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(54) Title: BIOREMEDIATION OF PETROCHEMICAL-CONTAINING SUBSTRATES USING FUNGI

(57) Abstract: The present disclosure provides fungal cultures, methods of maintaining same, and methods of using same in bioremediation of substrates containing a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt to produce biomass.

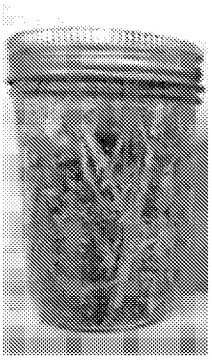

FIG. 1A

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BIOREMEDIATION OF PETROCHEMICAL-CONTAINING SUBSTRATES USING FUNGI

Priority Claim

This application claims priority to U.S. Provisional Patent Application Serial No. 62/655,276, filed September 11, 2018, the entire contents of which are incorporated herein by reference and relied upon.

Field

The present disclosure provides fungal cultures, methods of maintaining same, and methods of using same in bioremediation of substrates including a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt to produce biomass.

Background

Millions of tons of asphalt-including roofing materials are sent to landfills or incinerated each year, representing the fourth-largest volume of all construction and demolition waste streams. Due to high content of heavy metals and hydrocarbons, recycling and reuse options for these materials are limited, and many municipalities have banned or have implemented high fees to send asphalt- and/or PAH-containing materials to landfills. Existing recycling programs are not widely available and are costly to implement.

A need persists for economical, efficient and environmentally-friendly technologies for converting asphalt-containing substrates.

Summary

The present disclosure provides methods of bioremediating substrate that includes petrochemicals, such as a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt . In some embodiments, the method comprises contacting the substrate with fungal tissue to produce a bioremediated product.

In some embodiments, the present disclosure provides a composition comprising a substrate including a PAH and/or asphalt; a growth medium; and a fungal culture.

In some embodiments, the present disclosure provides a method of removing a target pollutant from a substrate that includes a PAH and/or asphalt, the method comprising contacting the substrate with a fungal culture for a period of time sufficient to produce a fungal culture comprising the target pollutant and a bioremediated product.

In some embodiments, the present disclosure provides a method of bioremediating a substrate that includes a PAH and/or asphalt, the method comprising mechanically reducing the substrate to produce a ground substrate; contacting the ground substrate with water for at least one hour to produce a hydrated ground substrate; combining the hydrated ground substrate with a growth medium comprising about 65% water to produce a pre-inoculation mixture; inoculating the pre-inoculation mixture with colonized grains of a fungal culture or a fungal block spawn at an inoculation rate of not more than about 20% to produce an inoculation mixture; and incubating the inoculation mixture at a temperature of about 70° F (about 21° C) for about one day to about one month to produce a bioremediated product.

These and other embodiments are described more fully in the following Detailed Description and accompanying Figures.

Brief Description of the Figures

FIG. 1A shows Substrate 1 of Examples 1-2 including a homogenized blend of a sawdust mixture, asphalt-containing shingle chips, and shredded wheat.

FIG. 1B shows Substrate 2 of Examples 1-2 including a homogenized blend of a sawdust mixture, asphalt-containing shingle chips, and shredded wheat.

FIG. 2A shows the mixture of FIG. 1A before homogenization.

FIG. 2B shows the mixture of FIG. 2A after homogenization.

FIG. 2C shows four containers of each of six fungal tissue-inoculated substrates of Example 2.

FIG. 3A shows an unsterilized container of Substrate 5 of Example 3 inoculated with Pearl Oyster fungal tissue.

FIG. 3B shows an unsterilized container of Substrate 6 of Example 3 inoculated with Pearl Oyster fungal tissue.

FIG. 3C shows an unsterilized container of Substrate 7 of Example 3 inoculated with Pearl Oyster fungal tissue.

FIG. 3D shows a container of Substrate 5 of Example 3 that was pasteurized at 160°F before inoculation with Pearl Oyster fungal tissue.

FIG. 3E shows a container of Substrate 6 of Example 3 that was pasteurized at 160°F before inoculation with Pearl Oyster fungal tissue.

FIG. 3F shows a container of Substrate 7 of Example 3 that was pasteurized at 160°F before inoculation with Pearl Oyster fungal tissue.

FIG. 3G shows a container of Substrate 5 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Pearl Oyster fungal tissue.

FIG. 3H shows a container of Substrate 6 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Pearl Oyster fungal tissue.

FIG. 3I shows a container of Substrate 7 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Pearl Oyster fungal tissue.

FIG. 4A shows shows an unsterilized container of Substrate 5 of Example 3 inoculated with Pearl Oyster fungal tissue.

FIG. 4B shows an unsterilized container of Substrate 6 of Example 3 inoculated with Turkey Tail fungal tissue.

FIG. 4C shows an unsterilized container of Substrate 7 of Example 3 inoculated with Turkey Tail fungal tissue.

FIG. 4D shows a container of Substrate 5 of Example 3 that was pasteurized at 160°F before inoculation with Turkey Tail fungal tissue.

FIG. 4E shows a container of Substrate 6 of Example 3 that was pasteurized at 160°F before inoculation with Turkey Tail fungal tissue.

FIG. 4F shows a container of Substrate 7 of Example 3 that was pasteurized at 160°F before inoculation with Turkey Tail fungal tissue.

FIG. 4G shows a container of Substrate 5 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Turkey Tail fungal tissue.

FIG. 4H shows a container of Substrate 6 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Turkey Tail fungal tissue.

FIG. 4I shows a container of Substrate 7 of Example 3 that was sterilized by autoclave at 250°F for one hour before inoculation with Turkey Tail fungal tissue.

The figures depict various embodiments of this disclosure for purposes of illustration only. One skilled in the art will readily recognize from the following discussion that alternative embodiments of the structures and methods illustrated herein may be employed without departing from the principles of embodiments described herein.

Detailed Description

Referring generally to FIGs. 1A-4I, the present disclosure provides methods of bioremediating an asphalt-containing substrate, and compositions comprising an asphalt-containing substrate; a growth medium; and a fungal culture.

1. Methods of Bioremediation

In general, methods consistent with the present disclosure comprise inoculating a substrate that includes a PAH and/or asphalt with a fungal culture, optionally in the presence of a growth medium.

