

The Biodegradation of Polystyrene by Soil Bacteria

by

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Dedication

This thesis and research are dedicated to my son, Austen, and to the rest of my “village.” The numerous family, friends, and professionals who have supported me on this longer and windier than expected road to success.

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I am forever grateful to my son, Austen Bossio, always patient when mom had to go to school or work on her own homework rather than doing something fun together; my wonderful boy who was always supportive and encouraging when it felt like it was all too much. My mother, Frances Brady, and my father and stepmother, John and Julie Brady, all of whom were tremendous sources of inspiration and unwavering support in innumerable ways; they have always supported me, and this journey was no exception.

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Abstract

The myriad of useful applications of plastic are ubiquitous across every aspect of modern life. Resistance to degradation, the hallmark property of many plastics including polystyrene, is paradoxically one of plastic's greatest threats to the environment. This study will focus on polystyrene, a plastic used in many private and commercial applications as a foam or solid. Studies dating back to the 1970's indicate polystyrene does not easily degrade naturally; thus polystyrene waste swept into the sea, found discarded within terrestrial ecosystems, or deposited in landfills persists in these environments for innumerable years impacting delicate ecosystems. In this study, soil bacteria species were screened for polystyrene degradation activity in order to reveal potential pollution intervention approaches. Soil microbes were recovered directly and by enrichment growth on Styrofoam packing peanuts. After single colony isolation on yeast extract peptone glucose (YEPG) agar, candidate microbes were grown in liquid carbon-free phosphate minimal medium (PMM) upon ninety-six well polystyrene plates for three weeks. Potential degrader status was assigned to isolates growing on polystyrene plastic dishes, (e.g. the sole carbon source), using a metabolic indicator dye that oxidizes a colorless substrate to a yellow color, water soluble tetrazolium (WST-1). Several intrinsic factors complicated the interpretation of this strategy including bacterial spore formation, stored carbon within polysaccharides, and metabolism of trace materials impregnated in polystyrene during manufacture. Mixed bacterial isolates were examined for sustained metabolic activity. Additional metabolic assessments included a candidate microbe's (2C73) use of monomeric styrene as a carbon source, and the influence of plant root exudates on metabolism and growth. A direct screening for survival and replication of 2C73 growing on

Styrofoam packing peanuts was conducted, with subsequent foam mass reduction noted. We isolated and characterized several rhizosphere bacteria from a residential site and an industrial site in terms of survival and growth on a polystyrene petri-dish (the sole carbon source). Candidates were characterized using WST-1, and colony forming units present after incubation in polystyrene as the only carbon source. 16S rDNA sequence analysis was utilized to determine genus. In summary: These results suggest some soil-borne bacteria have potential to motivate soil-born polystyrene degradation.

Chapter 1: Introduction

Polystyrene foam is designed for a useful life of minutes or hours, but it continues to exist in our environment for hundreds or thousands of years.

TheWayToGo 2008

Some of the very properties that make polystyrene a choice plastic for a multitude of applications, its low cost, light weight, durability, corrosion resistance, thermoplasticity, high thermal and electrical insulation and low biodegradability (Thompson et al., 2009), also make it very difficult to dispose of or recycle sustainably and contribute to its pervasiveness as pollution in both marine and terrestrial environments. Because of their chemical stability, plastics pose one of the greatest environmental challenges of today (Horton, 2022); disposal and recycling practices introduce physical and chemical pollutants to marine and land environments and incineration results in the release of harmful substances into the atmosphere (Adam et al., 2018; Barnes et al., 2009; Enck & Dell, 2022; Gautam et al., 2007; McDonough & Braungart, 2002; Thompson et al., 2009). However, we have come to rely on the many roles plastic plays in our lives daily and it will continue to play an invaluable role in day-to-day life in both expected and unexpected ways; for example, the increased production, consumption, and discard of personal protective equipment (PPE), respirators, thermometers, tubing, syringes, and other important health care items due to Covid 19 (De Sousa, 2020).

World production of plastics increased 25-fold between 1960 and 2000, though recovery of the material remained below 5% (Barnes et al., 2009; Lium, 2018; McAuley, 2016; Moore,

2008) and more plastics were produced between 2005 and 2017 than in the preceding 50 years (Geyer et al., 2017). The fastest growing component of the U.S. municipal waste stream between 1970 and 2003, plastics make up 60% to 80% of total marine litter (Mor & Sivan, 2008). Borrelle et al. (2020) estimated that 11 to 23 million tons of plastic waste, 11% of total plastic waste generated globally, entered aquatic ecosystems in 2016 alone and predict that amount to grow to 20 to 53 Mt/year if ambitious reduction targets are achieved, and an even greater amount if they are not.

Revered for its superior mechanical properties and the ease and cost effectiveness with which it is produced (Ojeda et al., 2009), polystyrene is used in the production of innumerable consumer products and packaging in both its foamed and solid states. Because foamed polystyrene is sturdy, lightweight, an excellent insulator, and costs less than alternatives, it is a popular choice for foodservice products such as packaging, takeaway containers, cups, and utensils – items that have a very short useful lifespan and are quickly discarded. The popularity and subsequent global use of this type of plastic has led to its accumulation in the environment. Landfills are rapidly filling, and incineration is associated with greater hazardous air emissions (Gautam et al., 2007). The United States Environmental Protection Agency (2021) reports that, in 2018, of the 35,680,000 tons of plastic municipal solid waste generated, 3,090,000 tons (8.7%) was recycled, and 5,620,000 tons (15.8%) was combusted with energy recovery, while 26,970,000 tons (75.6%) was landfilled (Figure 1-1, U.S. EPA, 2021). Sadly, the current waste management practices of plastics, including polystyrene, are unsustainable, end-of-pipe solutions struggling to keep up with the ever-increasing influx of polystyrene waste into the environment. Sustainable polystyrene waste management solutions are desperately needed to supplement current practices and to combat existing and future pollution of both terrestrial and aquatic ecosystems.

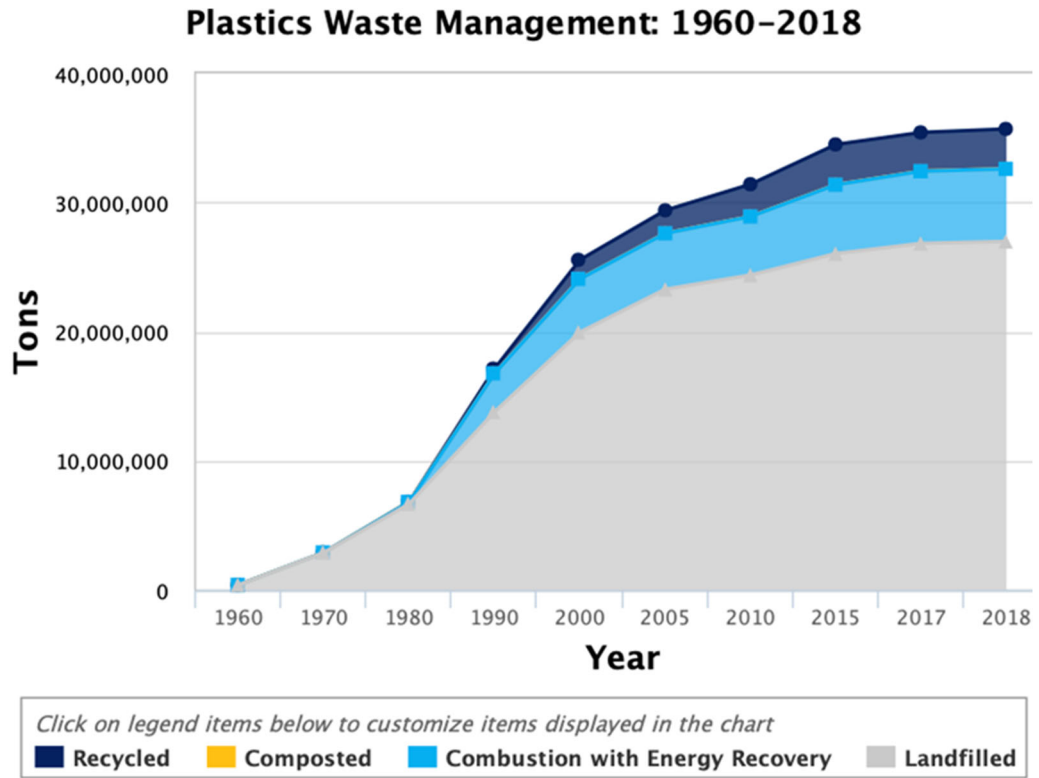


Figure 1-1: The generation of plastics versus the management of plastics from 1960 - 2018. (US EPA, 2021)

Chapter 2: This Study's Principal Impact to Environmental Sciences

Polystyrene, the polymerized styrene molecule, creates a durable thermoplastic generally considered to be non-biodegradable in the natural environment due to the very slow to non-existent rates of biodegradation that have been observed in studies (Kaplan et al., 1979; Otake et al., 1995). Most thermoplastics, like polystyrene, are hydrophobic in character, causing them to resist hydrolysis, which can affect how easily microorganisms can attach themselves (Ho et al., 2018).

Kim et.al. (2020) describes the ability of bacteria, colonized to form a biofilm, to secrete depolymerase enzymes that, in combination with other excretions, can alter a hydrophobic surface to become more hydrophilic, better allowing the attachment of bacteria. Hou and Majumder (2021) propose a multistep polystyrene degradation pathway involving main chain cleavage and side chain (aromatic rings) cleavage by multiple enzymes (Figure 2-1).

