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Green Disposal of Waste Bisphenol A

Abstract:
Bisphenol A (BPA) is an important precursor for polycarbonates and epoxy resins that are used to make products that many would consider essential for modern living. Unfortunately, BPA is an endocrine disrupter and more and more evidence is being uncovered about its negative effects on humans as well as on organisms in the environment. Sustainable chemistry means we take everything into account in designing a chemical process: the risks, the costs, the benefits, and so on. BPA is not likely to be replaced by a less toxic chemical in the foreseeable future, nor is our society willing to give up valuable polycarbonate products. As part of sustainable green chemistry, we want to assure that any toxic chemicals that leave a manufacturing plant pose a minimal risk to the environment. Using a green method to clean up effluent before it is released into the environment would be ideal. In this report, little bluestem seeds are shown to be capable of degrading BPA in aqueous solutions. This gives them the potential to be used in reducing the amount of BPA that may be in effluent. Additionally, an enzyme was isolated that was responsible for degrading BPA and this could also have potential for the treatment of effluent.

Keywords: Bisphenol A (BPA), little bluestem, green chemistry, sustainable chemistry, seed exudate, germination, enzymes, degradation

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1 Introduction

Bisphenol A (BPA) is an important industrial chemical that is used in the manufacture of epoxy resins and polycarbonates. According to the U.S. Environmental Protection Agency, BPA is considered a high-production volume chemical [1]. Merchant Research & Consulting published a report stating that the production of BPA in 2011 was approximately 4.4 million tons, increasing to 4.6 million tons in 2012, and predicted to be greater than 5.4 million tons by 2015 [2]. In order to make epoxy resins, BPA is combined with epichlorohydrin to make the monomer unit, then the resin is cured by reacting it with other chemicals to impart specific properties. Epoxy resin is used in making water pipes, flooring, wind rotor blades, marine coatings, automotive coatings, liners of food containers, fiber optics, and circuit boards. The other major use of BPA is in making the monomeric unit of polycarbonate plastics. Polycarbonate plastics are shatter resistant, are heat resistant, are lightweight, and have optical clarity. These properties have led to their use in safety equipment (helmets and visors), eye glasses, medical equipment, car components (headlamps and sunroofs), and coatings on CDs and DVDs. As of 2013, there was an annual production of 3.3 million tons of polycarbonate produced worldwide [3].

Epoxy resins and polycarbonates are an integral part of our daily lives and are not likely to be replaced with other materials in the foreseeable future. With concerns about BPA leaching out of products and having negative health effects, some have tried to green up the process of manufacturing plastics by looking for substitutes for BPA. For example, BPS (4,4′-sulfonyldiphenol) and BPF (4,4′-dihydroxydiphenylmethane) have both been used as alternatives to BPA but they also show endocrine disrupting effects like BPA [4, 5]. A novel substitute for BPA was proposed by Reno et al. [6] at an American Chemical Society meeting in 2014. They synthesized bisguaiacol F (BGF) from vanilyl alcohol and guaiacol which are lignin components. Polymers made with BGF had similar properties to those made with BPA, but rigorous testing of the polymers and of the health effects of BGF have not been completed. For now, it appears that the best approach is to try to keep BPA out of the environment and determine how to minimize human exposure to BPA.

Although landfills and household waste contribute to BPA in the environment, the largest contributor of BPA to the environment is industrial waste [1]. In making epoxy resin, BPA can be released when the monomer unit for epoxy resin is washed [7]. It is also released in the manufacture of products made from epoxy resin. The Epoxy Resin Committee commissioned an independent agency to determine potential emissions of BPA from the manufacture and use of epoxy resins [7]. They reported that in the manufacturing of marine coatings there is a maximum annual release of 194 kg of BPA. Maximum annual BPA releases for other manufacturing processes include 171 kg for flooring, 105 kg for automotive coating, 92 kg for wind rotor blades, and 11 kg

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for water pipes. The manufacturing of polycarbonates also produces BPA waste. In Nigeria, the effluents from plastic companies contained 107–162 µg/L BPA [8].