The substrate may be any substrate that includes a significant amount of a PAH and/or asphalt, also referred to as bitumen. The asphalt may be Type I asphalt, Type II asphalt, Type III asphalt, Type IV asphalt, or a combination of any two or more thereof, as determined by ASTM D312/.D312M-16a (2016). Non-limiting examples of asphalt-containing substrates include roofing shingles that include asphalt, pavement, blacktop, roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, rubberized asphalt, seal coat, fluid applied waterproofing, membrane waterproofing, asphalt-based coatings, asphalt coated materials, asphaltic mastics, asphalt impreganated felts, base sheets, interply adhesives, and other contaminated asphalt waste. In some embodiments, the substrate further includes an asphalt modifier, such as a filler, an extender, a rubber, a plastic, a rubber-plastic combination, a fiber, an oxidant, an antioxidant, a hydrocarbon, an antistripping agent, and/or a waste material. The filler may be, for example, a mineral filler, crusher fines, lime, portland cement, fly ash, and/or carbon black. The extender may be, for example, sulfur and/or lignin. The rubber may be, for example, natural latex, synthetic latex such as polychloroprene latex, a block copolymer such as styrene-butadiene-styrene (SBS), and/or reclaimed rubber such as crumb rubber from used tires. The plastic may be, for example, polyethylene/polypropylene, ethylene acrylate copolymer, ethyl-vinyl-acetate (EVA), polyvinyl chloride (PVC), ethylene propylene, ethylene propylene diene monomer rubber, and/or a polyolefin. The fiber may be, for example, a natural fiber such as asbestos and/or rock wool; or a manufactured fiber such as a polypropylene fiber, a polyester fiber, fiberglass, a mineral fiber, and/or a cellulose fiber. The oxidant may be, for example, a manganese salt. The antioxidant may be, for example, a lead compound, carbon, and/or a calcium salt. The hydrocarbon may be, for example, a recycled oil, a rejuvenating oil, a hard asphalt, and/or a soft asphalt. The antistripping agent may be, for example, an amine and/or lime. The waste material may be, for example, roofing shingles, recycled tires, and/or glass. In some embodiments, the asphalt modifier comprises coal tar pitch.

In some embodiments, the substrate includes polycyclic aromatic hydrocarbons (PAHs), such as pyrene, naphthalene, and anthracene. Non-limiting examples of substrates that include significant amounts of PAH include cigarette butts, incompletely combusted

coal, incompletely combusted petrol, incompletely combusted wood, incompletely combusted tobacco, charbroiled meat products, incompletely combusted trash, or incompletely combusted organic material.

In some embodiments, the substrate comprises an asphalt-contaminated material, such as clay tile onto which asphalt has adhered, or a substrate onto which an asphalt mastic has been applied (e.g., sprayed).

In some embodiments, the substrate (e.g., after a grinding process step) has a longest dimension (e.g., a longest edge length, or a diameter) not greater than about 5 inches, for example not greater than about 5 inches, not more than about 4.5 inches, not more than about 4 inches, not more than about 3.5 inches, not more than about 3 inches, not more than about 2.5 inches, not more than about 2 inches, not more than about 1.5 inches, not more than about 1 inch, not more than about 0.5 inches, or not more than about 0.25 inches. In some embodiments, the asphalt-containing substrate has a longest dimension of not more than about 1 inch. In some embodiments, the substrate is ground to produce a ground substrate wherein each piece of the ground substrate has a longest dimension (e.g., a longest edge length, or a diameter) not greater than about 5 inches, for example not greater than about 5 inches, not more than about 4.5 inches, not more than about 4 inches, not more than about 3.5 inches, not more than about 3 inches, not more than about 2.5 inches, not more than about 2 inches, not more than about 1.5 inches, not more than about 1 inch, not more than about 0.5 inches, or not more than about 0.25 inches. In other embodiments, the substrate is not ground, but is instead processed using a method disclosed herein without a step of mechanically disrupting the substrate prior to inoculation with the fungal culture.

The fungal culture may include any fungal species that is capable of colonizing an asphalt-containing substrate and converting the asphalt in the substrate to a biomass product(s). In some embodiments, the fungal species is selected from the group consisting of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

In some embodiments, the fungal species is *Pleurotus ostreatus*, commonly referred to as the pearl oyster mushroom or the tree oyster mushroom.

In some embodiments, the fungal species is *Pleurotus pulmonarius*, commonly referred to as the Indian oyster mushroom, the Italian oyster mushroom, the Phoenix mushroom, or the lung oyster mushroom.

In some embodiments, the fungal species is *Ganoderma lucidum*, commonly referred to as the Lingzhi mushroom.

In some embodiments, the fungal species is *Trametes versicolor*, commonly referred to as the turkey tail mushroom, and also known as *Coriolus versicolor* or *Polyporus versicolor*.

In some embodiments, the fungal species is *Pleurotus columbinus*, commonly referred to as the blue oyster mushroom.

In some embodiments, the fungal species is *Pleurotus eryngii*, commonly referred to as the king trumpet mushroom, the French horn mushroom, the king oyster mushroom, the king brown mushroom, boletus of the steppes, trumpet royale, or the ali'i oyster.

In some embodiments, the fungal tissue is a mixture of two or more fungal species selected from the group consisting of: *P. ostreatus*, *P. pulmonarius*, *G. lucidum*, *T. versicolor*, *P. columbinus*, and *P. eryngii*.

In some embodiments, the fungal tissue is a combination of *Trametes versicolor* and *Pleurotus ostreatus*.

The growth medium can be any growth medium that enables stable growth of the fungal culture. In some embodiments, the growth medium includes a sawdust mixture. In some embodiments, the sawdust mixture includes alder sawdust, wheat bran, and/or gypsum. In some embodiments, the sawdust mixture includes 50-100% (v/v) alder sawdust, 0-50% (v/v) wheat bran, and 0-50% (v/v) gypsum. In some embodiments, the sawdust mixture includes 60-90% (v/v) alder sawdust, 10-20% (v/v) wheat bran, and 5-15% (v/v) gypsum. In some embodiments, the growth medium comprises a lignin-containing material, such as paper, a lignin-based polymer, a lignin-based concrete additive, a dyestuff dispersant, animal feed, a lignin-based industrial binder, a lignin-based oil well drilling additive, and/or cigarette filters (e.g., cigarette butts).

In some embodiments, the inoculum for the petrochemical-containing material is a block spawn, pelletized spawn, or other spawn delivery form (collectively, "block spawn") comprising a support material and fungal tissue (mycelium). The source of mycelium may be sawdust spawn, compost spawn, straw spawn, grain block spawn, a liquid inoculum (e.g., a liquid suspension of mycelium), mycelium-on-agar, a fruiting block, or any other substrate that can serve as a vector for mycelium. In some embodiments, the support material is an agricultural biomass, such as sugarcane bagasse, corncob, naturally occurring sponge, an agro-waste material, or a lignocellulosic material such as sawdust, straw, or cottonseed hull. In other embodiments, the support material is a non-naturally occurring material such as a synthetic foam (e.g., polyurethane foam). A block spawn comprising an agricultural biomass support consistent with the present disclosure may be prepared by standard methods, for example, by removing any grain materials from the biomass, dividing the biomass (if necessary) into pieces approximately 1-2 inches in size, drying the biomass to a constant weight, optionally pasteurizing or sterilizing the biomass support material, and then inoculating the agricultural biomass support with a homogenized aqueous mycelium suspension.

