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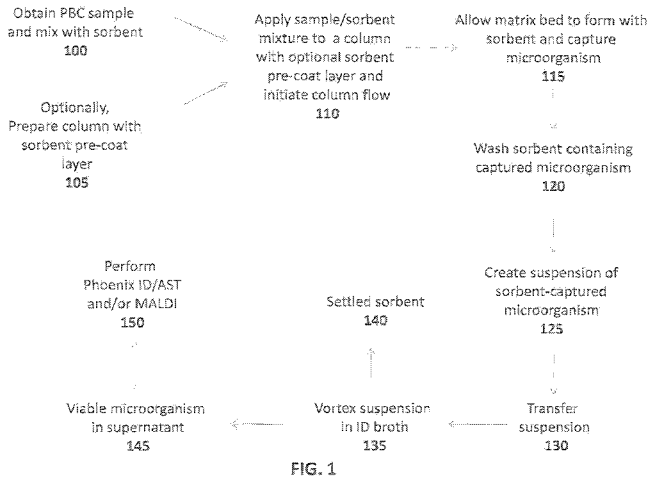

FIG. 1

(57) Abstract: The invention relates to methods for the isolation, purification, and concentration of microorganism (s) from a sample for use in downstream analyses. The methods involve obtaining a sample known to contain at least one microorganism, mixing the sample with a sorbent material (100), applying the microorganism-containing sample to a column (110) that optionally contains a sorbent material pre- coat layer (105), allowing the microorganism to be captured by the sorbent material (115) while washing away unwanted substances in the sample (120), and using the captured microorganism for subsequent analysis (150). The methods allow for maintaining the viability of the microorganism without the need for subculturing. In the alternative, the microorganism can be extracted while captured by the sorbent. These methods allow for the removal of substances in the sample that may interfere with downstream analyses, for example, the removal of blood components in a PBC sample that may interfere with identification methods.

WO 2014/018435 A1

ISOLATION AND RECOVERY OF MICROORGANISM USING SORBENT PARTICLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of the filing date of U.S. Provisional Application No. 61/674,564, filed July 23, 2012, entitled "Isolation and Recovery of Microorganism Using Sorbent Particles", the disclosure of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Sepsis is a serious medical condition caused by an overwhelming response of the host immune system to infection. It can trigger widespread inflammation, which can give rise to impaired blood flow. As sepsis progresses, the body's organs can be starved for oxygen and nutrients, causing permanent damage and eventual failure. Left improperly diagnosed or otherwise untreated, the heart weakens and septic shock can occur, leading to multiple organ failure and death. Blood cultures are required to detect and identify the presence of microorganism(s) in the patient and guide treatment. Current methods of isolating the microorganism often require sub-culturing the microorganism from the positive blood culture sample. Attempts have been made to use liquid separation methods that separate the microorganism from the sample without subculturing, but these methods often result in non-viable cells that are ineffective for antimicrobial susceptibility testing (hereinafter "AST") or other biochemical assays. In addition, current methods take at least 24 to 48 hours to perform, which results in many of the septicemia patients being initially treated with inappropriate antibiotics.

[0003] Positive blood cultures (hereinafter "PBCs") are often used for the detection/analysis of the microorganism(s) affecting the patient. PBCs have a high protein and salt content, contain a large amount of organic particles,

including various types of blood cells, as well as the sepsis-causing microorganism(s). Capturing microorganism from such complex samples in a form suitable for downstream testing, such as identification and AST, is very challenging, particularly for any filter-based methodology. In addition, certain downstream testing, for example AST, requires viable microorganism(s) in order to obtain accurate results.

[0004] Filtration-based methods using 0.2μm filters are commonly used in laboratories to sterilize liquids that may be contaminated with low levels of bacteria. Such filtration is, however, often not successful for capturing bacteria from PBCs or whole blood because these samples contain a large amount of organic particulate material such as blood cells along with the microorganism to be analyzed. These organic particulate materials often clog the filter, preventing adequate filtration, and ultimately interfere with downstream detection and/or analysis of the sample to determine the presence or absence of microorganism therein.

[0005] Recently, diatomaceous earth particles, including Celite®, have been used to separate microorganisms from various samples, including biological samples, followed by downstream detection and/or analysis of microorganism(s). U.S. Patent No. 5,369,011 describes methods for collecting, concentrating, detecting, and analyzing microorganisms from environmental samples, such as oil. Various diatomaceous earth particles are used to capture microorganisms from an environmental sample. The microorganism captured by the diatomaceous earth particles is recovered for detection and/or analysis by re-suspending the microorganism/diatomaceous earth complex with “detection fluid," or by lysing the microorganism by sonication to release intracellular components of the microorganism. These methods are inadequate for recovering viable microorganisms because sonication kills the microbial cells.