Although BPA has a relatively short half-life in the environment (4.5–4.7 days) [9] due to degradation by microorganisms [10, 11], there is a continual release of it into the environment giving a persistent detectable concentration. In sampling streams throughout the United States, BPA was found at a maximum concentration of 12 µg/L and the median detectable concentration was 0.14 µg/L [12]. There is no agreement on a minimum concentration of BPA which is safe for wildlife because toxic effects have been seen at very low levels for some organisms at specific stages of their development, while others require higher levels of BPA to see any effects. For example, when the crustacean, *Tigriopus japonicus* was exposed to 0.1 µg/L BPA for 4 weeks, its development was inhibited [13]. When the Western clawed frog (*Silurana tropicalis*) was exposed to 2.28 µg/L BPA for 9 days, spontaneous metamorphosis was inhibited [14]. Sufficient data regarding the toxicity of BPA to various organisms in the environment has accumulated [15] to convince Canada to require manufacturers to develop plans to deal with BPA in their waste, and propose a limit of 1.75 µg/L for emissions by manufacturers [16]. Environmental effects of BPA and mechanisms of action are outlined in a paper by Canesi and Fabbri [17]. They showed that BPA can cause developmental and reproductive effects in certain species of aquatic vertebrates; however, additional studies are necessary to establish dose–response curves and a more accurate picture of how aquatic organisms respond to BPA over the course of their life cycle.

Methods to remove BPA from wastewater include photodegradation using UV radiation with microwave and heat [18], biodegradation by white rot fungi and enzymes isolated from these fungi [19], and biodegradation by bacteria [20]. Plants can also degrade BPA. For example, cultured suspension cells of eucalyptus were shown to degrade BPA [21] and cultured suspension cells of tobacco as well as tobacco seedlings were found to take up and degrade BPA [22]. *Portulaca oleracea* was found to remove nearly all of 50 µM BPA from water in 24 h [23]. Water convolvulus, an aquatic plant, removed BPA from an aqueous medium and in 1 week the BPA was completely metabolized [24]. *Dracaena sanderiana*, a tropical evergreen plant, was able to take up BPA and was subsequently shown to be effective in removing BPA from landfill leachate [25].

In this study, we will be looking at little bluestem seeds (*Schizachyrium scoparium*) and their ability to degrade BPA as they germinate. Little bluestem is a perennial grass native to the United States and Canada and is used for prairie restoration [26]. It has also been used in phytoremediation studies for the degradation of petroleum hydrocarbons [27, 28]. This concept of seeds degrading chemicals as they germinate was first reported by Loffredo et al. [29] who used seeds of various plants and found that a number of them were able to degrade BPA as they were germinating.

2 Materials and Methods

2.1 Chemicals

BPA was obtained from Alfa Aesar, bis(4-hydroxyphenyl)methane (bisphenol F) was obtained from Aldrich, and BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) was obtained from Supelco. All other chemicals were reagent grade except for methanol which was HPLC grade. Little bluestem seeds were obtained from Outside Pride (Oregon).

2.2 Degradation/Uptake of BPA by Little Bluestem Seeds

Little bluestem seeds [10] were added to 6 mL of 46 mg/L BPA in small (60 × 15 mm) Petri dishes. Control plates contained 6 mL of BPA solution (46 mg/L) but no seeds. All experiments were run in quadruplicate. Plates were kept in the dark at 27 °C. After the incubation time, 1 mL of solution was removed, internal standard (BPF) was added, then the solution was extracted with 1 mL of ethyl acetate to recover BPA and BPF. The ethyl acetate was evaporated and the residue was derivatized for gas chromatography/mass spectrometry (GC/MS). Seeds were removed from each plate and extracted with methanol containing internal standard (1 mL methanol for 10 seeds) by shaking in a test tube for 10 min at 200 rpm. Methanol was decanted from seeds and evaporated before derivatization for GC-MS.

2.3 Quantification of BPA

Residue containing BPA was derivatized by adding 50 µL each of BSTFA and pyridine, as described by Gatidou et al. [30], then the derivatized BPA was quantified by GC-MS. The instrument was a Finnegan Trace GC Ultra

with Trace DSQ and AS 3000 autosampler (Thermo Electron Corp.). The column was a Phenomenex Zebron Inferno, ZB-5HT, 30 m × 0.25 mm i.d. and 0.25 µm. The inlet and the mass spec transfer line were at 280 °C. Injection was splitless and the carrier gas (He) flowed at 1.2 mL/min. The gas chromatograph program was 150 °C for 1 min, then the temperature was increased to 225 °C at a rate of 35 °C/min and held for 2 min. Finally, the temperature was increased to 325 °C at a rate of 25°/min and held there for 3 min. Detection mode was SIM, detecting positive ions with m/z of 179, 329, 344, 357, and 372.