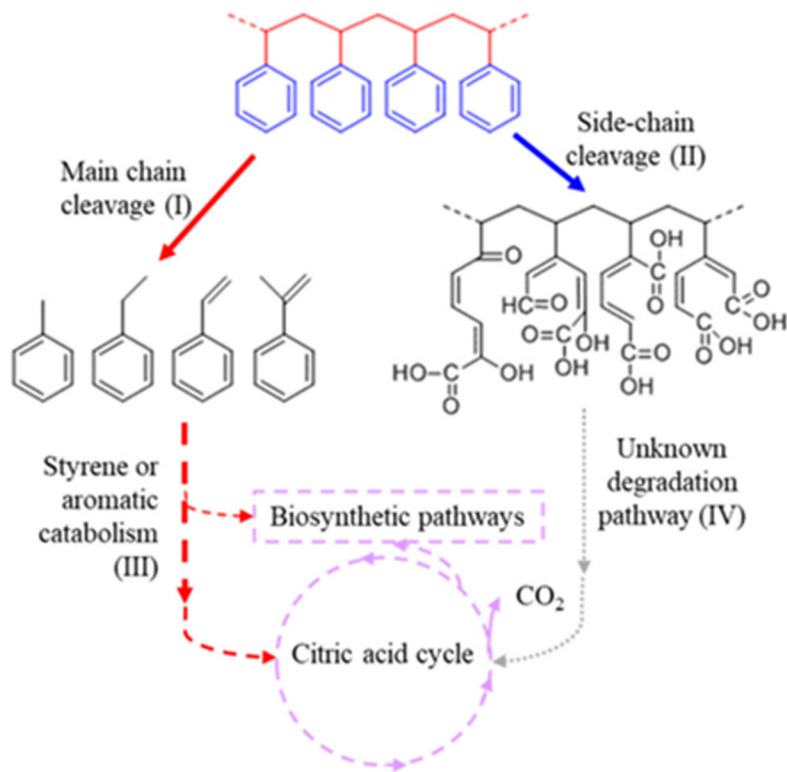


Figure 2-1: proposes a multistep polystyrene degradation pathway involving main chain cleavage and side chain (aromatic rings) cleavage by multiple enzymes (Hou & Majumder, 2021).

The use of additives in the manufacturing process such as antioxidants, flame retardants, processing lubricants, stabilizers, or antimicrobial agents can enhance the useful lifespan or esthetic of a polystyrene product but can further diminish the opportunity for breakdown by biological processes. Other types of plastics waste management practices such as recycling and combustion for energy recovery not only often pose environmental risk and concerns, but, as of 2018, accounted for less than 25% of municipal solid waste plastics waste management, with just over 75% of these plastics ultimately ending up in landfills (Figure 1-1, U.S. EPA, 2021). Judith Enck and Jan Dell of The Atlantic recently stated "Plastic Recycling Doesn't Work and Will Never Work" (Enck & Dell, 2022). Current plastics waste management techniques are unsustainable, end-of-pipe solutions in dire need of replacement by modernized and effective means of managing this material.

Methods for the recovery and re-manufacture of polystyrene foam currently exist but require extensive governmental and industrial cooperation and are often in opposition to the net cost or “bottom line” (McDonough & Braungart, 2002). Improved foam formulation and polymer design using green chemistry, “the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances” (U.S. EPA, 2023), offer some potential for decreasing waste plastic, but these approaches are largely beyond the scope of this investigation. Here, the focus is to screen soil-based consortia to discover bacteria that may effectively transform polystyrene (a technical nutrient) into a biological nutrient (biologically nontoxic substrates). The first steps in establishing the in-situ degradation of polystyrene in soil require the identification of one or more polystyrene degrading microorganisms, as well as optimal conditions for the degradation. Subsequent studies on mechanisms/chemical pathways of polystyrene degradation will represent future biochemical and bio-processing studies. The major environmental impact of this study will describe a novel conversion of a technical nutrient to a biological nutrient, averting toxicological end-of-cycle products.

Technical to biological nutrient conversion was previously thought impossible (McDonough & Braungart, 2002); however, one recent study (Sadler & Wallace, 2021) demonstrated the conversion of polyethylene terephthalate (PET) to vanillin by engineering a biosynthetic pathway in *Escherichia coli* able to convert the PET-derived monomer terephthalic acid to the flavor compound vanillin. Another, recent study (Acharjee et al., 2023) examines the potential of polyhydroxyalkanoates (PHAs) and polyhydroxybutyrates (PHBs) bioplastics as environmentally friendlier alternatives, as either a direct replacement to or as a blend with traditional petroleum-derived plastics.

The “white pollution” epidemic is overwhelming current polystyrene pollution management strategies. The landfill “Cradle to Grave” solution to polystyrene deposition is not a sustainable approach. Significant challenges to soil-based polystyrene biodegradation are emphasized by one study in which a polystyrene sheet was buried in soil and exhibited no evidence of biodegradation after 32 years (Otake et al., 1995). Likewise, using very sensitive radioactive tracer methods, polystyrene degradation was found “slow” (Guillet et al., 1974). ¹⁴C-labeled polystyrene exposed to fungi, soil invertebrates, and a variety of mixed microbial communities found about 0.5% degradation (measured as evolved ¹⁴CO₂) after 11 weeks (Kaplan et al., 1979).

Several studies completed in the years since the investigations described by Guillet et al. (1974), Kaplan et al. (1979), and Otake et al. (1995) have revealed greater potential in the ability of microbes to degrade plastics. One report describes how the enzymatic degradation of polystyrene to small, water-soluble molecules was accomplished by hydroquinone peroxidase enzyme extracted from *Azotobacter beijerinckii* HM121 (Gautam et al., 2007). When cultured on polystyrene flakes, *Rhodococcus ruber* C208 demonstrated a high affinity to the polystyrene and covered the flakes in a biofilm, leading to partial plastic degradation (Mor & Sivan, 2008). *Pseudomonas* have shown biodegradation capabilities at varying degrees of efficiency against polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PU), polyethylene terephthalate (PET), polyethylene succinate (PES), polyethylene glycol (PEG), and polyvinyl alcohol (PVA); for comparison to our focus on polystyrene, a 10% weight loss to 200 mg high-impact PS film was reported (Wilkes & Aristide, 2017). However, one glaring exception to these modest advances appears in an unverified report claiming effective polystyrene degradation with a microbial consortium of *P. putida*, *P. fluorescens* and *Streptomyces* sp. (Allard & Luong, 2010). In their non-peer-reviewed report, Allard and Luong (2010) claim that after an

incubation period of only two days, polystyrene was degraded 2.5%, and, after optimizing growing conditions, degradation increased to 69.5% after an incubation period of two weeks.

Our working hypothesis is that the degradation of polystyrene may be actualized by combining optimal culture conditions of polystyrene degraders with a preliminary abiotic attachment to the polymer. Incubation of *R. ruber* C208 with polystyrene as the sole source of carbon, resulted in biofilms exhibiting high adhesion and durability, and death of the planktonic (suspended) cells (Mor & Sivan, 2008; Zeraik & Nitschke, 2012). Besides culture optimization, substrate alteration may also promote biodegradation. Tissue-culture-treated polystyrene microtiter plates provide a surface appropriate for the attachment and proliferation of adherent bacterial cells and thus offer a suitable substrate for studying bacterial degradation of polystyrene (Therma Fisher Scientific, 2020). Plastic debris in the environment are indeed exposed to ultraviolet radiation and result in polystyrene photo-oxidation (Sivan, 2011), a physical breakdown of the polymer's molecular weight. Other abiotic breakdown processes such as photo- or thermo-oxidation were also proposed to result in efficient polystyrene degradation (Ward et al., 2019).

Predictions from Successful Identification of Polystyrene-Degrading Bacteria

Taken together from the literature, isolation of soil-derived microbial polystyrene degraders will display a substrate biofilm adhesion requirement, capable metabolic signaling to produced microbial degradative enzymes exposed to the extracellular environment, optimal physical conditions for enzymatic activity in soils, and little net toxicity derived from unknown metabolites of polystyrene degradation.

Research Objective

1. Isolation and identification of polystyrene-degrading bacteria from residential and industrial soil.

2. Determination of the effect of supplementation of bacterial suspensions on the biodegradation of polystyrene.

Expected Significance

Studies have revealed progress in polystyrene degradation by a variety of bacteria (Atiq et al., 2010; Mor & Sivan, 2008; Nikolic et al., 2014; Ward et al., 2006). Recently microbes from insect digestive systems were shown quite effective at polystyrene degradation into smaller biomolecules (Lou et al., 2020; Yang et al., 2015a; Yang et al., 2015b; Yang et al., 2020). These biotechnological approaches are applauded, and future studies should continue to characterize the metabolites and biosynthetic pathways of polystyrene degradation using these strategies; however, most present-day polystyrene waste is deposited in landfills. As a result, parallel efforts to examine soil-localized bacteria that also possess polystyrene-degrading capabilities may better contribute to future solutions for society's waste polystyrene.

This study comprised the isolation, identification, and characterization of potential polystyrene-degrading soil bacteria from soils. Supplementing bacterial suspensions with plant root exudate and copper sulfate on polystyrene biodegradation was also assessed. These results provide the basis for future work studying the biochemistry of polystyrene degradation in soil-borne microbes, in landfills, where most land-localized polystyrene is deposited.

Chapter 3: Methodology

Soil Samples

Soil samples were collected at the former Ford Rouge Steel Mill site in Dearborn, MI, latitude 42.3081/longitude -83.1662 (Google Earth, 2013), in early September 2005 at a depth of 10 - 15 cm. Similarly, soil from a Farmington Hills, MI residential yard, latitude 42.4420/longitude -83.3814 (Google Earth, 2013), was collected in June 2011. All material greater than 1 mm in diameter was discarded upon collection. Roots were noted at this depth at both locations.

Culture Media

Bushnell-Haas broth (257820) was purchased from Becton Dickinson Microbial Systems (Sparks, MS) and was used as a carbon-lacking culture media. YEPG (yeast extract peptone glucose) was used as a carbon-containing culture media which included glucose, and here is defined as: 0.05 g yeast extract, 0.05 g ammonium nitrate, 0.5 g BactoPeptone, 0.25 g glucose pH 7.0 (liquid) for culture broth, and, separately, with the addition of 15.0 g Bactoagar (DIFCO Laboratories; Detroit, MI, USA) for solid medium. Nutrient Broth (DIFCO Laboratories; Detroit, MI, USA) was used as a carbon-containing culture media which lacked glucose. Phosphate minimal media (PMM) is a balanced salt medium with no carbon source described in Johnsen et al. (2002). Kojima media is: 0.07% K_2HPO_4 , 0.15% KH_2PO_4 , 0.5% NH_4Cl , 0.1% yeast extract (DIFCO Laboratories; Detroit, MI, USA), and 0.02% $MgSO_4 \cdot 7H_2O$ pH 7.0 (Kojima et al., 1961). 15 g/L Bactoagar (DIFCO Laboratories; Detroit, MI, USA) with no yeast extract was used in plates for growth on styrene (monomer).

Isolation of Soil Bacteria

Soil samples from two separate locations were used for bacterial isolation: a residential backyard located in Farmington Hills, MI, latitude 42.4420/longitude -83.3814, collected 6 inches from the surface in June 2011; and the former Ford Rouge Steel Plant in Dearborn, MI, latitude 42.3081/longitude -83.1662 (Google Earth, 2013), collected 10 – 15 cm (Thomas et al., 2012). Ford Rouge Steel Plant site bacterial samples were previously isolated using dilution plating on YEPG medium (Thomas et al., 2012). These samples had been previously preserved at -80°C in 10% sterile glycerol.