[0006] International Patent Publication Nos. WO 2011/079038 to Kshirsagar (hereinafter "Kshirsagar I") and WO 2009/046191 to Kshirsagar *et al.* (hereinafter "Kshirsagar II") describe methods for concentrating microorganisms in a liquid sample in which the microorganisms remain viable for the purpose of detection and assay. Kshirsagar I describes the use of diatomaceous earth treated with an adsorption buffer-modified solution, such as a cation-containing salt, and complexed to a metal for concentrating microorganisms. Kshirsagar II describes the use of unmodified diatomaceous earth or diatomaceous earth modified with titanium dioxide, gold, or platinum as a concentration agent to bind microorganisms in a sample. In both references, once bound, the microorganism can be removed from the diatomaceous earth particles by sonication, or by chemical treatment with bovine serum albumin ("BSA"). However, such methods are harsh and often kill the microorganisms. Also, BSA interferes with certain downstream testing, such as identification by mass spectrometry. Sub-culturing is a time-consuming method which does not provide the needed fast determination for patients with suspected sepsis.

[0007] Therefore, there is a need for improved methods for rapidly concentrating and recovering microorganism(s) from a sample, such as a positive blood culture. These methods must retain the viability of the microorganism(s) for growth-based methods as well as eliminate any interfering substances.

BRIEF SUMMARY OF THE INVENTION

[0008] Methods described herein provide for the rapid isolation, purification, and concentration of microorganism(s) from various types of samples for use in downstream biochemical analyses and/or identification methods by removing or reducing interfering substances or particulate matter in the complex matrix of the sample.

[0009] The described methods include obtaining a sample known to contain at least one microorganism and mixing the

sample with sorbent material that is capable of capturing the microorganism. After mixing the sample with the sorbent material, the suspension is applied to a column where the microorganism is captured by the sorbent inside the column. The sorbent-captured microorganism is then washed to remove unwanted substances in the sample while retaining the microorganism. The captured microorganism is then used for subsequent analysis and characterization of the microorganism.

[0010] In certain embodiments, viable microorganism in the sample is recovered and further analysis is performed without a need to subculture the microorganism. In preferred embodiments, microorganism is first captured by a sorbent material which can be removed prior to downstream analysis of the microorganism. At least some of the microorganisms so removed remains viable afterwards. In yet another embodiment, if the downstream analysis does not require viable microorganisms, the constituents of the microorganism (*e.g.* proteins and nucleic acids) can be extracted while bound to the sorbent material. Therefore, the methods of the present invention contemplate the removal of substances in the sample that may interfere with downstream analyses (*e.g.* the removal of blood components in a PBC sample that may interfere with MALDI-TOF MS identification) in addition to preserving viability of microorganisms for downstream testing when required.

[0011] The methods described herein contemplate the use of a variety of methods and reagents used for the recovery and/or analysis of the microorganism once captured by the sorbent material. These methods and reagents can be customized for the particular downstream assay or analysis to be performed.

[0012] The methods described herein rapidly isolate and concentrate microorganism in a sample, *i.e.*, usually within 30 minutes, and do not require centrifugation of the sample

to achieve separation. Because of the brevity and repeatability of the methods described herein, the described methods are readily automated.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 illustrates one embodiment in which viable microorganism is isolated and concentrated from a PBC sample using sorbent material and subsequently used for AST testing and identification.

[0014] FIG. 2 illustrates another embodiment in which viable microorganism is isolated and concentrated from a PBC sample using sorbent material and subsequently used for AST testing and identification.

[0015] FIG. 3 illustrates one embodiment in which microorganism is isolated and concentrated from a PBC sample using sorbent material and subsequently undergoes on-column extraction of the bound microorganism followed by MALDI-TOF MS identification.

DETAILED DESCRIPTION

[0016] The methods described herein relate to the isolation, purification, and concentration of microorganism(s) from a sample for use in downstream analyses. The methods involve obtaining a sample known to contain at least one microorganism, mixing the sample with a sorbent material, applying the suspension to a column that may or may not contain a sorbent pre-coat layer, allowing the microorganism to be captured by the sorbent material while washing away unwanted substances in the sample, and using the captured microorganism for subsequent analysis. The methods allow for maintaining the viability of the microorganism without the need for subculturing. In the embodiments wherein intact and/or viable microorganisms are not required for downstream analysis, the microorganism can be extracted while captured by the sorbent.