In some embodiments, the inoculum for the petrochemical-containing material is created by inoculating a sterilized grain, such as hulled millet, with the fungal culture. Inoculation rate may vary based on the specific grain and specific species of fungal culture(s) employed. In general, however, methods of the present disclosure preferably include inoculating with the fungal culture at a rate of not more than about 20%, for example not more than about 20%, not more than about 19%, not more than about 18%, not more than about 17%, not more than about 16%, not more than about 15%, not more than about 14%, not more than about 13%, not more than about 12%, not more than about 11%, not more than about 10%, not more than about 9%, not more than about 8%, not more than about 7%, not more than about 6%, not more than about 5%, not more than about 4%, not more than about 3%, not more than about 2%, or not more than about 1%. In some embodiments, the grain is hydrated (e.g., to about 60% saturation), sterilized (e.g., by heating at 250°F for about one hour, followed by cooling to ambient temperature), and the sterilized grain is then inoculated with the fungal culture using standard aseptic fungal cultivation techniques.

In some embodiments, the substrate is pretreated by soaking in filtered water for about one hour, followed by sterilization (e.g., by heating at 250°F for about one hour, by pasteurization via steam bath, or by soaking in an alkaline solution at pH ~12) before inoculation with a fungal culture.

In some embodiments, the growth medium is pretreated by hydrating (e.g., to about 65% saturation), sterilized (e.g., by heating at 250°F for about one hour, by pasteurization via steam bath, or by soaking in an alkaline solution at pH ~12) before inoculation with the fungal culture.

In some embodiments, the growth medium is combined with the asphalt-containing substrate, and the combined mixture is then sterilized (e.g., by heating at 250°F for about one hour, by pasteurization via steam bath, or by soaking in an alkaline solution at pH ~12) before inoculation with the fungal culture.

In general, cultivation occurs in a manner that prevents competitive species from contaminating the cultivation mixture. In some embodiments, for example, cultivation occurs in a sealed container that includes an air filter.

In some embodiments, the inoculated substrate is cultivated by maintaining ambient temperature at about 70°F (about 21°C). In some embodiments, cultivation occurs under ambient light; in other embodiments cultivation occurs in the absence of light.

Cultivation occurs until the substrate has been consumed. In general, cultivation may require from about 2 weeks to about 6 weeks, depending on the inoculation rate, ambient temperature, and level of sterility of the substrate and/or growth medium prior to inoculation. In some embodiments, cultivation is complete within about 8 weeks, for example within about 8 weeks, within about 7 weeks, within about 6 weeks, within about 5 weeks, within about 4 weeks, within about 3 weeks, within about 2 weeks, or within about 1 week.

In some embodiments, the fungus produces a biomass product. In some embodiments, the biomass product includes a substantially reduced amount of a target pollutant (e.g., a heavy metal, a phthalate, and/or a polycyclic aromatic hydrocarbon) than found in the untreated substrate. Assessment of the amount of asphalt in substrate (e.g., before

and/or after bioremediation according to the present disclosure) may be accomplished using any suitable standard analytical methodology.

In some embodiments, the present disclosure provides a method of bioremediating a substrate, the method comprising contacting the substrate with a fungal culture to provide a bioremediated product, wherein the substrate includes asphalt and/or polycyclic aromatic hydrocarbons ("PAH"). In some embodiments, wherein the bioremediated product comprises water. In some embodiments, the bioremediated product comprises carbon dioxide. In some embodiments, the substrate consists essentially of substrate pieces each having dimensions of not greater than about 2.5 cm. In some embodiments, each substrate piece has a dimension of not greater than about 1 cm. In some embodiments, the method further comprises combining the substrate with a growth medium. In some embodiments, the method further comprises contacting the growth medium with a sterilizing agent before the step of combining the substrate with the growth medium. In some embodiments, the sterilizing agent comprises hydrogen peroxide. In some embodiments, the method further comprises contacting the substrate and the fungal culture with heat. In some embodiments, the method further comprises contacting the substrate and the fungal culture with water. In some embodiments, the method further comprises contacting the substrate and the fungal culture with air. In some embodiments, the method further comprises contacting the substrate and the fungal culture with light. In some embodiments, the method further comprises, after the step of contacting the substrate with the fungal culture, analyzing the fungal culture for a target pollutant. In some embodiments, the target pollutant is selected from the group consisting of: a heavy metal, a phthalate, and a polycyclic aromatic hydrocarbon. In some embodiments, the growth medium comprises one or more of: lignin-based material, paper, cigarette waste, sawdust, paper, cardboard, straw, wheat bran, and gypsum. In some embodiments, the asphalt-containing substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt. In some embodiments, the PAH-containing substrate comprises pyrene, naphthalene, and/or anthracene. In some embodiments, the PAH-containing substrate comprises cigarette butts, incompletely combusted coal, incompletely combusted petrol,

incompletely combusted wood, incompletely combusted tobacco, charbroiled meat products, incompletely combusted trash, or incompletely combusted organic material. In some embodiments, the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*. In some embodiments, the fungal culture comprises *G. lucidum*. In some embodiments, the fungal spore comprises *T. versicolor*. In some embodiments, the fungal culture comprises *P. ostreatus*. In some embodiments, the step of contacting the substrate with the fungal culture comprises inoculating the substrate with colonized grains of the fungal culture or a fungal block spawn at at an inoculation rate of not more than about 20%. In some embodiments, the inoculation rate is not more than about 5%. In some embodiments, the method further comprises incubating the mixture resulting from the step of contacting the substrate with the fungal culture for a period of time sufficient to produce the biomass. In some embodiments, the step of incubating occurs at a temperature of about 70° F (about 21° C). In some embodiments, the period of time is about one day to about one month. In some embodiments, the period of time is about two weeks to about six weeks. In some embodiments, the biomass comprises mycobased fillers, particles, strands, pieces for use within the manufacture of new biobased products or readily available in new form for recycle or disposal. In some embodiments, the ratio of the growth medium to the substrate is about 10:1 to about 1:10. In some embodiments, the ratio is about 3:1.