2.4 Degradation of BPA by Seed Exudate

Seed exudate was prepared by soaking little bluestem seeds in deionized water in the dark at 27 °C for 6–10 days. After the incubation time, the seeds were removed and the remaining solution was the seed exudate.

Exudate solution (0.5 mL) was added to 0.5 mL of 0.100 M potassium phosphate pH 6.5 containing BPA giving a final concentration of 30 mg/L BPA in the incubation mixture. Controls contained deionized water instead of exudate solution. All were done in quadruplicate. Solutions were incubated at 27 °C for 4–8 days. After the incubation time, internal standard (BPF) was added, then the solution was extracted with 1 mL of ethyl acetate as mentioned earlier and derivatized for GC/MS analysis.

2.5 Sephadex G-75

Freeze-dried exudate (3.7 mg) was applied to a Sephadex G-75 column (34 × 2 cm). The column was eluted with 0.100 M potassium phosphate pH 6.5 and 1 mL fractions were collected. Absorbance was measured at 280 nm. Fractions obtained from the column were dialyzed against water and freeze-dried before being tested for their activity to degrade BPA. Blue dextran and DNP-aspartate were the standards used to determine the void volume and total volume of the column.

2.6 Bradford Protein Assay

Protein was quantified with the Bradford assay using bovine gamma globulin as the standard [31].

2.7 Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [32]. The gels were BioRad mini-protean TGX precast gels 4–20 %. They were stained with Coomassie Blue.

2.8 Solid-Phase Extraction

A C-18 solid-phase extraction (SPE) cartridge (Alltech) was used to purify Fraction 2. One mg of Fraction 2 dissolved in 1 mL of water was applied to the column. The brown color was adsorbed to the cartridge and was eluted with 0.5 mL methanol.

2.9 FT-IR

The Fourier transform infrared (FT-IR) spectrum was obtained by scanning 16 times on a neat film and with a resolution of 16 cm^{-1} using a Shimadzu FT-IR Miracle ATR in reflectance mode.

2.10 Statistics

Differences between samples were tested for significance with one-way ANOVA.

3 Results and Discussion

3.1 Degradation of BPA by Little Bluestem Seeds

Little bluestem seeds were incubated in a solution containing BPA (46 mg/L) for 6 days. After 6 days, the amount of BPA remaining in the solution was determined and compared with the control solution that did not contain seeds. When seeds were present the amount of BPA in the solution was decreased by 65 % (Figure 1). To determine whether BPA was taken up by the seeds, the seeds were extracted, and the amount of BPA extracted from the seeds was 3 % of the control. This indicates that 62 % of the BPA was either metabolized or was taken up by the seeds and could not be extracted. There is precedence for BPA becoming inextractable following absorption as shown by a study that traced BPA's absorption and metabolism in a plant, water convolvulus (*Ipomoea aquatica*) [24]. The authors concluded that over 50 % of the absorbed BPA was either tightly bound to plant material or had polymerized. This is unlikely to be the case for little bluestem seeds, however, because the exudate from little bluestem seeds is able to degrade the BPA, and this would probably occur before the BPA could be incorporated into the seeds.

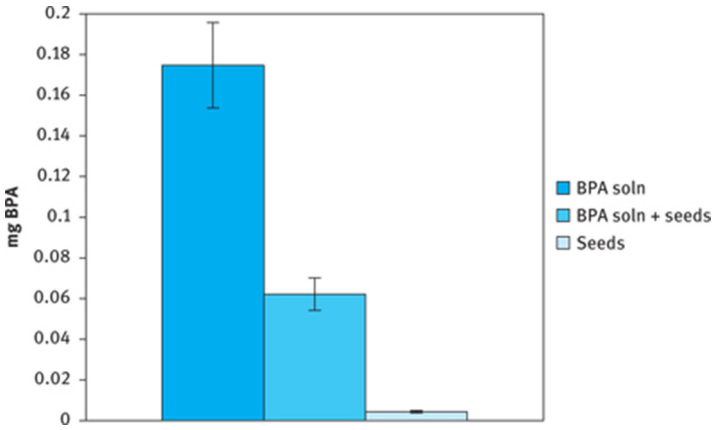

Figure 1: BPA solution (46 mg/L) was incubated with and without little bluestem seeds for 6 days, after which the solution was analyzed for the amount of BPA present. Seeds were extracted with methanol and the amount of BPA in the methanol extract was measured. The solid bar is the control (BPA solution without any seeds present), the hashed bar is the BPA solution that contained seeds, and the unfilled bar is the BPA extracted from seeds. Error bars represent the standard deviation of four replicates.