[0017] Various sorbent materials are known in the art and selection of a particular sorbent material depends upon the nature of the sample to be analyzed. It is to be understood that the capture of the microorganism by the sorbent material refers to the ability of the sorbent to trap and/or bind the microorganism without adversely affecting viability. Throughout the specification, "binding" refers to a chemical interaction such as those caused by a surface modification of the sorbent material which causes the microorganism to be captured by the sorbent through chemical interactions. In the alternative, "trapping" refers to the microorganism being captured by the sorbent material due to size restriction: *i.e.*, the size of the microorganism, compared to the particle size of the sorbent material, enables the microorganism to be physically trapped by the sorbent material rather than "bound" by chemical interactions.

[0018] More particularly, the sorbent materials, which may be used in the practice of the methods described herein, are composed of various particles sizes and various shapes. Without being bound by theory, when a liquid suspension of the sorbent material containing various particle sizes and shapes is applied to a column, and when column flow is initiated, the sorbent material will begin to stack and form a tight cake containing microscopic channels that are capable of trapping various sized microorganisms in the sample, while allowing the liquid to pass through the channels.

[0019] In addition, the sorbent materials that may be used are micro-porous. Without being bound by theory, sorbent materials containing micropores allows for the formation of channels between the sorbent material particles as well as through the particles. The microorganism is trapped by the majority of the channels between the sorbent material while `the liquid from the sample is able to flow through the pores in the sorbent material particles. This allows for the processing of larger sample volumes. In one embodiment, the

pore size in the sorbent material particle is smaller than the size of the microorganism to be captured.

[0020] Furthermore, the sorbent materials that may be used in the described methods are physically rigid, *i.e.*, the sorbent material cake formed in the column cannot be easily compressed under normal filtration pressure, allowing flow channels to remain open.

[0021] In addition, the sorbent materials that may be used are optionally, chemically inert. If the sorbent materials are not inert, *i.e.*, contain surface modifications that would cause the microorganism to bind to the sorbent material, rather than be trapped by it, removal of the microorganism from the sorbent material may require harsher reagents that could decrease viability and/or interfere with downstream analysis. However, if the downstream analysis does not require intact cells to be released from the column or cell viability to be maintained, non-inert sorbent materials can be used. It is also possible that non-inert sorbents may be blocked by components from the complex sample and thus be used in these methods.

[0022] The amount of sorbent material used in the methods described herein is based on the volume of the sample to be processed. The more sample to be analyzed, the more volume of sorbent material is required to capture the microorganism in the sample.

[0023] The ratio of sorbent material to sample volume affects the flow of the column and thus the capture efficiency of the microorganism. Each type of sorbent material has a different capacity for trapping a certain amount of microorganism in the sample per unit volume of sorbent material used. Therefore, the methods described herein contemplate the use of various ratios of sorbent material volume to sample volume, depending on sample complexity and the particular sorbent material being used.

[0024] In one embodiment, the sorbent material comprises diatomaceous earth particles. In another embodiment, the sorbent material is the sorbent material commercially available under the trademark Celite®, which is a registered trademark of the Celite Corporation. In yet another embodiment, the sorbent material comprises cellulose fibers or particles.

[0025] FIG. 1 illustrates one exemplary embodiment in which the viability of microorganism is preserved after being isolated and concentrated from a PBC sample using sorbent material. A sample known to contain at least one microorganism is obtained in step 100, for example a PBC sample, and mixed with a suspension containing sorbent material. The sample mixed with sorbent in step 100 is applied to a column in step 110 and flow is initiated in order to allow the microorganism in the sample obtained in step 100 to be captured by the sorbent material inside the column in step 115. Optionally, the column can be prepared with a sorbent material pre-coat layer in step 105 by adding the sorbent material in a liquid suspension to the column containing a frit and allowing the liquid from the suspension to exit the column while the sorbent material is retained by the frit. After applying the sample mixed with sorbent material in step 100 to the column in step 110, other organic or liquid material in the sample can be separated from the microorganism by allowing the sample liquid to flow through the column, and/or by washing the column 120 containing the sorbent-captured sample in step 120. A suspension of the sorbent-captured microorganism is prepared in step 125 by adding a liquid reagent, for example Phoenix® ID broth, to the column and transferring the suspension in step 130 to a separate container. The suspension of step 125 is vortexed on step 135 to release the microorganism from the sorbent material, allowing the sorbent material to settle in step 140 at the bottom of the container while viable microorganism

remains suspended 145 in the supernatant. Downstream analysis 150, for example BD Phoenix® ID/AST and/or MALDI-TOF MS identification, of suspended microorganism in step (from step 145) in the supernatant is performed in order to characterize the microorganism in sample obtained in step 100.

[0026] Sample can be any sample known to contain at least one microorganism. Possible types of samples include, but are not limited to, body fluids, whole blood samples, positive blood cultures, urine, cerebrospinal fluid, environmental samples such as soil, and industrial samples.

[0027] The type of microorganism in the sample is not limited. The methods described herein can be used to prepare and analyze various types of microorganisms in the sample, including, for example, bacteria, yeast, fungi and mycobacteria.