In some embodiments, the present disclosure provides a method of removing a target pollutant from a substrate, the method comprising contacting the substrate with a fungal culture for a period of time sufficient to produce: (a) a fungal culture comprising the target pollutant, and (b) a bioremediated product. In some embodiments, the bioremediated product comprises the target pollutant in an amount significantly less than an amount of the target pollutant in the substrate. In some embodiments, the target pollutant is a metal element or metalloid element having an initial oxidation state before the step of contacting the substrate with the fungal culture, and wherein the metal or metalloid has a different oxidation state after the step of contacting the substrate with the fungal tissue. In some embodiments, the target pollutant comprises one or more of: a heavy metal, a phthalate, and a polycyclic aromatic hydrocarbon. In some

embodiments, the step of contacting comprises inoculating the substrate with colonized grains of the fungal culture or a fungal block spawn at an inoculation rate of not more than about 20%. In some embodiments, the period of time is about one day to about one month. In some embodiments, the step of contacting occurs at a temperature of about 70° F (about 21° C). In some embodiments, the method further comprises contacting the substrate with water. In some embodiments, the method further comprises contacting the substrate with a growth medium. In some embodiments, the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum. In some embodiments, the asphalt-containing substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt. In some embodiments, the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

In some embodiments, the present disclosure provides a method of bioremediating a substrate, the method comprising: mechanically reducing the substrate to produce a ground substrate; contacting the ground substrate with water for at least one hour to produce a hydrated ground substrate; combining the hydrated ground substrate with a growth medium comprising about 65% water to produce a pre-inoculation mixture; inoculating the pre-inoculation mixture with colonized grains of a fungal culture or a fungal block spawn at an inoculation rate of not more than about 20% to produce an inoculation mixture; and incubating the inoculation mixture at a temperature of about 70° F (about 21° C) for about one day to about one month to produce a bioremediated product wherein the substrate comprises a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt. In some embodiments, the substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt. In some embodiments, the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*. In some embodiments, the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum. In some embodiments, the method further comprises sterilizing the pre-inoculation mixture before the step of inoculating. In some

embodiments, the step of sterilizing comprises contacting the pre-inoculation mixture with a sterilization agent selected from the group comprising: a chemical sterilizing agent, and heat. In some embodiments, the chemical sterilizing agent comprises hydrogen peroxide.

2. Compositions

The present disclosure also provides compositions comprising an asphalt-containing substrate; a growth medium; and a fungal culture.

The asphalt-containing substrate may be any substrate that includes a significant amount of asphalt, also referred to as bitumen. Non-limiting examples of asphalt-containing substrates include roofing shingles that include asphalt, pavement, blacktop, roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, rubberized asphalt, seal coat, fluid applied waterproofing, membrane waterproofing, asphalt-based coatings, asphalt coated materials, asphaltic mastics, asphalt impregnated felts, base sheets, interply adhesives, and other contaminated asphalt waste. In some embodiments, the asphalt-containing substrate includes polycyclic aromatic hydrocarbons ("PAHs"). In some embodiments, the substrate includes phthalates.

In some embodiments, the asphalt-containing substrate has a longest dimension (e.g., a longest edge length, or a diameter) not greater than about 5 inches, for example not greater than about 5 inches, not more than about 4.5 inches, not more than about 4 inches, not more than about 3.5 inches, not more than about 3 inches, not more than about 2.5 inches, not more than about 2 inches, not more than about 1.5 inches, not more than about 1 inch, not more than about 0.5 inches, or not more than about 0.25 inches. In some embodiments, the asphalt-containing substrate has a longest dimension of not more than about 1 inch.

The fungal species may be any fungal species that is capable of colonizing an asphalt-containing substrate and converting the asphalt in the substrate to a biomass product(s). In some embodiments, the fungal species is selected from the group

consisting of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

In some embodiments, the fungal species is *P. ostreatus*, commonly referred to as the pearl oyster mushroom or the tree oyster mushroom.

In some embodiments, the fungal species is *P. pulmonarius*, commonly referred to as the Indian oyster mushroom, the Italian oyster mushroom, the Phoenix mushroom, or the lung oyster mushroom.

In some embodiments, the fungal species is *G. lucidum*, commonly referred to as the Lingzhi mushroom.

In some embodiments, the fungal species is *T. versicolor*, commonly referred to as the turkey tail mushroom, and also known as *Coriolus versicolor* or *Polyporus versicolor*.

In some embodiments, the fungal species is *P. columbinus*, commonly referred to as the blue oyster mushroom.

In some embodiments, the fungal species is *P. eryngii*, commonly referred to as the king trumpet mushroom, the French horn mushroom, the king oyster mushroom, the king brown mushroom, boletus of the steppes, trumpet royale, or the ali'i oyster.

In some embodiments, the fungal species is a mixture of two or more fungal spores selected from the group consisting of: *P. ostreatus*, *P. pulmonarius*, *G. lucidum*, *T. versicolor*, *P. columbinus*, and *P. eryngii*.

The growth medium can be any growth medium that enables stable growth of the fungal culture. In some embodiments, the growth medium includes a sawdust mixture. In some embodiments, the sawdust mixture includes alder sawdust, wheat bran, and/or gypsum. In some embodiments, the sawdust mixture includes 50-100% (v/v) alder sawdust, 0-50% (v/v) wheat bran, and 0-50% (v/v) gypsum. In some embodiments, the sawdust mixture includes 60-90% (v/v) alder sawdust, 10-20% (v/v) wheat bran, and 5-15% (v/v) gypsum.

In some embodiments, the biomass includes a substantially reduced amount of a target pollutant (e.g., a heavy metal, a phthalate, and/or a polycyclic aromatic hydrocarbon)

than found in the untreated asphalt-containing substrate. Without wishing to be bound by theory, it is believed that target pollutants, especially heavy metals, tend to concentrate in fungal tissue (e.g., the fruiting body of the fungus), sequestering the target pollutant from any produced biomass product.

In some embodiments, the present disclosure provides a composition comprising: a substrate comprising a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt; a growth medium; and a fungal culture. In some embodiments, the substrate consists essentially of substrate pieces having a maximum dimension of not greater than about 2.5 cm. In some embodiments, the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum. In some embodiments, the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*. In some embodiments, the composition further comprises water. In some embodiments, the composition further comprises a biomass product produced by the fungal spore.

Examples

Example 1.

Organic hulled millet was hydrated to approximately 60% saturation, and then sterilized at 250°F (about 121°C) for one hour. After cooling, the millet grains were inoculated with *Pleurotus ostreatus* (PO, Pearl Oyster) or *Pleurotus pulmonarius* (PP, Phoenix Oyster) using standard aseptic cultivation techniques. The inoculated millet grains were incubated for three weeks until fully colonized by mycelium

The following two asphalt-containing substrates were prepared:

Substrate Component	Substrate 1	Substrate 2
Sawdust mixture*	50% v/v	75% v/v
Shingle chips**	25% v/v	25% v/v
Shredded wheat straw**	25% v/v	0% v/v

* Sawdust mixture included 75% alder sawdust, 15% wheat bran, 10% gypsum, and was pre-hydrated to ~65% saturation with water.