In this initial phase, residential soil microbes were isolated by suspending residential soil in growth media treatments. Growth media treatments used in this study were chosen based on the carbon available for bacterial metabolism: Bushnell Haas broth, completely lacking carbon; yeast extract peptone glucose (YEPG), a rich carbon source; and nutrient broth, also a rich carbon source, but lacking in glucose. 20 mL treatments of each growth media alone, 1:1 ratio mixes (10 mL Bushnell Haas broth: 10mL nutrient broth and 10mL Bushnell Haas broth/10mL YEPG), and 1:9 ratio mixes (1mL nutrient broth: 9 mL Bushnell Haas broth and 1mL YEPG/9 mL Bushnell Haas broth) were used to culture soil samples (Figure 3-1). Approximately 1 g of residential soil was suspended in each 20 mL growth media treatment, along with one drop of sterile mineral oil used as a non-ionic surfactant, and 0.001 mL CuSO₄. One sterilized polystyrene foam-packing peanut was also added to each growth media treatment (Figure 3-2). Each culture was replicated in triplicate and one control vial (containing soil sterilized by autoclave) was prepared and monitored. All test vials were incubated at room temperature (20-24 °C) for up to three months.



Figure 3-1: Selection of cultivatable soil microbes. Notation refers to ratios of culture media. For example, 1YEPG: 1BH signifies a 1:1 mixture of the media YEPG and BH.



Figure 3-2: Soil, liquid enrichment growth media, non-ionic mineral oil, and CuSO₄ were incubated in Falcon (Blue Max 352098) 50 mL sterile tubes for up to three months to isolate soil microbes.

After incubation of up to three months, 0.1 mL was removed from each vial and used for serial dilutions. Due to its carbon-lacking nature, serial dilutions were performed in Bushnell Haas culture media. Following the serial dilutions, 50 μ L of the 1×10^{-4} dilution and 50 μ L of the 1×10^{-6} dilution were plated on YEPG agar medium and allowed to culture at room temperature (20 – 24 °C) for 24 hours (Figure 3-3). The flame-loop method was then used to transfer microbial

isolates from the culture plates to 2.5 mL YEPG in glass test tubes tilted 270° on a gyratory shaker at 100 - 125 rpm at 20 – 24 °C for two days.

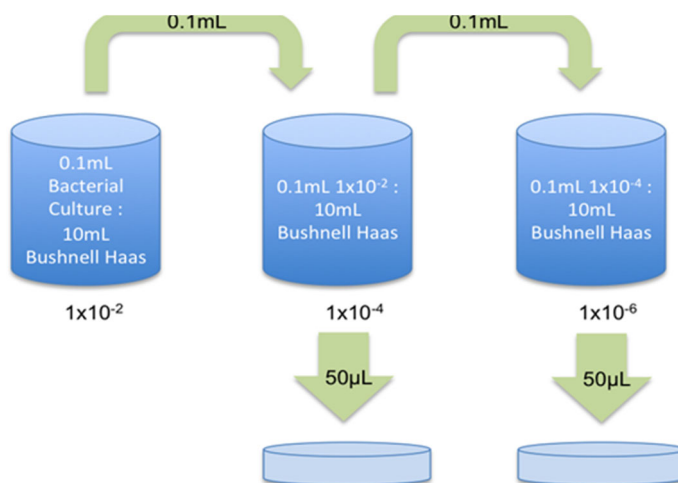


Figure 3-3: Serial dilution scheme for polystyrene enrichment cultures, plated for colony recovery on YEPG agar dishes (light blue).

Metabolic Assessment Studies

Individual isolates' ability to metabolize polystyrene was evaluated via WST-1 assessment (Johnsen et al., 2002; Thomas et al., 2011). Each purified microbial isolate was centrifuged, re-suspended in phosphate minimal medium (no carbon source) and cultured for 21 days in 96-well polystyrene microplates (Costar 3595 96-well Tissue Culture Treated, Corning Inc or Cyto One 96 well Non-treated, USA Scientific) where the microplate acted as the sole source of carbon available for mineralization. Each microplate well was inoculated with 10 uL bacterial suspension and 190 uL carbon-free phosphate minimal medium (PMM) (Kirner et al., 1996). The 96-well microplates were incubated for 21 days at room temperature (20 -24 degrees C) with no exposure to light.

Following 21 days of incubation, 50 uL of electron donor solution [0.07 g glucose, 0.05 g succinate, 0.05 g sodium pyruvate, in 25 ml of 25 mM Tris (pH 8)], and 10 uL of WST-1 reagent

(Roche Diagnostics Company, Mannheim, Germany)(used as a colorimetric assay to detect cell proliferation and viability) was added to each well and plates were incubated from 12-14 hours at 25° C before being photographed or undergoing spectrophotometric assay, performed at 415 nm/655 nm (Johnsen et al., 2002).

Isolates were considered able to survive on and/or mineralize the polystyrene plate as their sole source of energy if a yellow color was produced. Cells unable to mineralize the polystyrene microplate lacked a yellow color (Figure 3-4a). Negative controls (*Agrobacterium tumefaciens* LBA4404) and wells containing no bacteria were considered negative controls and exhibited a pink tint. Use of blue light provided the most visual contrast for culture assessment (Figure 3-4b). Generally, the results were recorded at 12 hours because negative controls (no bacteria) and LBA4404 (*Agrobacterium tumefaciens*) remained the light pink color of the WST-1 indicator substrate for 12 hours. Results at 12 hours were photographed and compared qualitatively based on the production of dye in each well and recorded using a binary method: metabolic activity was considered a positive result and the absence of dye, indicating a lack of metabolic activity, was recorded as a negative result.

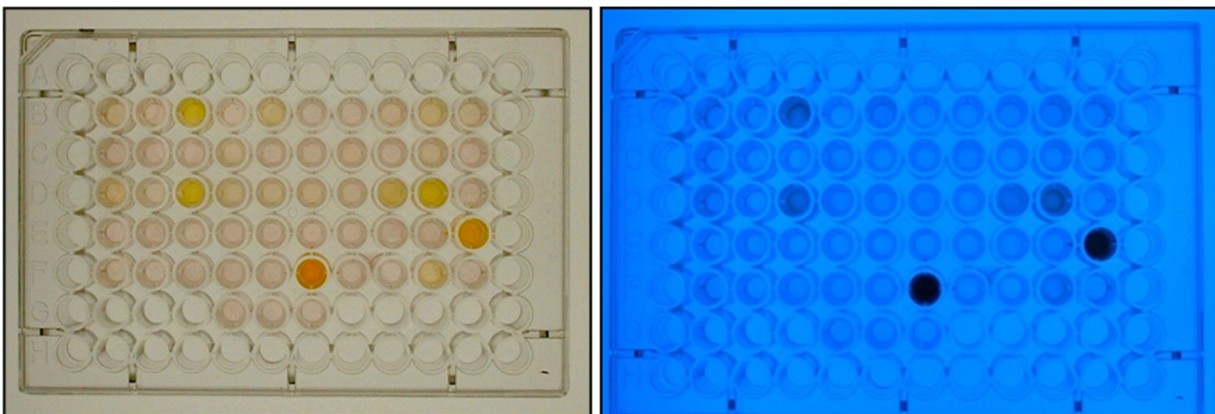


Figure 3-4: (2-4a, left) Proof of concept. Metabolism on polystyrene plates: random selection of isolated soil microbes and metabolic assessment using WST-1 in white light. (2-4b, right) The same plate was photographed with blue illumination to better visualize different intensities of (+) metabolism (darker spots = greater metabolism).

WST-1 Assessment of Impact of Root Exudate and Copper Sulfate

The influence of *Solidago patula*, commonly known as Swamp Goldenrod, plant root exudate and copper sulfate on specific microbial isolates was also evaluated. Swamp Goldenrod root exudates, composed largely of reducing sugars, amino acids, and trace amounts of proteins, have been found to promote the degradation of polycyclic aromatic hydrocarbons (Thomas and Rugh, 2007) (Thomas & Dabkowski, 2011). Copper sulfate may stimulate the oxidation-catalyzing enzyme laccase (Kellner et al., 2008; Morozova et.al, 2007). Swamp Goldenrod plant root exudate was added to microbial isolate PMM suspensions in 96-well polystyrene microtiter plates, at 0.035% (v/v) and 0.35% (v/v) concentrations. The influence of one hundred micromolar copper sulfate solution on WST-1 analysis was concurrently assessed. Protocol for WST-1 assessment of supplementation on the metabolism of isolates was identical to the WST-1 assessment protocol described above, with the addition of the supplementation of select wells with 35 uL Swamp Goldenrod root exudate [0.35% (v/v)], 3.5 uL Swamp Goldenrod root exudate [0.035% (v/v)], or 100 uL copper sulfate.

Determination of Doubling Times Using 96-well Polystyrene Plates as a Carbon Source

Polystyrene 96-well microplates (tissue culture grade) were used as a carbon source, and, rather than developing the cultures for WST-1 (as above), serial dilutions of three-week-old cultures were done and colony forming units on YEPG agar were determined by subsequent culture on YEPG medium. Colony counting was done 3-5 days post-plating. Doubling time was estimated from the original cultured density in colony forming units as compared to the final bacterial colony forming units.

Comparison of "tissue culture-treated" versus non-treated used identical bacterial source samples, and WST-1 activity was quantified using a scanning multi-welled spectrophotometer at 415 nm/655 nm (Johnsen et al., 2002). The 655 nm reading takes particulates into account. Data was analyzed using ANOVA as each sample contained triplicate treatments.

Polystyrene – Isolate Incubation on Polymeric Substrate (Packing Peanuts)

The environmental isolate found to perform best in WST-1 testing, 2C73, was next used as the focus of experimentation to assess the presence of polystyrene digestion via loss of mass using sterilized foamed polystyrene, Styrofoam, packing peanuts (one bag of Styrofoam packing peanuts obtained from Postal Annex, Livonia, MI, USA). An overnight culture of 2C73 in YEPG medium was incubated on Styrofoam packing peanuts (sterilized using 70% ethanol, rinsing, and drying) placed in BH or BH:YEPG treatments for 15 months. The weight of the packing peanuts was measured before and after the incubation period to determine whether loss of mass, presumed to indicate polystyrene digestion, had occurred. It bears mentioning that the expanded polystyrene foam (EPF) manufacturing process may introduce unknown residual impurities or additives to the final product, here Styrofoam packing peanuts.