[0028] In one embodiment, the sample is mixed with a suspension of sorbent material prior to the applying step 110. The sorbent material suspension can comprise, for example, a suspension buffer containing water or a saline solution. In another embodiment, the suspension buffer may include a lysis buffer containing, for example, saponins, Tweens®, Tritons™, ammonium chloride, lytic culture medias, or mixtures thereof.

[0029] In one embodiment, the optional pre-coat layer is washed with a wash buffer prior to mixing with the sample. In another embodiment, the wash buffer is compatible with the downstream assay, *i.e.*, the wash buffer does not interfere with obtaining accurate and consistent results. In one exemplary embodiment the wash buffer is a saline solution or water. In another embodiment, the wash buffer may include a lysis buffer containing, for example, saponins, Tweens®, Tritons™, ammonium chloride, lytic culture medias, or mixtures thereof.

[0030] In one embodiment, microorganism captured by the sorbent material in step 115 is washed in step 120 with a wash buffer prior to creating a suspension of sorbent-captured microorganism in step 125. This wash step may help provide more accurate results by removing unbound substances in the sample that interfere with the downstream assay and by using a wash buffer that is compatible with the downstream assay. In one exemplary embodiment, the wash buffer is BD Phoenix® ID broth or water. Phoenix® is a registered trademark of Becton Dickinson and Company. In another exemplary embodiment, the wash buffer may include a lysis buffer containing, for example, saponins, Tweens®, Tritons™, ammonium chloride, a lytic culture, or mixtures thereof. The washing step can comprise a single wash or multiple rounds of washing. Multiple rounds of washing can be accomplished with a single type of wash buffer or with multiple wash buffers.

[0031] Once the microorganism is captured by the sorbent material, the methods described herein contemplate optimizing the subsequent reagents and processing steps for the particular downstream assay to be used. The methods include recovering viable or non-viable microorganism from the sorbent material. The type of downstream assay which can be conducted is not limited, and can include, for example, growth-based and non-growth-based methods, biochemical assays, identification techniques such as MALDI-TOF MS and Phoenix® ID, AST, and molecular tests such as PCR, SDA, and BD MAX™. BD MAX™ is a trademark of Becton Dickinson and Company. The reagents and processing steps used in the methods described herein should be compatible with the particular downstream assay or assays to be used.

[0032] The methods described herein can prepare a common sample containing one or more microorganisms (*e.g.* bacteria) for multiple downstream analyses, including both growth-based and non-growth based methods. In one embodiment, one sample known to contain microorganisms is divided into portions,

each portion is mixed with a sorbent material, each of the sorbent-microorganism suspensions is applied to a separate column, and each of the sorbent-captured microorganism eluents is used for a different downstream analysis. In another embodiment, one sample known to contain microorganisms is mixed with a sorbent material, the sorbent-microorganism suspension is applied to a single column, the microorganism is eluted from the sorbent material, and a portion of the microorganism-containing eluent is used for one downstream analysis while another portion is used for a different downstream analysis.

[0033] In one embodiment, microorganism captured by the sorbent material in the column is recovered from the sorbent material by creating a suspension by adding a suspension buffer to the microorganism captured in step 115 by the sorbent material while the column exit is closed. In one embodiment, cell viability is maintained upon addition of the suspension buffer. Suspension buffers can include, for example, water or BD Phoenix® ID broth at various concentrations, or detergent-containing lysis buffers. In another exemplary embodiment, the suspension buffer contains buffers known to those skilled in the art that support viability, growth and detection of the microorganism and are not described in detail herein.

[0034] In one embodiment, the suspension is transferred in step 130 to another container and vortexed in step 135 in order to separate the microorganism from the sorbent material. After vortexing the supernatant contains viable microorganism while the sorbent material settles at the bottom of the container. In another embodiment, the suspension can be vortexed or mixed while still inside the column.

[0035] FIG. 2 illustrates one exemplary embodiment in which the viability of microorganism is preserved after being isolated and concentrated from a PBC sample using sorbent

material. A sample known to contain at least one microorganism is obtained in step 200. The sample, for example a PBC sample, is mixed with a suspension of sorbent material. The sample mixed with sorbent material in step 200 is applied to a column in step 210 in order to allow the microorganism in the sample to be captured by the sorbent material inside the column in step 215. Optionally, the column can be prepared with a sorbent pre-coat layer (step 205) by adding the sorbent material in a liquid suspension to the column containing a frit and allowing the liquid from the suspension to exit the column while the sorbent material is retained by the frit. After applying the sample mixed with sorbent from material from 200 to the column in step 210, other organic or liquid material in the sample can be separated from the microorganism by allowing the sample liquid to flow through the column, and/or by washing the column (step 220) containing the sorbent-captured sample. The captured microorganism is released from the sorbent material by adding a buffer in step 225 to the sorbent containing captured microorganism (washed in step 220) to create a suspension of sorbent material and released microorganism. The sorbent material is allowed to settle in step 230 and the viable, concentrated microorganism is retrieved from the supernatant 235. Downstream analysis of the microorganism is performed in step 245 (*e.g.*, Phoenix ID/AST and/or MALDI-TOF MS identification).