** Pre-hydrated by soaking for 1 hour in filtered water.

About 8 ounces (about 235 mL) of each Substrate was added to each of four 8-ounce glass jars (FIGS. 1A-2B), which were then sterilized at 250°F (about 121°C) for one hour. After cooling to ambient temperature, each jar was inoculated with the inoculated millet grains at an inoculation rate of about 5%. Each jar was covered with a lid that included an air filter to prevent influx of foreign microbes.

After incubation at 70°F (about 21°C) for one week, abundant fungal growth was observed in each jar. Fungal tissue growth was observed as most abundant in Pearl Oyster (PO) and Phoenix Oyster (PP), followed by *Ganoderma lucidum* (GL), Turkey Tail (TV), Blue Oyster (PC), and King Oyster (PE). Substrate 1 appeared to increase the appearance of mycelium in most species compared to Substrate 2.

Example 2.

Organic hulled millet was hydrated to about 60% saturation and sterilized at 250°F (about 121°C) for one hour. After cooling, the millet grains were separated into six batches and each batch was inoculated with one of the following fungal species:

A) *Pleurotus ostreatus* (PO, Pearl Oyster)

B) *Pleurotus pulmonarius* (PP, Phoenix Oyster)

C) *Pleurotus columbinus* (PC, Blue Oyster)

D) *Pleurotus eryngii* (PE, King Oyster)

E) *Ganoderma lucidum* (GL, Reishi)

F) *Trametes versicolor* (TV, Turkey Tail)

Each batch was then incubated for three weeks until fully colonized by mycelium.

The following three substrates were prepared:

Substrate Component	**Substrate 1**	**Substrate 2**	**Substrate 3 (no-asphalt control)**
Sawdust mixture*	50% v/v	25% v/v	75% v/v

Shingle chips**	25% v/v	50% v/v	0% v/v
Shredded wheat straw**	25% v/v	25% v/v	25% v/v

* Sawdust mixture includes 75% alder sawdust, 15% wheat bran, 10% gypsum, and was pre-hydrated to ~65% saturation with water.

** Pre-hydrated by soaking for 1 hour in filtered water.

Eighteen 8-ounce glass jars were filled with about 8 ounces (about 235 mL) of each Substrate. Eighteen jars of each Substrate were sterilized at 250°F for one hour; eighteen jars of each Substrate were sterilized by steam bath pasteurization; and eighteen jars of each Substrate were not sterilized.

Once all jars reached ambient temperature, each of the six inoculated millet grain batches were added to nine jars at an inoculation rate of about 5% using standard aseptic cultivation techniques as follows:

1) Substrate 1, sterilized to 250°F for one hour via autoclave

2) Substrate 1, pasteurized by 160°F water bath

3) Substrate 1, not sterilized

4) Substrate 2, sterilized to 250°F for one hour via autoclave

5) Substrate 2, pasteurized by 160°F water bath

6) Substrate 2, not sterilized

7) Substrate 3, sterilized to 250°F for one hour via autoclave

8) Substrate 3, pasteurized by 160°F water bath

9) Substrate 3, not sterilized

The inoculated substrate jars were incubated at 70°F (about 21°C) for one week. Fungal growth was assessed each day as a function of the degree of colonization observed (see FIG. 2C). Samples were collected for analytical testing at mix-up of the

three mixes, when each mix of species, substrate, and sterilization treatment reached about 50% colonization, and again when each jar reached 100% colonization.

Example 3.

Two top-performing fungal species from Example 2 were selected for scale-up testing. Each species will be inoculated onto organic hulled millet that was hydrated to about 60% saturation, sterilized at 250°F (about 121°C) for one hour, and cooled to ambient temperature.

Six Substrates were prepared as follows:

Substrate Component	Substrate 4	5	6	7	8	9
Sawdust mixture*	0% v/v	10% v/v	0% v/v	10% v/v	25% v/v	50% v/v
Shingle chips**	100% v/v	90% v/v	90% v/v	75% v/v	50% v/v	0% v/v
Shredded wheat straw**	0% v/v	0% v/v	10% v/v	15% v/v	25% v/v	50% v/v

* Sawdust mixture included 75% alder sawdust, 15% wheat bran, 10% gypsum, and was pre-hydrated to ~65% saturation with water.

** Pre-hydrated by soaking for 1 hour in filtered water.

Twenty-four 5-L containers were filled with each Substrate, for a total of 144 5-L containers. For each Substrate, six containers were sterilized at 250°F (about 121°C) for one hour; six were pasteurized using a 160°F water bath, six were soaked in a strongly alkaline (pH 12) solution, and six were not sterilized.

For each combination of Substrate and sterilization method, three containers were inoculated with each species of incubated grain spawn under aseptic conditions, as follows:

Fungal Species #1—Pearl Oyster (n=72 containers):

	250°F @ 1 hour	Pasteurization	Alkaline Soak	No Sterilization
Substrate 4	3 containers	3 containers	3 containers	3 containers
Substrate 5	3 containers (FIG. 3A)	3 containers (FIG. 3D)	3 containers	3 containers (FIG. 3G)
Substrate 6	3 containers (FIG. 3B)	3 containers (FIG. 3E)	3 containers	3 containers (FIG. 3H)
Substrate 7	3 containers (FIG. 3C)	3 containers (FIG. 3F)	3 containers	3 containers (FIG. 3I)
Substrate 8	3 containers	3 containers	3 containers	3 containers
Substrate 9	3 containers	3 containers	3 containers	3 containers

Fungal Species #2—Turkey Tail (n=72 containers):

	250°F @ 1 hour	Pasteurization	Alkaline Soak	No Sterilization
Substrate 4	3 containers	3 containers	3 containers	3 containers
Substrate 5	3 containers (FIG. 4A)	3 containers (FIG. 4D)	3 containers	3 containers (FIG. 4G)
Substrate 6	3 containers (FIG. 4B)	3 containers (FIG. 4E)	3 containers	3 containers (FIG. 4H)
Substrate 7	3 containers (FIG. 4C)	3 containers (FIG. 4F)	3 containers	3 containers (FIG. 4I)
Substrate 8	3 containers	3 containers	3 containers	3 containers
Substrate 9	3 containers	3 containers	3 containers	3 containers

After inoculation, each container was incubated at 70°F (about 21°C) for three weeks, with fungal growth progression observed and documented daily as a function of degree of colonization. Samples were collected for analytical testing at mix-up of the three mixes, when each mix of species, substrate, and sterilization treatment reached about 50% colonization, and again when each jar reached 100% colonization. Collected data was analyzed using analysis of variance (ANOVA) with repeated measured on the second factor. A Fisher's LSD analysis was performed for any significant effects. The statistical significance level was set at $p < 0.05$. Statistical analysis was performed using SPSS software and data was interpreted hierarchically.