Of the BPA that was not recovered from seeds or solution (62 %), some of it may have been taken up by the seeds but was not extractable, some of it may have been taken up by the seeds and metabolized, and some of it may have been metabolized by something that leached out of the seeds, a seed exudate. The next experiment tested for the latter: can seed exudate degrade BPA? Seeds were soaked in water for 9 days and the aqueous solution, which now had a brown color, was collected and used as the seed exudate. Seed exudate was incubated with BPA to determine if it could metabolize BPA. The exudate caused a 74 % decrease in the amount of BPA after 5 days (Figure 2). To determine whether the active component in seed exudate was sensitive to heat, a portion of the exudate was boiled before it was incubated with BPA. Although there was some decrease in the amount of BPA, it was not significantly different than the control. This experiment indicates that little bluestem seeds exude something into the water that is capable of breaking down BPA. The active component in the exudate can be inactivated by heat. This is consistent with the idea that an enzyme is exuded from the seeds as they are germinating. A Bradford protein assay indicated that the exudate was 25 % protein by weight also supporting the hypothesis that it is an enzyme. Loffredo et al. [29] had similar results with seed exudate from perennial ryegrass which was found to degrade BPA. They also hypothesized that it was enzymatic activity.

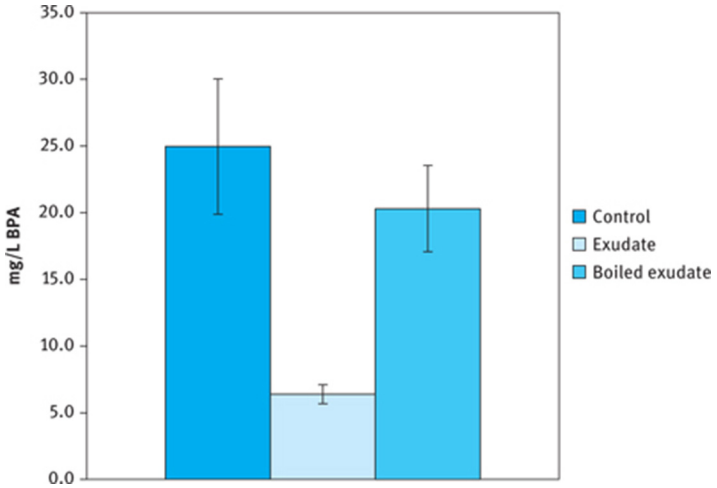

Figure 2: Little bluestem seed exudate was collected after 9 days. A portion of it was placed in boiling water for 10 min to give "boiled exudate." Exudate and boiled exudate were incubated with BPA (30 mg/L) for 5 days after which the solution was analyzed to determine the BPA concentration. The control contained water instead of exudate. Error bars represent the standard deviation of four replicates. The solid bar is the control, the unfilled bar is the BPA solution incubated with exudate, and the bar with diagonal lines is the BPA solution incubated with boiled exudate.

With the understanding that something in the exudate was able to degrade BPA, attempts were made to purify the substance. It was successfully separated by size exclusion chromatography (Sephadex G-75) to give two fractions (Figure 3). The second fraction was notable in that it was a very broad peak and was brown in color. The first fraction was clear. When standards were run on this column (blue dextran and DNP-aspartate), the total volume of the column was determined to be 28 mL. This means that even the smallest protein should elute in 28 mL. The elution volume of the second fraction from seed exudate was about 35 mL, suggesting that the compound was actually binding to the Sephadex G-75 rather than simply traveling through the pores of the size exclusion resin as would be expected for a small protein.