[0036] In one embodiment, the microorganism is extracted while captured by the sorbent material inside the column using an on-column extraction method. One exemplary embodiment of this method is illustrated in FIG. 3. Captured microorganism is obtained in step according to any of the methods described herein using a column that may optionally contain a sorbent pre-coat layer. Captured microorganism from step 300 is washed with an organic solvent in step 310, such as for example, 70% ethanol in water by applying the

ethanol to the column, allowing the ethanol to flow over the sorbent-bound microorganism and through the column, and discarding the flow-through. Captured and washed microorganism is then extracted in step 320 directly in the column by applying an acid, such as, for example, 70% formic acid in water, to the column. The formic acid comes in contact with sorbent-captured microorganism, thereby extracting the microorganism, and flows through the column. The flow-through from extraction in step 320 is collected in step 330 and analyzed in step 340 by MALDI-TOF MS for microbial identification.

[0037] In another embodiment, as further illustrated in FIG. 3, subsequent to extraction step 320 with formic acid, a second extraction step 350 is optionally performed by applying a 1:1 solution of 70% formic acid in acetonitrile to the column and allowing the solution to come in contact with any residual washed sorbent-bound microorganism from step 310 not extracted during first extraction step 320. The formic acid/acetonitrile comes in contact with any residual microorganism, extracting the residual microorganism and flows through the column. The flow-through from second extraction step 350 is collected in step 360, combined with the flow-through from 330 from the first extraction step 320, and analyzed in step 340 by MALDI-TOF MS for microbial identification. In an alternative embodiment, flow-through from the second extraction step 350 is collected and analyzed by MALDI-TOF MS identification in step 370 without combining the flow-through from the first and second extractions before identification.

[0038] In one embodiment, the sample known to contain at least one microorganism is whole blood or a positive blood culture sample. In this embodiment, the red blood cells in the sample are lysed prior to performing capture by the sorbent material. In an alternative embodiment, capture of the microorganism by the sorbent material in the whole blood

or PBC is performed on un-lysed blood samples. In this embodiment, after capture of the microorganism by the sorbent material, lysis of the blood cells can optionally be performed in the column. Methods for lysing red blood cells are well known to those skilled in the art and are not described in detail herein.

EXAMPLES

[0039] EXAMPLE 1: Capture of *Escherichia coli* (*E. coli*) from a saline solution using Celite® followed by MALDI-TOF MS identification

Capture of Microorganism

[0040] A suspension (5 ml) of *E. coli* (ATCC catalog number 25922) in BBL normal saline (Becton Dickinson and Company catalog number 221818) at a concentration of 1 McFarland was mixed with 1 ml of a Celite® 500 suspension (50% suspension in water). The *E. coli*/Celite® mixture was then added to the Celite® pre-coat layers column prepared below in three portions. The liquid was withdrawn from the column at a rate of 1 to 2 ml per minute with the aid of a syringe. A Celite® cake containing the trapped bacteria was formed at the bottom of the column. The Celite®-trapped bacteria was washed with 2 ml of BD Phoenix® ID broth (Becton Dickinson and Company catalog number 246001) followed by a wash with 2 ml of sterile water. The wash cycle with BD Phoenix® ID broth and water was repeated one time.

Preparation of Column with Pre-Coat Layer

[0041] A suspension (1 ml) of 50% Celite® 500 fine particles with 13 μm mean particle size (Sigma-Aldrich catalog number 22145) and 50% of a solution of 70% ethanol in water was added to a 5 ml disposable plastic column containing a 30 μm pore size polyethylene frit disc. The liquid was withdrawn from the bottom of the column forming a layer of Celite® particles on the column frit. The Celite® particle layer was washed with 10 ml of Dulbecco's phosphate

buffered saline (DPBS) to stabilize and equilibrate the particle layer. The DPBS was withdrawn from the bottom of the column to form a Celite® pre-coat layer.

Isolation and Identification of Viable Microorganism

[0042] The washed Celite®-trapped bacteria was removed from the column by adding 1 ml of sterile water to the column containing the Celite®-trapped bacteria to create a suspension, and the Celite®-bacteria suspension was transferred to a clear glass tube. The glass tube containing the Celite®-bacteria suspension was vortexed briefly to remove the bacteria from the Celite® particles and to resuspend the captured bacteria in the water. After vortexing, the mixture was allowed to stand for approximately 10 to 60 minutes to allow the Celite® particles to settle to the bottom of the tube. The resulting supernatant contained resuspended, viable bacteria.