Packing peanuts were sterilized via 1 hour of agitation in a sterile beaker in 70% (v/v) ethanol, followed by extensive rinsing with sterile distilled water. The sterilized peanuts were then placed under a biological safety hood (no UV light) and allowed to dry. Peanuts were weighed individually and labeled (one, two or three marks with a Sharpie™ marker) before being placed (in groups of three) into commercial jam jars (Bonne Maman, www.bonnemaman.us) previously cleaned and autoclaved with aluminum foil tops (foil tops were secured with rubber bands post autoclaving).

Culture jars were prepared with 100 mL of one the following treatments: 100% Bushnell Haas (BH); 90% BH and 10% YEPG; and 50% BH and 50% YEPG. Four jars were prepared for each treatment (12 jars total). Three of the four jars for each treatment were inoculated with 500 uL of the prepared 2C73 culture. A single jar for each treatment was not inoculated with the 2C73 culture, these were considered the controls. Three sterilized, pre-weighed and labeled (one, two or three marks with a Sharpie™ marker) Styrofoam packing peanuts were then added to all culture jars including controls.

All samples were incubated at room temperature (20 - 24 °C) and low light for 15 months with occasional shaking. At approximately 6 months, 20 ml of sterile BH medium was added to each culture to compensate for medium evaporation. At the experiment conclusion, packing peanuts were washed four times in distilled water, and allowed to air dry for one week. Dry packing peanuts were weighed, and any net loss of Styrofoam mass was determined by subtracting peanuts' end weights from the initial weights. Any changes were compared to the control; weight changes in the peanuts incubated in the corresponding medium (non-inoculated). The total number of cultured peanuts was twelve per experimental treatment (four jars per medium mixture). A previous pilot experiment with only 2-3 peanuts per treatment was done to develop the feasibility of the 15-month experiment described above.

Environmental Isolate Incubation on Monomeric Styrene Substrate

The monomer substrate styrene was evaluated as a potential growth promoting carbon source for isolated bacteria using described methods (O'Connor et al.,1997). Styrene-agar plates were used to ascertain whether environmental bacterial isolates were able to survive and reproduce on styrene alone. Plates were prepared by adding 0.15% (v/v) liquid styrene (Sigma/Aldrich, St. Louis, MO) in Kojima media (Kojima et al., 1961). A 1.5% styrene treatment was prepared in the

same way for a pilot experiment. Bacteria were spread upon the styrene-agar medium via the flame-loop method and inoculated styrene-agar plates were incubated in a chemical hood at room temperature for 7 - 56 days. Isolate growth was analyzed qualitatively and recorded at two, four, six, and eight weeks of growth.

Styrene Monooxygenase (SMO) Enzymatic Assay

SMO Enzymatic indole to indigo conversion (O'Connor et al., 1997) was used to analyze bacterial degradation of polystyrene and styrene monooxygenase (SMO) activity. The enzymatic conversion tested isolates' use of one well-studied biochemical pathway for styrene metabolism (Gursky et al., 2010). For indole metabolism, bacteria suspended in YEPG medium were inoculated with indole stock solution and cultured at room temperature up to 24 hours. Cells were centrifuged ($3,000^\circ \times g$) for 15 min and the pelleted cells re-suspended in 100 μ l of 50 mM potassium phosphate buffer (pH 7) containing 1 mM indole. Suspended cells were incubated for 24 hours (longer than the 1 to 4 hours in O'Connor et al. [1997]) at 30° C with shaking at 200 rpm. Following cell lysis in DMSO, supernatant optical density was measured spectrophotometrically at 610 nm (O'Connor et al., 1997).

Bacterial Genus Identification

Genus and species for WST-1-positive isolates were determined via DNA sequence analysis. Identities of WST-1-positive bacteria were determined using genomic rDNA isolation, PCR with 16S rRNA gene primers 8F and 926R followed by DNA sequence analysis (Liu et al., 1997). Genomic DNA from 2-day grown cultures of bacteria was isolated according to Yeates et al. (1998). rDNA was sequenced, the sequence edited using 4Peaks v. 1.7.2 (Griekspoor and Groothuis, 2006), and the sequence identification determined using rDNA databases, including the Ribosomal Database for Microbes at Michigan State University (<http://rdp.cme.msu.edu/>) and the

NCBI database (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al., 1990). For isolate 2C73, purified 16S rDNA was subjected to PCR using primers 8F and 927R (Liu et al., 1997), the PCR products separated using a PCR clean-up kit (Qiagen, Hamburg, Germany), and ligated into an *E. coli* vector plasmid vector (pGEM Easy, Promega, Madison, WI). Six different plasmid clones containing the 2C73 amplified rDNA were prepared and submitted to the DNA Sequencing Core, University of Michigan Ann Arbor (Dr. Robert Lyons). Sequencing primers included 8F, 927R, and M13F and M13R (Liu et al., 1997). Sequence overlaps provided the final sequence submitted to Genbank (Accession KF110992). Each read was sequenced and analyzed using 4Peaks, <http://mekentosj.com/4peaks>.

Chapter 4: Results

Isolation of Soil Bacteria (Results from this Section from Methodologies)

Incubation of growth media treatment suspensions, followed by serial dilutions and plating, resulted in the isolation of twenty-two individual purified bacterial colonies from the yard soil samples (Figure 4-1). A small number of fungi were also realized; however, further study was confined to bacterial isolates. Microbes from the Ford Rouge soil had been previously refined to individual isolate colonies and identified to the genus and species through rDNA sequencing. The Ford Rouge soil samples were previously found to contain bacterial isolates mainly of the *Arthrobacter* genus; a bacterial genus whose strains are known for their ability to survive in harsh environments and industrially contaminated soils (Mongodin et al., 2006). Experimental comparison of the yard soil sample isolates to the Ford Rouge isolates indicated a greater diversity of species among the yard soil isolates compared to the Ford Rouge isolates; while a greater diversity of species was exhibited in the yard soil sample isolates, ultimately, no *Arthrobacter* species were identified in the yard soil isolates.

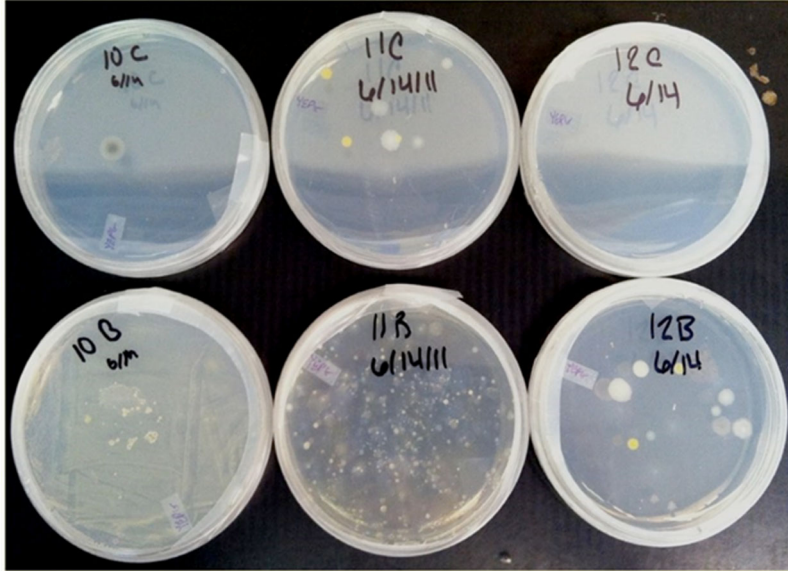


Figure 4-1: Distribution of diluted colonies from enrichment culture of yard soils.

Polystyrene – WST-1 Assessment to Identify Metabolic Activity Post-Incubation

Residential soil bacterial isolates [isolated by completing serial dilutions and single colony isolation from original consortia suspensions (Figure 3-1) followed single colony isolation and then suspension of isolates in 2.5 mL YEPG (flame loop method) in 16 x 25 mm sterile glass test tubes slanted 270° on a gyratory shaker at 100 - 125 rpm at room temperature (20 – 24 °C) for 2 days] and Ford Rouge Steel Mill soil bacterial isolates (also suspended in YEPG) were subjected to WST-1 testing. Ten microliter samples of these suspensions were incubated for 21 days in 190 µL of PMM on 96-well tissue culture polystyrene plates.

In total, fifty isolates were tested for the presence of metabolic activity after 21 days of incubation in the carbon-free PMM on 96-well polystyrene microtiter plates. Twelve isolates from the residential soil samples and fifteen isolates from the Ford Rouge Steel Mill soil samples were identified as exhibiting significant metabolic activity without additional carbon sources in the PMM medium. WST-1 analysis after 21 days of incubation in PMM in 96-well tissue culture

plates was ultimately performed 17 separate times with multiple replicates for select isolates to verify these results.

Polystyrene – WST-1 Assessment of Isolate Suspension Supplementation

The influence of *Solidago patula*, Swamp Goldenrod, plant root exudate on metabolic activity was also explored via WST-1 assessment, as plant root exudates are known to contain materials that may facilitate the degradation of polycyclic hydrocarbons (Thomas & Dabkowski, 2011) or may simply provide needed carbon-based derivatives which may act as a carbon source itself. Suspensions of 10 uL bacterial isolates suspensions and 190 uL PMM were assessed without additives, with 0.035% or 0.35% Swamp Goldenrod plant root exudate, 0.1 mM CuSO₄, and a mixture of 0.35% plant root exudate with 0.1 mM CuSO₄. The copper addition was used because one common bacterial oxidase (laccase) requires a copper cofactor for high enzyme activity (Kellner et al., 2008; Morozova et al., 2007). Results for each isolate were recorded using the same binary method as describe previously. Most bacterial isolates were found to exhibit some level of metabolic activity in both the 0.035% Swamp Goldenrod plant root exudate and 0.35% Swamp Goldenrod root exudate treatments after three weeks of incubation. Assay results for bacterial isolates incubated in the 0.1 mM CuSO₄ treatment most often revealed an absence of metabolic activity at the conclusion of the 21-day incubation period (Figure 4-2). Control LBA4404, (*Agrobacterium tumefaciens*) tested negative in all WST-1 trials except in four instances when Swamp Goldenrod plant root exudate, very active towards polyaromatic hydrocarbon breakdown was added to the isolate/PMM suspension (Thomas & Dabkowski, 2011). Likewise, environmental isolates comprising the Pseudomonads 2C50, 2C57, 2C59, 12B-9LY, 12B-3W, *Arthrobacter* 2C73, UPD1, UPD6, and *P. putida* consistently exhibited metabolic

activity when subjected to WST-1 testing after incubation for 21 days on exudate-supplemented polystyrene microtiter plates in carbon-free PMM medium.