Pleurotus ostreatus (Pearl Oyster) exhibited the greatest growth rates on Substrate 7 that had been pasteurized. Colonization of this combination reached 50% after 8 days; 100% colonization was achieved after 13 days.

Claims

What is claimed is:

1. A method of bioremediating a substrate, the method comprising contacting the substrate with a fungal culture to provide a bioremediated product, wherein the substrate includes asphalt and/or polycyclic aromatic hydrocarbons ("PAH").

2. The method of Claim 1, wherein the bioremediated product comprises water.

3. The method of Claim 1 or Claim 2, wherein the bioremediated product comprises carbon dioxide.

4. The method of any one preceding claim, wherein the asphalt consists essentially of asphalt-containing substrate pieces each having dimensions of not greater than about 2.5 cm.

5. The method of Claim 4, wherein each asphalt-containing substrate piece has a dimension of not greater than about 1 cm.

6. The method of any one preceding claim further comprising combining the substrate with a growth medium.

7. The method of Claim 6 further comprising contacting the growth medium with a sterilizing agent before the step of combining the substrate with the growth medium.

8. The method of Claim 7, wherein the sterilizing agent comprises hydrogen peroxide.

9. The method of any one preceding claim further comprising contacting the substrate and the fungal culture with heat.

10. The method of any one preceding claim further comprising contacting the substrate and the fungal culture with water.

11. The method of any one preceding claim further comprising contacting the substrate and the fungal culture with air.

12. The method of any one preceding claim further comprising contacting the substrate and the fungal culture with light.

13. The method of any one preceding claim further comprising, after the step of contacting the substrate with the fungal culture, analyzing the fungal culture for a target pollutant.

14. The method of Claim 13, wherein the target pollutant is selected from the group consisting of: a heavy metal, a phthalate, and a polycyclic aromatic hydrocarbon.

15. The method of any one of Claims 6-14, wherein the growth medium comprises one or more of: lignin-based material, paper, cigarette waste, sawdust, paper, cardboard, straw, wheat bran, and gypsum.

16. The method of any one preceding claim, wherein the asphalt-containing substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt.

17. The method of any one preceding claim, wherein the PAH-containing substrate comprises pyrene, naphthalene, and/or anthracene.

18. The method of any one preceding claim, wherein the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

19. The method of Claim 18, wherein the fungal culture comprises *G. lucidum*.

20. The method of Claim 18, wherein the fungal culture comprises *T. versicolor*.

21. The method of Claim 18, wherein the fungal culture comprises *P. ostreatus*.

22. The method of any one preceding claim, wherein the step of contacting the substrate with the fungal spore comprises inoculating the substrate with colonized grains of the fungal culture or a fungal block spawn at an inoculation rate of not more than about 20%.

23. The method of Claim 22, wherein the inoculation rate is not more than about 5%.

24. The method of any one preceding claim, wherein the method further comprises incubating the mixture resulting from the step of contacting the substrate with the fungal spore for a period of time sufficient to produce the bioremediated product.

25. The method of Claim 24, wherein the step of incubating occurs at a temperature of about 70° F (about 21° C).

26. The method of any one preceding claim, wherein the period of time is about one day to about one month.

27. The method of Claim 26, wherein the period of time is about 2 weeks to about 6 weeks.

28. The method of any one of Claims 6-27, wherein a ratio of the growth medium to the substrate is about 10:1 to about 1:10.

29. The method of Claim 28, wherein the ratio is about 3:1.

30. A composition comprising:

an substrate comprising a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt;

a growth medium; and

a fungal culture.

31. The composition of Claim 30, wherein the substrate consists essentially of substrate pieces having a maximum dimension of not greater than about 2.5 cm.

32. The composition of Claim 30 or Claim 31, wherein the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum.

33. The composition of any one of Claims 30-32, wherein the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

34. The composition of any one of Claims 30-33 further comprising water.

35. The composition of any one of Claims 30-34 further comprising a biomass product produced by the fungal culture.

36. A method of removing a target pollutant from a substrate, the method comprising contacting the substrate with a fungal culture for a period of time sufficient to produce: (a) a fungal culture comprising the target pollutant, and (b) a bioremediated product.

37. The method of Claim 36, wherein the bioremediated product comprises the target pollutant in an amount significantly less than an amount of the target pollutant in the substrate.

38. The method of Claim 36 or Claim 37, wherein the target pollutant comprises one or more of: a heavy metal, a phthalate, and a polycyclic aromatic hydrocarbon.

39. The method of any one of Claims 36-38, wherein the step of contacting comprises inoculating the substrate with colonized grains of the fungal culture or a fungal block spawn at an inoculation rate of not more than about 20%.

40. The method of any one of Claims 36-39, wherein the period of time is about one day to about one month.

41. The method of any one of Claims 36-40, wherein the step of contacting occurs at a temperature of about 70° F (about 21° C).

42. The method of any one of Claims 36-41 further comprising contacting the substrate with water.

43. The method of any one of Claims 36-42 further comprising contacting the substrate with a growth medium.

44. The method of Claim 43, wherein the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum.

45. The method of any one of Claims 36-44, wherein the substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt.

46. The method of any one of Claims 36-45, wherein the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

47. A method of bioremediating a substrate, the method comprising:

mechanically reducing the substrate to produce a ground substrate;

contacting the ground substrate with water for at least one hour to produce a hydrated ground substrate;

combining the hydrated ground substrate with a growth medium comprising about 65% water to produce a pre-inoculation mixture;

inoculating the pre-inoculation mixture with colonized grains of a fungal culture or a fungal block spawn at an inoculation rate of not more than about 20% to produce an inoculation mixture; and

incubating the inoculation mixture at a temperature of about 70° F (about 21° C) for about one day to about one month to produce a bioremediated product,

wherein the substrate comprises a polycyclic aromatic hydrocarbon ("PAH") and/or asphalt.

48. The method of Claim 47, wherein the substrate comprises one or more of: roofing shingles, built-up roofing including bitumen, interply of fiberglass and/or polyester, modified bitumen, and rubberized asphalt.

49. The method of Claim 47 or Claim 48, wherein the fungal culture comprises one or more of: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, *Ganoderma lucidum*, *Trametes versicolor*, *Pleurotus columbinus*, and *Pleurotus eryngii*.

50. The method of any one of Claims 47-49, wherein the growth medium comprises one or more of: sawdust, paper, cardboard, straw, wheat bran, and gypsum.

51. The method of any one of Claims 47-50 further comprising sterilizing the pre-inoculation mixture before the step of inoculating.

52. The method of Claim 51, wherein the step of sterilizing comprises contacting the pre-inoculation mixture with a sterilization agent selected from the group comprising: a chemical sterilizing agent, and heat.