[0043] A portion of the supernatant (1 to 2 µl) containing the resuspended bacteria was analyzed by MALDI-TOF MS on a Bruker Microflex MALDI-TOF for bacterial identification. The MALDI matrix solution was prepared by dissolving 2.5 mg of HCCA in 250 µL of a solution that is 2.5% trifluoroacetic acid, 47.5% deionized water, and 50% acetonitrile. All of the mass spectrometry data was recorded on Bruker Microflex LT with Biotyper software with the following MALDI score key definition. A MALDI ID score of greater than 2.0 indicates a satisfactory identification to the Genus and Species level. A MALDI ID score of 1.7-1.999 indicates a satisfactory identification to the Genus level. A MALDI ID score of less than 1.699 indicates an unacceptable or not reliable identification.

[0044] In order to obtain a viability count, flow-through liquid from the capture and wash steps was analyzed by diluting and plating discrete volumes on agar plates and counting the resulting colonies.

Results

[0045] MALDI-TOF analysis resulted in a positive identification of *E. coli* ATCC 25922 with a score of 2.2 indicating identification to the species level. Flow-through analysis of the capture and wash steps did not reveal any viable bacterial counts, indicating efficient capture of *E. coli* by the Celite® particles. Comparison of the viability count of the Celite®-bacteria suspension with that of the original sample indicated at least 47% of the viable counts from the original sample are present in the suspension. These results illustrate that the sorbent materials described herein can be used to isolate and concentrate viable bacteria from a dilute suspension. Vortexing the Celite®-bacteria suspension did not appear to adversely affect microbial viability. In addition, a positive MALDI-TOF MS identification was obtained because interfering substances, *i.e.*, salt from the saline in the sample, were removed prior to MALDI-TOF MS analysis.

[0046] EXAMPLE 2: Capture of *Staphylococcus aureus* (*S. aureus*) from a blood sample using Celite® followed by Phoenix® identification and AST.

Capture of Microorganism

[0047] A BACTEC plus aerobic/F bottle (Becton Dickinson and Company catalog number 442192) was spiked with 10 ml human blood and sufficient *S. aureus* (ATCC 29213) to create a 1 McFarland suspension. A portion of this suspension (5 ml) was withdrawn from the BACTEC bottle and mixed with 5 ml of lytic reagent with 0.66% Triton™, then 1 ml of sterile Celite® 535 (50% suspension in saline). The Celite®/bacteria suspension was added to the pre-coated Celite® column prepared below in three portions. The liquid was withdrawn from the column at a rate of 1 to 2 ml per minute with the aid of a syringe. A layer of Celite®-trapped bacteria was formed above the pre-coat layer at the bottom of the column. The Celite®-trapped bacteria was washed with 2 ml of Phoenix®

ID broth followed by a wash with 2 ml of sterile water. The wash cycle with BD Phoenix ID broth and water was repeated one time.

Preparation of Column with Pre-Coat Layer

[0048] A Celite® pre-coat layer was prepared as in Example 1 except that Celite® 535 particles containing <94.3 μm particle size (Sigma Aldrich catalog number 22138) were used.

Isolation, Identification and AST Testing of Viable Microorganism

[0049] The Celite®-trapped bacteria was removed from the column by adding 4.5 ml Phoenix® ID broth to the column to create a suspension, and the Celite®-bacteria suspension was transferred to a clear glass tube. The glass tube containing the Celite®-trapped bacteria suspension was vortexed briefly to remove the bacteria from the Celite® particles and to resuspend bacteria in the Phoenix® ID broth. After vortexing, the mixture was allowed to stand for approximately 10 to 60 minutes to allow the Celite® particles to settle to the bottom of the tube. The resulting supernatant contained resuspended, viable bacteria. A portion of the supernatant (approximately 4 ml) containing the resuspended bacteria was directly used to inoculate the gram positive ID-105 ID panel of the BD Phoenix® ID/AST system followed by a standard dilution to inoculate the AST panel. The Phoenix® ID/AST System is described in, *e.g.*, U.S. Patent Nos. 5,922,593, 6,096,272, 6,372,485, 6,849,422, and 7,115,384, the contents of which are hereby incorporated by reference in their entirety.

[0050] Flow-through liquid from the capture and wash steps was also analyzed by plate count as described in Example 1.

Results

[0051] Phoenix ID/AST testing resulted in a positive *S. aureus* identification and an expected AST profile for *S. aureus*. Analysis of the flow-through from the capture and

wash steps indicated a capture efficiency of approximately 78%. These results illustrate that sorbent materials as described herein can be used to isolate and concentrate bacteria from a complex sample, such as a blood sample, maintain microbial viability for AST testing, and remove interfering substances for Phoenix® identification.