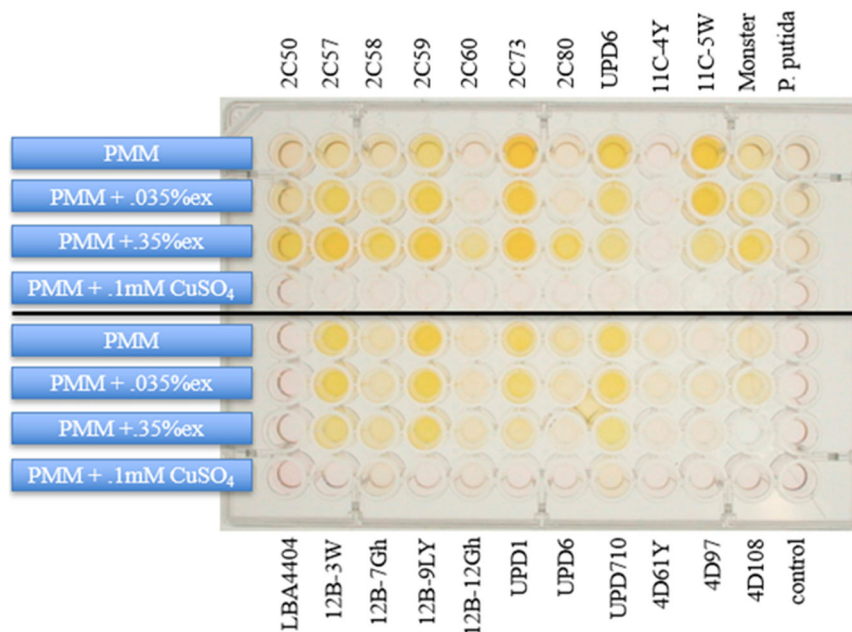


Figure 4-2: Role of root exudates [0.35% (v/v) and 0.035% (v/v)] from Swamp Goldenrod (*Solidago patula*), and 0.1 mM CuSO₄. Each sample was screened at least ten separate times. Note: 2C73 activity is largely unaltered by any treatment. This is a representative result, color development at 12 hours.

Most bacterial isolates were found to exhibit some level of metabolic activity in both the 0.035% Swamp Goldenrod plant root exudate and 0.35% Swamp Goldenrod root exudate treatments after three weeks of incubation; overall, these studies demonstrated that 0.35% (v/v) exudate promoted the greatest WST-1 response.

Under this assay condition we were unable to determine if the WST-1 augmentation was the result of increases in cell number or net metabolic activity. Nevertheless, the lack of WST-1 activity (except for the 0.35% exudate) suggested that the LBA4404 *Agrobacterium tumefaciens* organism was utilizing the exudate for carbon sourcing. The inability of the same organism to use the higher exudate dose may likewise reflect negative growth compounds amongst the various materials composing exudate (Thomas & Dabkowski, 2011). Moreover, the inhibition of WST-1

activity by the addition of 0.1 mM CuSO₄ was observed in all samples (Figure 4-3). 2C73 and 12B-91Y demonstrated enhanced WST-1 activity in both exudate doses.

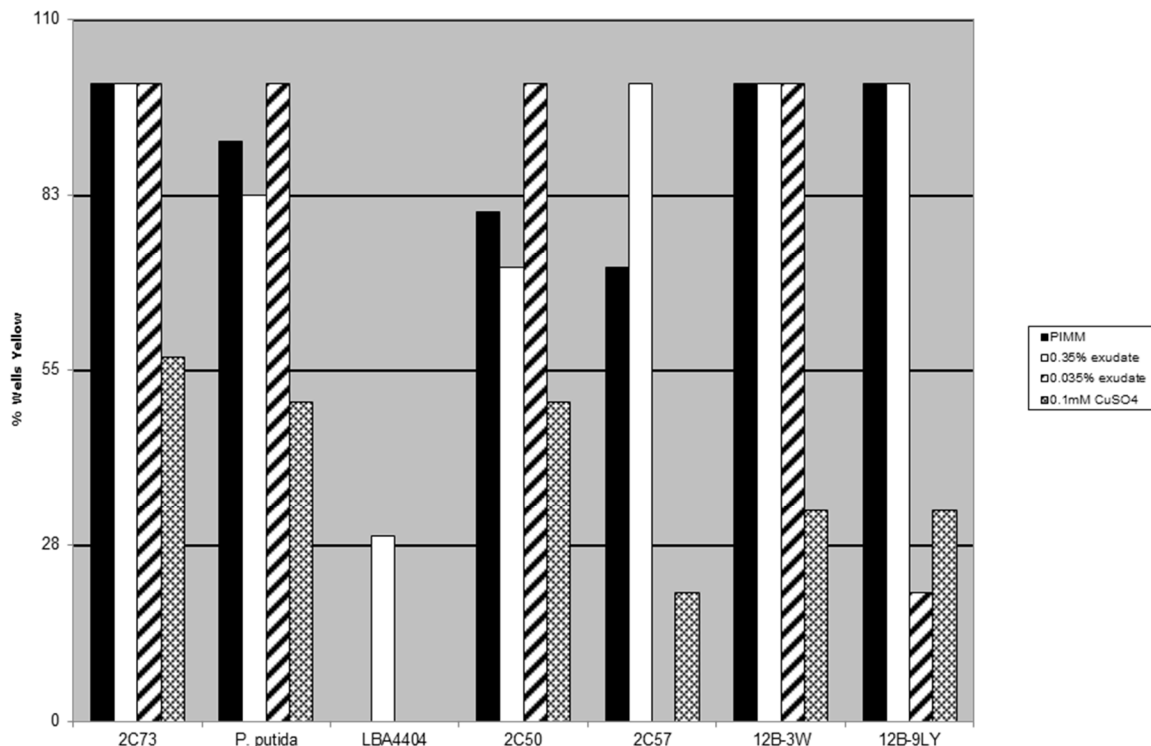


Figure 4-3: Summary of all WST-1 analysis for individual bacterial isolates. Temperature variances within the laboratory during growth or WST-1 incubation, inoculate size, and/or contamination/cross contamination may have caused variation between experiments.

Polystyrene – Serial Dilutions and Cell Counting

Many environmental bacteria are capable of long-term survival without undergoing cell replication. To determine whether isolates cultured for 21-days on polystyrene microtiter plates were undergoing cell division or simply surviving nutrient elimination, cell numbers were estimated for 2C73, 2C57, *P. putida*, UPD1 and LBA4404 before and after 21 days incubation in 96-well polystyrene microtiter plates. Serial dilutions of 1×10^{-5} to 1×10^{-8} were prepared for each isolate incubated in PMM alone as well as for each isolate incubated in PMM plus 0.35% Swamp Goldenrod plant root exudate on day 1 and day 21. Inoculated plates were incubated at room

temperature for 4 days. Visible CFUs were counted and recorded as CFU/mL using the calculation $\frac{\#ofCFUs \times DilutionFactor}{PlatedVolume(mL)}$. *P. putida* isolates incubated in PMM alone produced an average of 6.0×10^7 CFU/mL after seven days. These experiments were performed in triplicate and the experiment repeated 3 times or 4 times. For each isolate, the average number of cells on day 21 was divided by the average number of cells on day 0; the result represented the average number of divisions undergone by that isolate during the 21-day incubation period.

Preliminary cell counting of cultures in individual wells indicated most cultures were capable of some cell division within the 21-day incubation. As shown in Figure 4-4, 2C73 was able to divide twice in carbon free medium in the presence or absence of root exudate. Cell division of the laboratory strain of *A. tumefaciens* was restricted in the absence of an available carbon source, while *P. putida* responded to root exudate with four cell divisions (Figure 4-4).

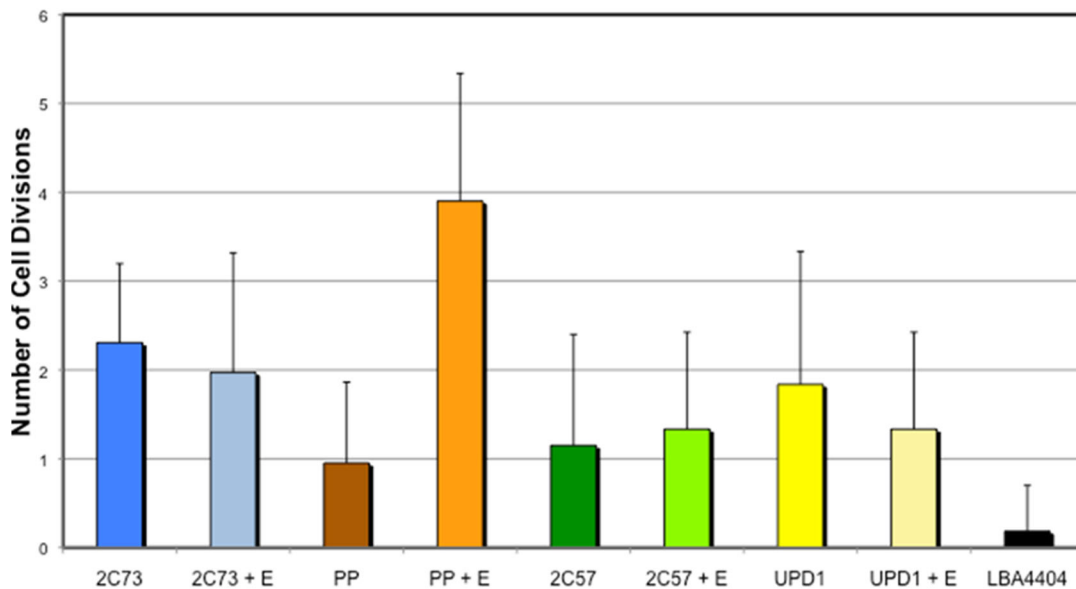


Figure 4-4: Ratio of cell division in some individual bacterial isolates in the presence (white bars) or absence (black bars) of 0.35% Swamp Goldenrod root exudates. LBA4404 (*Agrobacterium tumefaciens*) with exudates was not tested. PP: *Pseudomonas putida* G7.

Polystyrene: Packing Peanut Digestion?