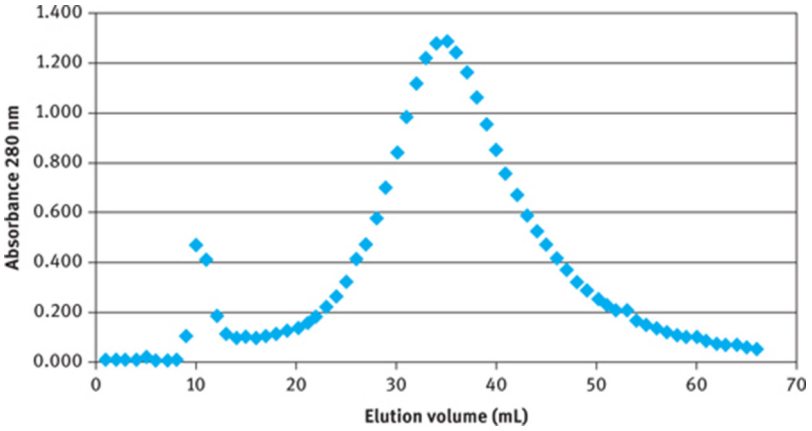

Figure 3: Little bluestem seed exudate (collected after 8 days and freeze-dried) was applied (3.7 mg) to a Sephadex G-75 column (34 × 2 cm). The column was eluted with 0.100 M potassium phosphate buffer pH 6.5. Eluant was collected in 1 mL increments and the absorbance was measured at 280 nm. The first eluting component is fraction 1 and the second one is fraction 2.

To determine which of these fractions contained the active component for degrading BPA, each fraction (1 and 2) was incubated with BPA to determine whether it could degrade BPA. Neither of the fractions alone could degrade BPA, but when they were combined, BPA degradation occurred (Figure 4). This indicates that both fractions must be present in order to degrade BPA.

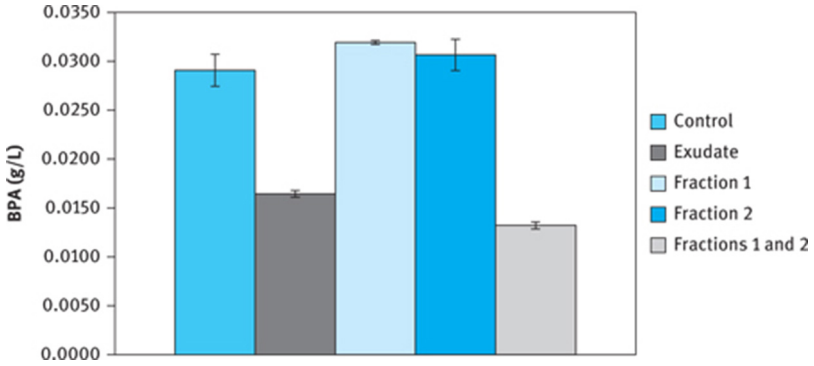

Figure 4: Little bluestem seed exudate (collected after 7 days) was freeze-dried, and a portion of it was separated by Sephadex G-75. Freeze-dried exudate and column fractions were incubated with BPA (30 mg/L). The concentration of BPA was determined after 6 days. The control contained water instead of exudate. Error bars represent the standard deviation of four replicates. The solid bar is the control, unfilled bar is freeze-dried exudate, bars with vertical and horizontal lines are fractions 1 and 2, respectively, and the bar with diagonal lines is a combination of the two fractions.

In order to characterize the components of fractions 1 and 2, they were subjected to electrophoresis. Fraction 1 was successfully separated by SDS-PAGE showing multiple protein bands with molecular weights ranging from 7.5 to 68 kDa (Figure 5). Fraction 2 could not be separated by SDS-PAGE because it appeared to interact with the gel in a way that inhibited its migration.