[0052] EXAMPLE 3: Capture of *Staphylococcus aureus* (*S. aureus*) from a blood sample using Celite® followed by on-column extraction and MALDI-TOF MS identification.

Capture of Microorganism

[0053] *S. aureus* 29213 was prepared and captured on the Celite® 535 pre-coat layer prepared below and as in Example 2.

Preparation of Column with Pre-Coat Layer

[0054] A Celite® 535 pre-coat layer was prepared as in Example 2.

On-column Extraction and Identification of Captured Microorganism

[0055] An on-column extraction of the bacteria was performed directly on the Celite® column containing the captured *S. aureus*. First, the column was washed with 1 ml of 75% ethanol in water. The ethanol was slowly withdrawn through the column with the aid of a syringe over a 2 minute period until all of the ethanol was eliminated from the column and discarded. The Celite®-trapped bacteria was extracted by adding 1 ml of 70% formic acid in water to the column and slowly withdrawing the formic acid through the column with the aid of a syringe over a 2 minute period until all of the formic acid was eliminated from the column and collected. A portion (1 to 2 μl) of the formic acid flow-through was analyzed for bacterial identification by MALDI-TOF MS using a Bruker Microflex MALDI-TOF MS as described above. A second on-column extraction step was performed in the same manner as the first extraction step except that a

1:1 ratio of acetonitrile/70% formic acid in water was used. A portion (1 to 2 µl) of the flow-through from the second extraction step was also analyzed for bacterial identification by MALDI-TOF MS.

Results

[0056] MALDI-TOF MS analysis of the flow-through from the first extraction step with formic acid alone resulted in a positive identification of *S. aureus* ATCC 29213 with a score of 2.0 indicating identification to the species level. MALDI-TOF MS analysis of the flow-through from the second extraction step with acetonitrile and formic acid also resulted in a positive identification of *S. aureus* ATCC 29213 with a score in the range of 1.7 - 2.0. These results indicate that sorbent materials as described herein can be used to isolate and concentrate bacteria from a complex sample, such as a blood sample, while removing substances that interfere with MALDI-TOF MS identification. In addition, these results illustrate that bacteria samples can be extracted while trapped by the sorbent material inside the column without the need for removing the bacteria from the column and performing a separate extraction procedure. A single extraction step with formic acid alone is sufficient to extract sorbent-captured *S.aureus* and obtain a positive identification by MALDI-TOF MS.

[0057] EXAMPLE 4: Capture of *S. aureus* from a single PBC sample using Hyflo Super Cel followed by Phoenix ID/AST and MALDI-TOF MS identification

Capture of Microorganism

[0058] A BACTEC plus aerobic/F blood culture bottle was inoculated with 10-100 cfu *S. aureus* ATCC 29213 and 10 ml blood and allowed to incubate until positive in a BACTEC FX instrument. A first portion of this suspension was applied to one of the Celite® pre-coat layer columns prepared below and used for Phoenix ID/AST while a second portion of the

suspension was applied to the second Celite® pre-coat layer column prepared below and used for MALDI-TOF MS identification.

Preparation of Column with Pre-Coat Layer

[0059] Two disposable plastic columns (5 ml each) were pre-coated with Hyflo® Super Cel® Celite® particles (Sigma Aldrich catalog number 56678) using the same method as described in Example 1 above to form a Celite® pre-coat layer.

[0060] The following steps were conducted in the duplicate columns: a portion (5 ml) of the bacterial suspension was withdrawn from the Bactec bottle and mixed with 5 ml of 0.66% Triton™ X-100 in water and 1 ml Hyflo® Super Cel® (50% suspension in DPBS). The Celite®-*S. aureus* suspension was added to the Celite® pre-coat layer column prepared above. The sample liquid was withdrawn from the column with the aid of a syringe at a flow rate of approximately 1 to 2 ml per minute. The Celite®-trapped bacteria was washed with 2 ml Phoenix® ID broth followed by 2 ml of 0.3% Triton™ X-100 in water. The wash cycle with BD Phoenix ID broth and water was repeated one time.

Column 1: Isolation, Identification, and AST Testing of Viable Microorganism

[0061] The Celite®-trapped bacteria was removed from the columns by adding 4.5 ml BD Phoenix® ID broth to the column to create a Celite®-bacteria suspension, and transferring the Celite®-bacteria suspension to a clear glass tube. The glass tube containing the Celite®-bacteria suspension was vortexed briefly to remove the bacteria from the Celite® particles and to resuspend the bacteria in the Phoenix® ID broth. After vortexing, the mixture was allowed to stand for approximately 10 to 60 minutes to allow the Celite® particles to settle at the bottom of the tube. The resulting supernatant contained resuspended, viable bacteria. A portion of the supernatant (approximately 4 ml) containing the resuspended bacteria was

then used to directly inoculate the gram positive ID-105 ID panel and diluted according to standard protocol for inoculating the AST panel as described above.