Many environmental studies use the loss of mass as an indication of polystyrene digestion (Priyanka & Archana, 2011). To examine this activity, 2C73 cells were added to Bushnell Haas and YEPG mixtures, and Styrofoam packing peanut mass loss was measured.

Enrichment cultures along with three labeled and pre-weighed polystyrene peanuts were incubated at room temperature with occasional shaking for fifteen months. Bushnell Haas medium, absent isolates, was used as a negative control culture. At the conclusion of fifteen months, a 100 μ L sample was removed and plated on YEPG-agar plates. 2C73 colonies were verified using rDNA sequence. Additionally, the cultures had a similar yellow/white color and growth habit (opaque/watery) on the YEPG-agar plates after three days incubation at room temperature. No contaminant cells (non-2C73 cells) were recovered from these cultures (Figure 4-5, Figure 4-6 data not shown).

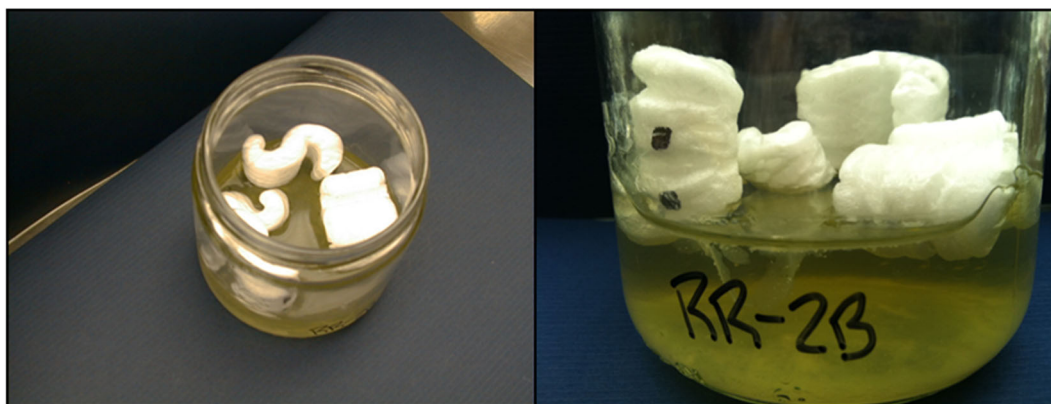


Figure 4-5: Culture jars containing polystyrene packing peanuts. Note the righthand image where apparent biofilm connects the peanuts and the culture medium.

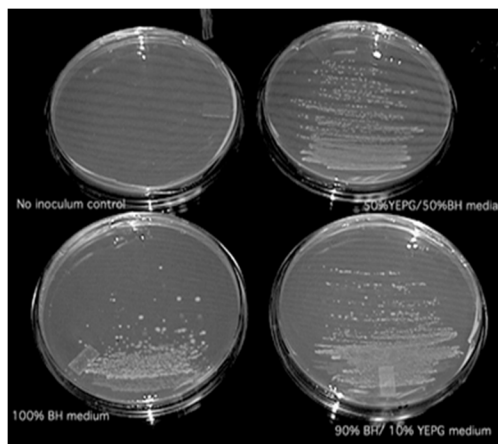
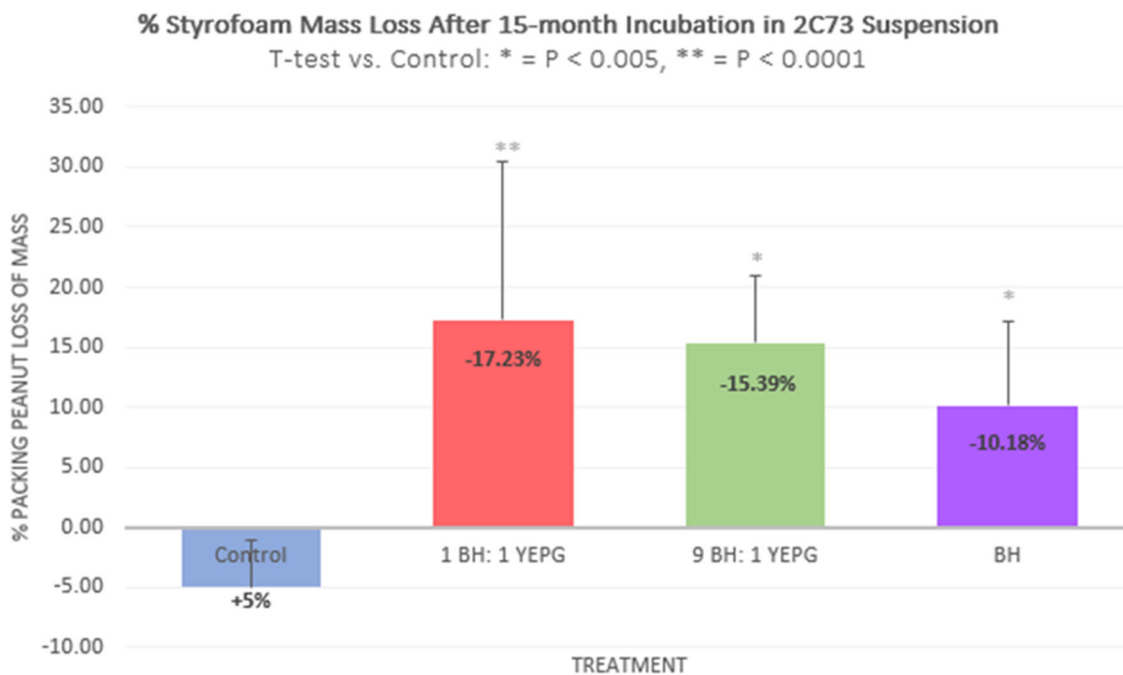


Figure 4-6: Remaining 2C73 bacteria after 15 months on polystyrene packing peanuts. All organisms contained yellow pigmentation identical to the original inoculum (2C73). Each plate was inoculated with one sterile loop of the culture medium.

In the subsequent scaled up experiment, three culture media (100% BH, 75% BH & 25% YEPG and 50% BH & 50% YEPG) were tested with an inoculum of 2C73 grown overnight in YEPG, with non-inoculated treatments considered as controls. Because the cultures were grown at room temperature for an extensive time, addition of BH medium was added to compensate for evaporation (see Materials and Methods). Overall, each medium mixture demonstrated an average loss of mass; however, ANOVA analysis did not find significant differences in weight loss between the culture media treatments (Figure 4-7). No detectable green (likely algae) was observed in any culture. Recovery of existing bacteria after the culture period showed a preponderance of 2C73 remaining in the inoculated cultures (Figure 4-6). Controls (non-inoculated cultures) did not contain any viable microbes capable of growth on YEPG after the 15-month culture duration.



| Treatment | 50% BH: 50% YEPG | 90% BH: 10% YEPG | 100% BH |
|-----------|----------------------|---------------------|---------------------|
| Mass Loss | -17.23% +/- 13.18 | -15.39% +/- 5.55 | -10.18% +/- 7.00 |

Figure 4-7: Loss of mass in polystyrene packing peanuts after 15 months of continuous co-culture in 2C73. Data represents the mean and standard deviation calculated between twelve peanuts. Initial weight of each peanut was approximately 0.05g. In comparison to n non-inoculated controls, results were not significant. (ANOVA p=0.05 level).

Monomer Styrene as a Carbon Source

The catabolic breakdown of polystyrene by depolymerase enzymes has been reported to produce styrene as a metabolite of depolymerization (Parthasarathy et al., 2022). The ability of soil microbes to maintain metabolic pathways which use the monomer styrene as an effective carbon source is well-documented (Sielicki et al., 1978; Ward et al., 2005). Our assumption in considering possible biochemical pathways for degrading polystyrene was that the monomer styrene may partner with unknown depolymerizing activities of styrene-related compounds. To determine which bacterial isolates were able to survive on the monomer styrene substrate, isolates

were plated on 0.15% styrene-agar plates after suspension for two days in YEPG liquid medium. Isolates were incubated on multiple plates and plates were cultured non-simultaneously. Growth was analyzed using a qualitative numbering system rating isolates relative to one another. Where no colonies/growth were observed: 0. If a sample grew quickly and was observed as little as 2 days after incubation at room temperature, the rating was 3. The averaged data for each like time point was then averaged for each isolate (Figure 4-8). Subsequently, a single averaged data point was extrapolated for each isolate at two, four, six, and eight weeks. Except for *P. putida*, all other isolates were viable throughout the entire eight weeks of the study; at eight weeks of incubation no colonies were visible for *P. putida*. In contrast, at week eight, 2C73 was the only isolate for which the ranking did not drop from 3 to 1 (Figure 4-8).

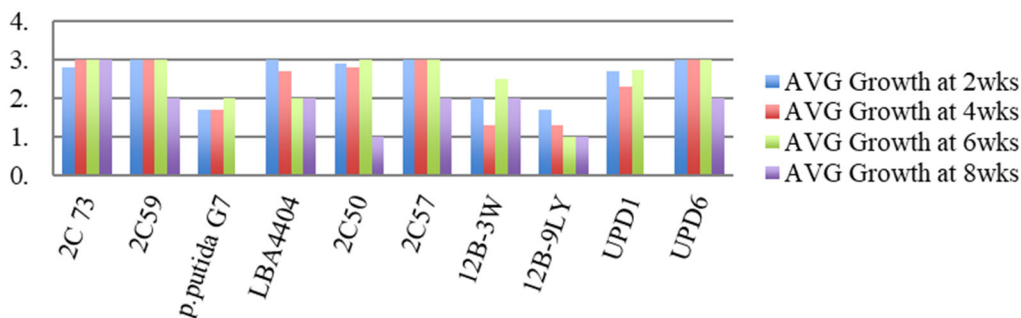


Figure 4-8: Environmental bacteria were plated on Kojima basal medium with 0.15% styrene and 1.5% (w/v) agar plates after suspension culture for two days in YEPG liquid medium. Ascending qualitative ranking of the previous week's growth was used for each isolate.

In one pilot experiment, isolates were also plated on styrene-agar plates where the styrene concentration was increased to 1.5%. At two weeks the average growth rates of 2C73, 2C59, 2C50, 2C57, and 12B-9LY were comparable to those recorded for the 0.15% styrene treatment at two weeks. *P. putida* and 12B-3W survived but experienced significantly decreased average growth rates compared to those recorded for the 0.15% styrene treatment, and no viable CFUs were observed for LBA4404 after two weeks incubation on the 1.5% styrene-agar.