53. The method of Claim 52, wherein the chemical sterilizing agent comprises hydrogen peroxide.

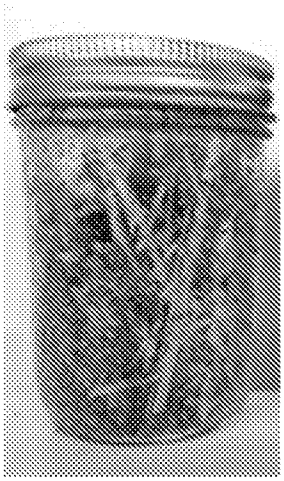

FIG. 1A

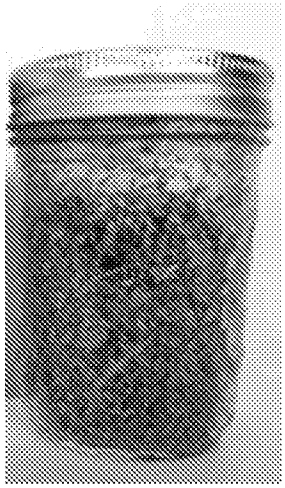

FIG. 1B

FIG. 2A

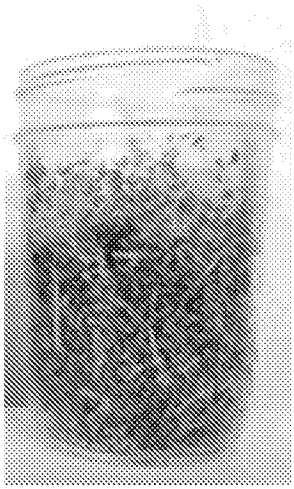

FIG. 2B

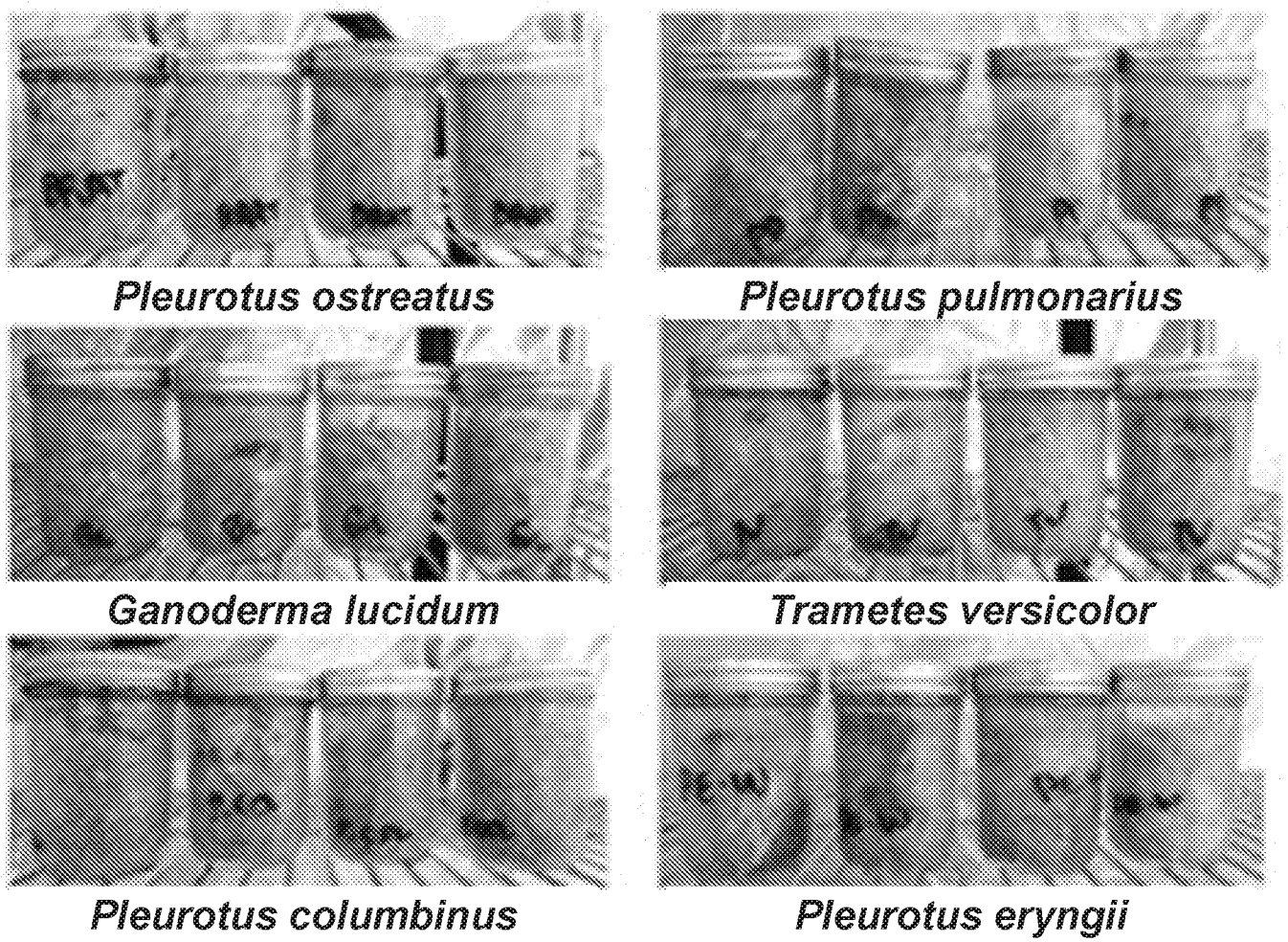
Pleurotus ostreatus
Pleurotus pulmonarius
Ganoderma lucidum
Trametes versicolor
Pleurotus columbinus
Pleurotus eryngii

