded out of the four found in BTEX. It was degraded more rapidly when it was the sole carbon source. Our findings that strain Wilcox can degrade hydrocarbons under both aerobic and anaerobic conditions is important for the treatment of subsurface brine at oil and gas production sites and anoxic produced water. However, more studies are needed as to its capability to degrade other aliphatic and aromatic compounds and optimal conditions for maximal degradation.

Conclusions and Future Directions

A future direction would be to explore strain Wilcox's genome and search for genes related to known anaerobic pathways, biosurfactant production genes, and genes for heavy metal resistance. Possible future experiments include growing strain Wilcox under anaerobic conditions and then introducing oxygen to see if the rate of degradation is affected. Strain Wilcox should also be screened for its potential to degrade a variety of other hydrocarbon compounds.

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