Styrene Monooxygenase Enzymatic Assay: Analogous Indole to Indigo Conversion

Enzymatic indole to indigo conversion (O'Connor et al., 1997) was used to analyze SMO activity, utilizing methods developed for a known pathway for styrene metabolism (Gursky et al., 2010). For indole metabolism, bacteria suspended in YEPG medium were inoculated with indole stock solution and cultured at room temperature. After incubation, centrifugation, and re-suspension of pelleted cells in potassium phosphate buffer containing indole, cells were again incubated at room temperature with shaking and lysed in DMSO. The average formation of indigo from 1mM indole incubation with cultures of environmental bacterial isolates is summarized in Figure 4-9 below. Y axis is the OD at 610nm. While *P. putida* (G7) was the most active in these 3 experiments, its SMO activity was 100 to 1,000 times less active than the cultures described by O'Conner et al. (1997).

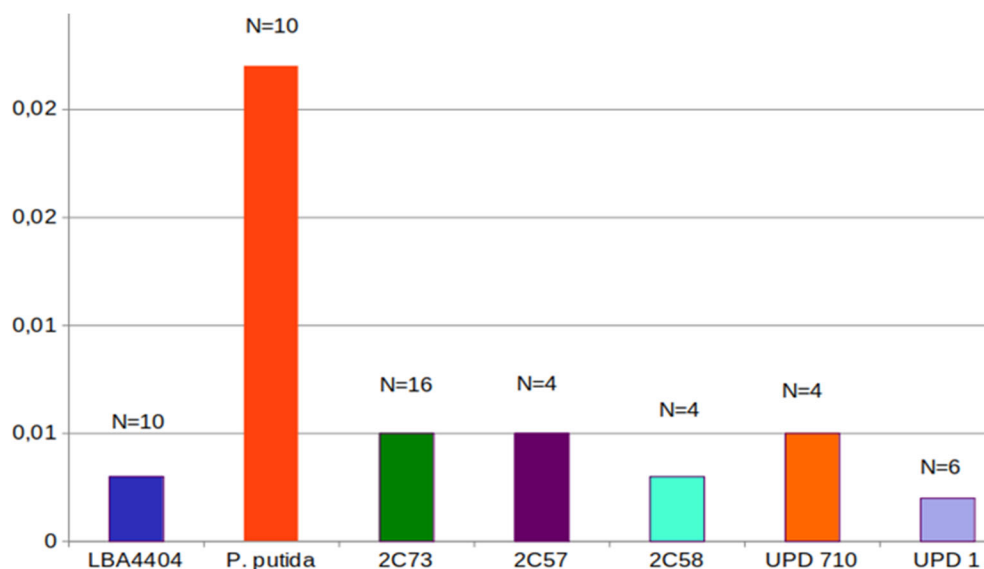


Figure 4-9: Average formation of indigo from 1mM indole incubation with triplicate cultures of environmental bacterial isolates. The Y axis represent Optical Density at 610 nm.

Bacterial Genus Identification

Genomic DNA isolation, PCR with 16S rDNA primers (8F and 926S) and DNA sequence analysis using the Ribosomal Database for Microbes at Michigan State University (<http://rdp.cme.msu.edu/>) and the NCBI database (Altschul et al., 1990) identified the classification of isolates to the genus (Table 4-1). Isolates identified as members of the genus *Arthrobacter* included: 2C47, 2C50, 2C57, 2C59, 2C60, 2C73, 2C80, UPD1, UPD6 (GenBank accession number GU973892), 4D61Y, and 4D97. Isolates identified as members of the genus *Pseudomonas* sp. included: 11C-5W, 12B-3W, and 12B-9LY. Isolates identified as members of the genus *Acinetobacter* included: 11C-8Gh and 12B-7Gh. Isolates identified as members of the genus *Chryseobacterium* included: 42 and 11C-4Y. 12B-2W was identified as a *Duganella*, UPD710 was identified as a *Rhodococcus* (Thomas & Dabkowski, 2011, Genbank Accession HQ230308), and 12B-1Y was identified as a *Pedobacter*. *Psuedomona putida* was previously obtained from James Tiedje (Michigan State University, East Lansing, Michigan). LBA4404 was purchased from Strategene (Tory Pines, CA) and was identified as *Agrobacterium tumefaciens* lacking the Ti plasmid. Further sequence analysis was done to confirm the genus and species of the best performer in this study, isolate 2C73.

Table 4-1: Summary of purified bacterial colonies isolated from Ford Rouge soil samples and yard soil samples.

| Isolate Code | DNA Analysis | Microbe Origin |
|--------------|---------------------------|-------------------|
| 2C47 | Arthrobacter sp. | Ford Rouge |
| 2C50 | Arthrobacter sp. | Ford Rouge |
| 2C57 | Arthrobacter sp. | Ford Rouge |
| 2C59 | Arthrobacter sp. | Ford Rouge |
| 2C60 | Arthrobacter sp. | Ford Rouge |
| 2C73 | Arthrobacter aurescens | Ford Rouge |
| 2C80 | Arthrobacter sp. | Ford Rouge |
| 4D 97 | Arthrobacter sp. | Ford Rouge |
| 4D 61Y | Arthrobacter sp. | Ford Rouge |
| Monster | Arthrobacter sp. | Ford Rouge |
| UPD1 | Arthrobacter sp. | Ford Rouge |
| UPD6 | Arthrobacter sp. | Ford Rouge |
| UPD710 | Rhodococcus sp. | Ford Rouge |
| 42 | Chryseobacterium sp. | Ford Rouge |
| P. putida | Pseudomonas putida | obtained from MSU |
| LBA4404 | Agrobacterium tumefaciens | Negative Control |
| 11C-8GH | Acinetobacter sp. | Yard Soil |
| 12B-7GH | Acinetobacter sp. | Yard Soil |
| 11C-4Y | Chryseobacterium sp. | Yard Soil |
| 12B-2W | Duganella sp. | Yard Soil |
| 12B-1Y | Pedobacter sp. | Yard Soil |
| 11C-5W | Pseudomonas sp. | Yard Soil |
| 12B-3W | Pseudomonas sp. | Yard Soil |
| 12B-9LY | Pseudomonas sp. | Yard Soil |

Using the 16S rDNA amplified from genomic DNA of 2C73, 8F and 926R gene primers were used to recover the DNA sequence. The purified PCR product was cloned in pGEM Easy and submitted to University of Michigan DNA Core Facility (Dr. Robert Lyons) for analysis. From 3 clones submitted for analysis, 5 reads at greater than 1,000 base pairs per read were returned. Each read was edited and aligned to 100% redundancy using MacVector version 12.7 software. Isolated 2C73 rDNA was subjected to a NCBI database search (Altschul et al., 1990)

and results suggested the identity to be *Arthrobacter aurescens*. Sequenced rDNA for 2C73 assigned GenBank accession number is KF110992 (Figure 4-10).

```
BLAST BLASTN 2.2.28+ Results Description List

BLAST Analysis for Sequence: Final Overlap 4
Search from 1 to 906
Expect: 10          Low complexity filter: on
Genetic Code: n/a   Matrix: n/a
Open cost: 5        Gapped search: on
Extend cost: 2

Database: nr (1,914,789,577 residues in 26,000,382 sequences)
Karlin-Altschul Statistics:
  Kappa = 0.41, Lambda = 0.625, Entropy = 0.78

1. gi|315189987|gb|HQ597008.1| Arthrobacter aurescens strain ABRIINW
23 16S ribosomal RNA gene, partial sequence

  Length = 1511
  Score = 1604.5 bits (1778), Expect = 0
  Identities = 896/898 (99%), Positives = 896/898 (99%), Gaps =
2/898 (0%)
```

Figure 4-10: Examples of BLAST searches used to place 2C73 in the *Arthrobacter* genus, with several comparisons with previously described organisms. Sequenced rDNA for 2C73 assigned GenBank accession number is KF110992.

Chapter 5: Discussion

Summary

The goal of this scientific investigation was twofold: I.) Identification of soil microorganisms capable of biodegrading polystyrene, and II.) Determination of the effect of supplementation of bacterial suspensions on polystyrene biodegradation. Soil bacteria capable of surviving 21-day incubation periods on the sole carbon source provided by polystyrene were identified by enrichment cultures, colony isolation and purification, and WST-1 viability testing when microbes were grown on polystyrene 96 well plates. Species determination used 16S ribosomal RNA primers and procedures coupled to DNA gene sequencing (reference to primers). Additionally, proliferation was surveyed with the monomer styrene as an alternative carbon source. Overall, the results revealed several species originating from soils at a former steel plant or in a residential backyard are indeed capable of proliferation given only polystyrene as a useable source of carbon. While the metabolic details need to be further researched, these findings help contribute to existing research identifying biological means to degrade waste polystyrene, a plastic derived from petroleum with an extremely long half-life in landfills (Ojeda et al., 2009; Yoshito et al., 1995). Combined with eco-friendly manufacturing either using polymers more easily degraded in the environment, incorporating degradative enzymes within the plastic polymer, or alternative packaging, all represent considerable progress minimizing nuisance waste plastic in assorted ecosystems.

Microbial growth parameters are important to consider when determining appropriate conditions to promote polystyrene degradation. Adhesion of microbes to polystyrene in biofilms

seems to play a significant role in positioning plastics and microbes within an environment conducive for biodegradation (Mor & Sivan, 2008; Ebcida & Gnanamani, 2020; Han, et al., 2020; Zeraik & Nitschke, 2012). Microbial group communication (e.g., quorum sensing) is known to regulate the metabolism of planktonic (freely swimming) cells, inducing microbial aggregation and forming biofilms, causing increased pathogen virulence (Melaugh et al., 2016). In preliminary experiments with selected environmental microbes growing in shaker cultures, microorganism aggregates from planktonic/free cells have been observed up to mm in diameter, suggesting biofilm formation may be a prelude to promote polystyrene degradation (Melaugh et al, 2016). Conglomerate biofilms represent distinct metabolic commitments, differing from their previous planktonic growth metabolism. The resultant subsets of member cells in a biofilm alter metabolic processes in heterogeneities mixtures of nutrients and the variable oxygen concentration, creating a multicellular biochemical unit that is hypothetically metabolically diverse and potentially capable of polystyrene destruction (Stewart, 2003; Wentland et al., 1996).

With recent data suggesting light-mediated attack of polystyrene (Kim et al., 2013; Tofa et al., 2019; Ward et al., 2019), light-mediated scarring of polystyrene surfaces may function as an initial microbial docking site, later promoting effective biofilm development, to remediate waste polystyrene. Given that a large amount of waste polystyrene currently resides in landfills, obviously protected from light-mediated scarring, this study attempts to understand how soil borne microbes may indeed permit polystyrene destruction within poorly illuminated soils yet in exposed light the entire polystyrene molecule can be deteriorated (Ward et al., 2019).