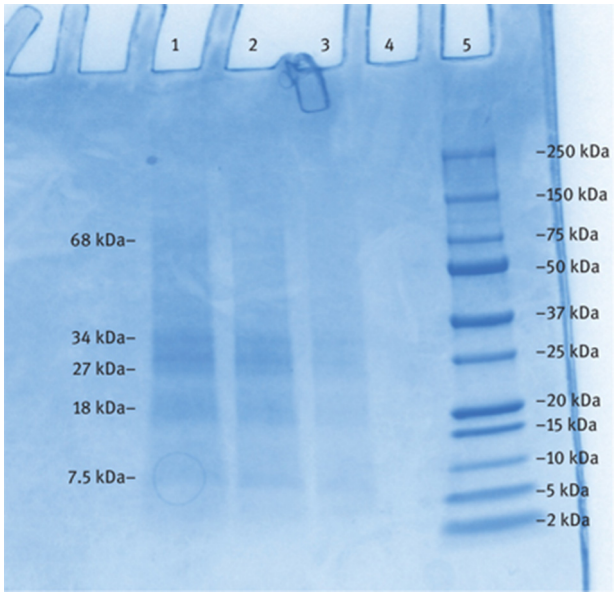

Figure 5: SDS-PAGE of fraction 1 from little bluestem seed exudate. Lanes 1, 2, and 3 are decreasing amounts of fraction 1, lane 4 is empty, and lane 5 is the molecular weight standards.

Fraction 2 is a brown substance that is very water soluble. Often it is polyphenols in plant extracts that impart a brown color. In this case, however, we know this compound is not phenolic because it was not positive with the Prussian Blue reagent [33]. To attempt to identify the substance in fraction 2, it was further separated using a solid phase C-18 extraction cartridge. Fraction 2 was applied to the cartridge, and then the brown color was eluted with methanol. The methanol eluent was analyzed with FT-IR (Figure 6). The IR spectrum indicates that fraction 2 does not contain protein because there is no carbonyl stretch (1,870–1,540 cm^{-1}). However, there is evidence that it contains an aromatic ring with C–H stretching around 3,100 cm^{-1}, C–H bending around 1,000 cm^{-1} and C=C ring stretching in the 1,500–1,400 cm^{-1} region. There is also evidence that it contains an amine with an N–H stretch at 3,402 cm^{-1}. The aromatic ring is also consistent with the compound's absorbance at 280 nm when it eluted from the Sephadex G-75 column.

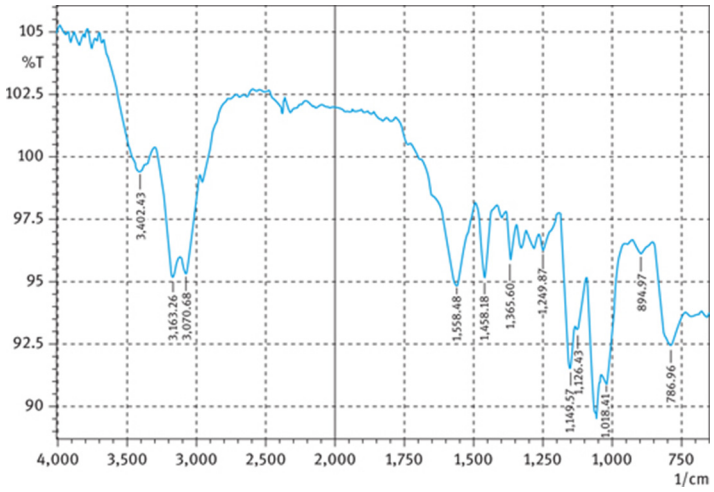

Figure 6: IR spectrum of fraction 2 of little bluestem seed exudate that was purified using a C-18 SPE cartridge.

It is likely that fraction 2 is some sort of cofactor needed by the protein in fraction 1 for the enzyme activity that degrades BPA. It remains a challenge to determine the structure of the cofactor and the source of the brown color.

4 Conclusion

In summary, little bluestem seeds can remove BPA from an aqueous solution as they are germinating. Most of the BPA is degraded by seed exudate that is present in the aqueous solution after the seeds have soaked in water for 6–10 days. The component of seed exudate that degrades BPA appears to be an enzyme that requires a cofactor that has yet to be fully characterized.

Little bluestem seeds have the potential to remove BPA from industrial waste effluent. They would need to be tested on a larger scale and in the presence of the other components of the effluent. The concept of using seeds to clean up contaminated water is also being pursued by Gattullo et al. [34] who used radish seedlings and showed that they could degrade BPA and other endocrine disrupters in samples of water. Alternatively, the enzyme from seed exudate could be isolated and used to degrade BPA in the effluent. Using enzymes in water treatment has been reported by Cabana et al. [35] who used a mixture of fungal enzymes to remove BPA from wastewater and Tsutsumi et al. [36] who used the enzyme laccase to successfully remove BPA and nonylphenol from water. Little bluestem seeds are readily available and it would be worth pursuing their use in cleaning up BPA from effluent.

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