FIG. 2C

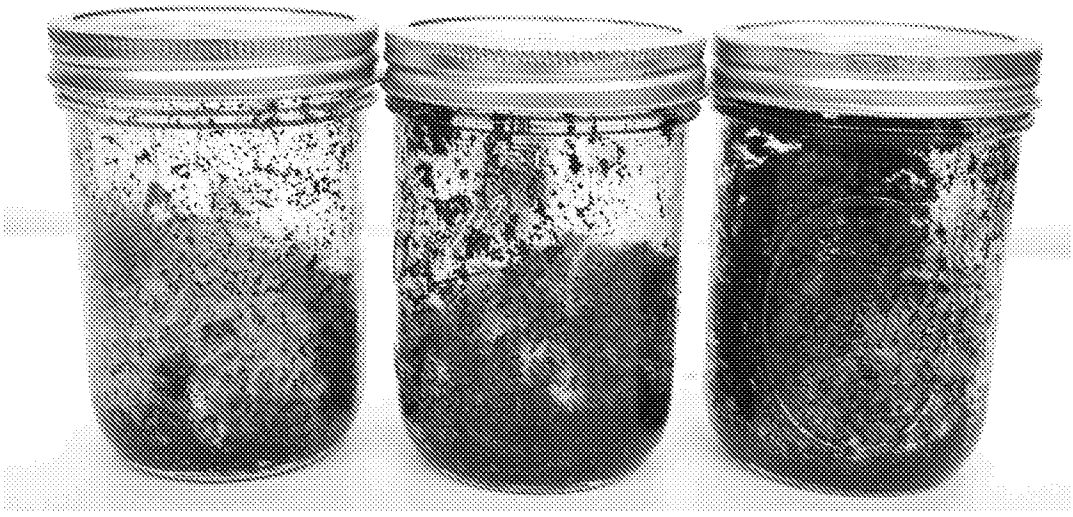

FIG. 3A FIG. 3B FIG. 3C

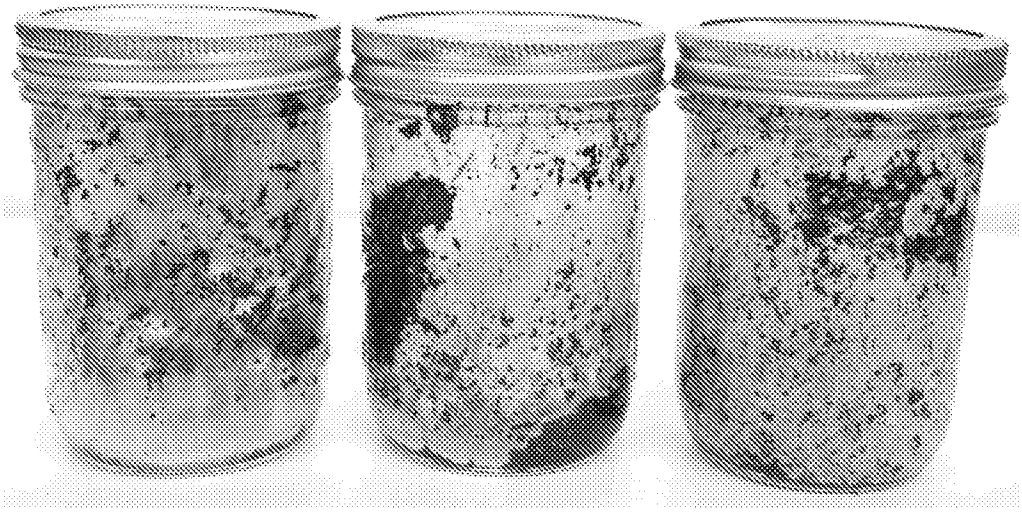

FIG. 3D FIG. 3E FIG. 3F

FIG. 3G FIG. 3H FIG. 3I

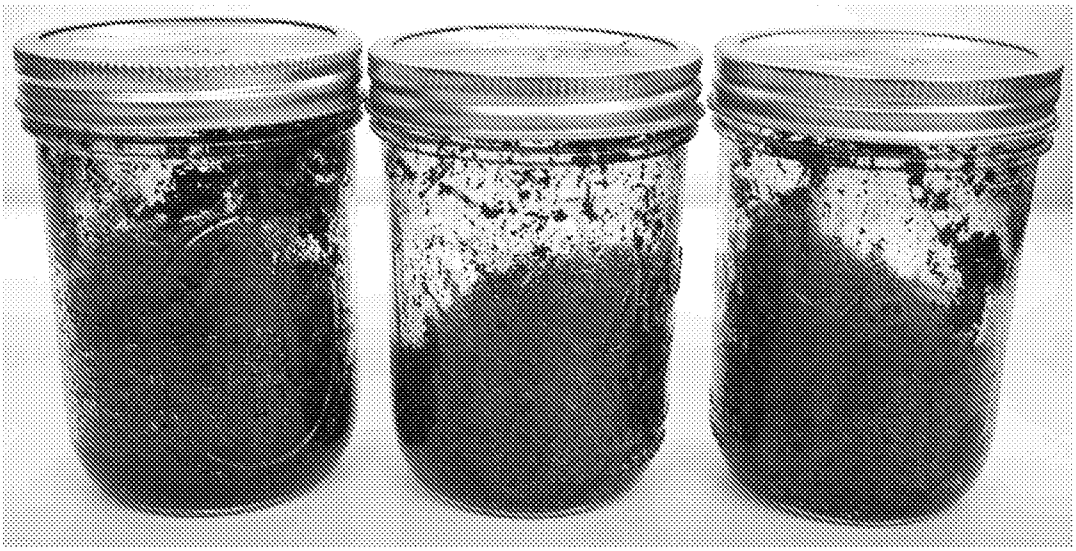

FIG. 4A FIG. 4B FIG. 4C

FIG. 4D FIG. 4E FIG. 4F

FIG. 4G FIG. 4H FIG. 4I

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/50128

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-29, 33-35, 39-46, 50-53
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/50128

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - B01D 15/08; C02F 1/28; C07C 17/38 (2019.01)
CPC - B01D 15/3828; C02F 1/286; C02F 2101/327

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2004/0023362 A1 (Stanley et al.) 5 February 2004 (055.02.2004) Abstract; para [0025], para [00105], para [00106], para [0024], para [0019], para [0058], para [0016], para [0062], para [0058], para [0054], Table 2 and entire document	1-3,30-32,36-38,47-48 --- 49
Y	WO 92/13960 A1 (Mycotech Corporation) 20 August 1992 (20.08.1992) Abstract, pg 5 ln 30-35; pg 18 ln 10-25, pg 6 ln 1-5)	49
Y	JP 6234720 B2 (Daiwa House Ind) 22 November 2017 (22.11.2017) Abstract; para [0002], para [0023], para [0030]-[0040]	1-3,30-32,36-38,47-49
A	Davis et al.; "Field Evaluation of the Lignin-Degrading Fungus Phanerochaete sordida to Treat Creosote-Contaminated Soil", Environ. Sci. Techolog. Vol 27 No 12; pp 2672-2676 (1993), Abstract; Fig 1;	1-3,30-32,36-38,47-49
A	Mueller et al; "Bioremediation of environments contaminated by PAH" pp 125-194, Chapter in Book "Bioremediation:Principles and Applications" Edited by Ronald Crawford and Don Crawford; Cambridge University Press(1996)	1-3,30-32,36-38,47-49
Y	US 6,204049 B1 (Bennett et al.) 20 March 2001 (20.03.2001) Abstract; col 6 ln 35- col 11 ln 60	1-3,30-32,36-38,47-49

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 2019	04DEC 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774