WST-1: Can A Metabolic Indicator Also Reveal Polystyrene Degraders?

Key to this study was the ability to screen isolated species to survive given polystyrene as the sole source of carbon was analyzed using the WST-1 cell assay. WST-1 is a colorimetric test wherein the stable tetrazolium salt in WST-1 is bio-reduced to produce formazan dye at the cell surface, a reaction highly dependent on the glycolytic production of NADPH in living cells, thus the amount of formazan dye formed is directly correlated to the metabolic activity of cells (Ishiyama et al., 1995). Analysis of the WST-1 results was completed using a binary method where production of the dye was considered a positive result and lack of visible dye production counted as a negative result. The formazan product of WST-1 indicates “metabolism” and an active citric acid cycle, where positive isolates were undergoing aerobic respiration. We cannot rule out isolates that may undergo anaerobic fermentation, as no oxygen-free environments were used to assess this assumption and respiration processes are likely dominant in an oxygen atmosphere. Recently, studies with *Exiguobacterium* sp. RIT 594, a soil bacterium, and the same genus as those from insect guts fed polystyrene, found that unlike aerobic cultivation, no polystyrene degradation was observed using FTIR spectral analysis (Parthasarathy et al., 2022). Lack of sufficient oxygen in landfill soils likely explains delayed degradation by soil localized microbes.

Polystyrene “metabolism” indicated by WST-1 has the caveat that the *Arthrobacter* genus, under starvation conditions, use their vast stored polysaccharides as an energy source, subsequently demonstrating long term survival in resource depleted soils (Boylen, 1973; Mongodin et al., 2006). 2C73 demonstrated cell division independent of the presence of root exudate, perhaps because of polysaccharide storage generated during the initial overnight culture

in YEPG prior to subculture on carbon free medium (Boylen, 1973; Mongodin et al., 2006). *P. putida* was responsive to additional carbon sources within the exudates, as extensive carbohydrate and amino acid materials have been demonstrated in this particular exudate preparation (Thomas et al., 2017). This result is not entirely surprising as many other bacteria were exclusively positive in the presence of this root exudate, being fed by the various carbon-containing nutrients found in root exudates (Thomas et al., 2013; Thomas et al., 2017). Only the addition of CuSO₄ was found to be inhibitive, underlying the toxic nature of copper introduction stimulating free radial attack (Hochstein et al., 1980), also an inhibitor to all cultures tested (Figure 4-5). When incubated on 0.15% styrene-agar plates, not only was 2C73 found capable of both survival and proliferation, but this isolate was also found to be the strongest performer overall. When evaluated in the 14 month packing peanuts experiment, mass loss caused by 2C73 also suggested this isolate capable of polystyrene breakdown.

Pseudomonads, prevalent in industrial soils, may also be of interest as they are found in industrial soils and may contain polystyrene degradation potential (Thomas et al., 2012). LBA4404, an *Agrobacterium tumefaciens*, was used as the negative control in this study. This isolate is often used in plant genetic engineering because the Ti plasmid was disarmed (Komari et al., 1996). LBA4404 consistently exhibited low levels of WST-1 indicated metabolism after incubation (Figure 4-5). Likewise, styrene (monomer) used as a carbon source revealed that LBA4404 can survive, but not thrive, on styrene (Figure 4-6).

Is Mass Loss of Packing Peanuts a Viable Index of Degradation?

Larger cultures with greater liquid media volumes were screened after a 14-month cultivation (Table 2). As noted in Figure 4-5, isolate 2C73 was recovered following the 14-month cultivation on minimal medium with packing peanuts. *Pseudomonas putida* was used as a positive

control but may demonstrate catabolite repression of styrene metabolism in non-limiting concentrations of succinate and glutamate as reported by O'Connor et al. (1996). Moreover, *P. putida* is not capable of degrading styrene because the accumulation of the toxic metabolite 3-vinylcatechol deactivates catechol-2,3-dioxygenase, an aromatic ring-cleaving enzyme (George & Hay, 2012). Our results support George and Hay's (2012) findings confirming *P. putida* as incapable of maintaining long-term growth on styrene-agar plates because of toxic metabolic side products. The apparent positive WST-1 results after 21 days incubation on polystyrene may indicate 3-vinylcatechol may be insufficient to limit metabolism. The only deviation from this trend was with 0.1 mM CuSO₄ treatment (Figure 4-2), a well know Fenton metal that produces toxic free radicals (Hochstein et al., 1980). Cell counting studies revealed that *P. putida* experienced an average of one division during the 21 days incubation on polystyrene and an average 4 divisions during 21 days incubation in the presence of 0.35% Swamp Goldenrod plant root exudate [consistent with exudate stimulation of cell divisions in this species (Thomas & Dabkowski, 2011)]. Unlike LBA4404, *A. tumefaciens*, the influence of Swamp Goldenrod exudate may influence metabolism but not directly stimulate cell division in *Pseudomonas* (Figure 4-3).

Is 2C73 a Unique or Special Isolate with Respect to Polystyrene Biological Activity?

Polystyrene culture, on otherwise carbon-free growth, of *Arthrobacter* and *Pseudomonas* isolates consistently responded positively in WST-1 tests. While strong performers when incubated on polystyrene, *Pseudomonas* isolates *p. putida*, 12B-9LY, and 12B-3W were the three worst performers when incubated on styrene-agar plates. Though inconclusive due to small sample size and the specificity of the WST-1 for metabolism and only indirectly at polystyrene degradation, perhaps these two isolates can metabolize a small portion the hydrocarbon backbone (perhaps the terminal end of the polymer) but are incapable of polymers internal ring cleaving and

utilizing the attached ring structure as described recently in another soil-based bacteria (Parthasarathy et al., 2022).

One experimental challenge interpreting WST-1 96-well plate tests limited our conclusions because after 3 weeks in the dark at room temperature, the culture volume showed significant evaporation. Thus WST-1 analysis was useful in the identification of the presence of live cells post three-week incubation periods, but did not delineate between cell survival and proliferation, nor did it indicate whether polystyrene had been utilized as a carbon source. Also arguing for the extensive survival using alternative carbon sources stored by some cells (e.g., polysaccharides), positive WST-1 results may indicate metabolism from impurities trapped by the polystyrene biosynthesis, or metabolism of stored polysaccharides. Cell counting at day 0 and day 21 of incubation on polystyrene in PMM alone or PMM plus 0.35% plant root exudate had simply survived nutrient starvation or had multiplied during incubation.

What Is the Nature of An Unknown and Uncharacterized Polystyrene Carbon Harvesting Biochemical Pathway?

It is well known that many soil microbes maintain metabolic pathways which use the monomer styrene as an effective carbon source (Sielicki et al., 1978; Ward et al., 2005). Our assumption in considering possible biochemical pathways degrading polystyrene was that the monomer styrene may partner with unknown depolymerizing activities of styrene-related compounds. Environmental bacteria recovered here showed that styrene growth was generally coincident with the WST-1 positive results after 21 days incubation. The notable exception was *Agrobacterium tumefaciens* (LBA 44040) (Figure 4-8).

In one breakthrough study, the larvae of mealworms known to efficiently digest polystyrene (*Tenebrio molitor Linnaeus*) was found to live as well on a diet composed solely of

Styrofoam for one month as mealworms fed the normal bran diet (Yang et al., 2015a; Yang et al., 2015b). Since then, multiple insect-based gut microbes have been shown able to utilize polystyrene as a carbon source (Lou et al., 2020; Urbanek et al., 2020). Recently, *Exiguobacterium* species from soils under an oak tree also demonstrated a unique “oxygenase,” that has activity against polystyrene. In this report, Parthasarathy et al. (2022) used Fourier transform infrared (FTIR) spectroscopy analysis and revealed that during biodegradation in *Exiguobacterium* species an increase of carboxyl and hydroxyl groups and unconjugated C-C double bonds (e.g., polystyrene de-aromatization). *Exiguobacterium* species growing on polystyrene demonstrate created holes and small polystyrene fragments, as well as their cell shape being transformed into an elongated shape; reminiscent of studies in carbon-limitation in this species and original EM images (Yang et al., 2015a; Yang et al., 2015b).

Chapter 6: Conclusion

The useful life of most polystyrene applications is minuscule in comparison to the hundreds or thousands of years these products can persist in the environment as pollutants and a threat to wildlife and humans alike; a new approach to managing this type of waste is needed. Polystyrene's most celebrated qualities, its inertness and durability, are also the basis of its resistance to degradation in the biotic environment. Discovering a pathway to convert technical nutrients such as polystyrene to biological nutrients may provide a pathway to begin to mitigate technical end-of-cycle toxicologic concerns (McDonough & Braungart, 2003).

The goal of this research was to identify and characterize soil bacteria capable of biodegrading polystyrene. In this study, individual microbial isolates were established from yard soil samples (Farmington Hills, MI) and from soil of the Ford Rouge Steel plant (Dearborn, MI). WST-1 testing was used to identify isolates demonstrating substantial metabolism when cultured on polystyrene as the sole carbon source. The influence of plant root exudates and/or 0.1 mM CuSO_4 on microbial isolate metabolism were then examined. The most metabolically active microorganisms were found to derive from the genera *Arthrobacter* and *Pseudomonas*, determined using rDNA sequence identification. *Arthrobacter* 2C73 was the most likely candidate, of the soil microbes evaluated, to perform well in further experimentation to identify bacteria capable of consistently exhibiting metabolic activity. A recent report of a soil isolate able to effectively degrade polystyrene, *Exiguobacterium* sp. RIT 594 (Parthasarathy et al., 2022), lends further support to the idea that environmental bacteria are indeed capable of polystyrene degradation in the presence of oxygen.

Our study examined the behavior of purified bacterial isolates removed from the diverse populations of bacterial species within which they would be found in a natural environment. Other recent research (Cao et al., 2022) has found microbial consortia better able to degrade complex compounds than bacterial isolates. Our study did not consider the potential synergistic effect of consortia and associated enzymes; however, advancements in metabolic engineering and synthetic biology may lead to the possibility of the design and construction of artificial microbial consortia.

The research of a variety of biotechnical approaches to the biodegradation of complex, petroleum-derived compounds such as polystyrene is applauded and should continue to explore biosynthetic pathways of plastics degradation. The results of this study support future work researching the biochemistry of polystyrene degradation in soil-borne microbes.

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