Column 2: On-column Extraction and Identification of Captured Microorganism

[0062] An on-column extraction protocol was applied directly to the Celite®-trapped bacteria in the second column as described in Example 3 except that only one extraction step with 70% formic acid alone was performed. The flow-through liquid from the formic acid extraction step was analyzed for bacterial identification by MALDI-TOF MS as described above.

Results

[0063] Phoenix® ID/AST testing resulted in a positive identification and an expected AST profile for *S. aureus*. MALDI-TOF MS positively identified *S. aureus* ATCC 29213 with a score of 2.2 indicating a positive identification to the species level. These results illustrate that a single sample can be processed for both non-growth based methods such as identification by MALDI-TOF MS and Phoenix, as well as for growth-based methods such as AST, using the methods and reagents described herein.

[0064] Example 5: Lysis of whole blood containing microorganisms followed by capture of the microorganisms with sorbent material.

Preparing and Lysing Whole Blood Samples

[0065] Whole blood was spiked with enough *E. coli* to make a concentration of 3000 cfu/ml. The blood cells of a portion (5 ml) of the whole blood containing the bacteria were lysed by mixing the whole blood with 5 ml of one of the following buffers for 10 minutes:

1	0.6% Triton™ X-100 in Phoenix ID Broth
2	0.6% Triton™ X-100 in

	Mycolytic Broth
3	0.6% Triton™ X-100 in 0.3M NH_4Cl
4	0.3M NH_4Cl in 9% Saponin

Capture of Microorganism with Sorbent Material

[0066] A suspension of sorbent material at a concentration of 50% was prepared with Celite® 535 sorbent material (Sigma-Aldrich catalog number 22138). A portion of the sorbent material suspension (2 ml) was added to 10 ml of the lysed whole blood prepared above and loaded in three portions onto the column prepared below containing a sorbent material pre-coat layer. The liquid flow-through was collected and the volume of flow-through was measured. The column was then washed with 2 ml of Phoenix® ID broth. The flow-through from the wash step was collected, combined with the flow-through from the sample application step, and analyzed for cell viability before being discarded.

Preparation of Column with Pre-coat Layer

[0067] A 50% suspension (1 ml) of Celite® 535 sorbent material in 70% ethanol in water was added to a 5 ml disposable plastic column containing a 30 μm pore size polyethylene frit disc. The liquid was withdrawn from the bottom of the column forming a layer of sorbent material on the column frit. The sorbent material coating layer was washed with Dulbecco's phosphate buffered saline (DPBS) to stabilize and equilibrate the layer.

Isolation and Counting of Viable Microorganism

[0068] The washed, sorbent-trapped bacteria were removed from the column by adding 3 ml of Phoenix® ID broth to the column to create a suspension. Three portions of 100μl each of the suspension were used to perform a plate count of viable colonies.

Results

[0069] The results are summarized in Table 1 below. The capture efficiency of viable bacteria when using Celite® 535 as a sorbent material was in the range of approximately 62 to 93%. In addition, 48 to 61% of the original viable cells were recovered from the Celite® particles. These results indicate that various lysis buffers for lysing whole blood are compatible with the methods described herein and do not interfere with the capture and recovery of viable microorganism from a complex sample.

Table 1: Percent Recovery of Viable Microorganism

Lysing reagent	Volume Sample passed (ml)	Equivalent Blood Volume (ml)	Viable Capture Efficiency (%)	Celite Recovery of Viable Cells (%)
1	5	2.08	84%	53%
2	3.5	1.46	93%	61%
3	6.6	2.75	62%	48%
4	1	0.42	82%	52%

[0070] Example 6: Capture and Recovery of Viable Bacteria Using Cellulose

Preparation of PBC Samples and Capture of Microorganism by Sorbent Material

[0071] A portion of a PBC sample (20 ml) containing *E. faecalis* was withdrawn from a PBC bottle and treated with 2 ml of 20% choline chloride in water for 5 minutes. A lysis buffer (20 ml) containing 0.66% Triton™ X-100 in lytic-10 broth was added to the sample and allowed to incubate for 5 minutes in order to lyse the blood cells in the PBC sample. A portion of the lysed sample (10 ml) was combined with 2 ml of a 50% suspension of either Sigmacell® Cellulose (50μm) or Sigmacell® Cellulose (20μm). The sample/cellulose mixture (12 ml) was added to each of the three columns prepared below to capture the microorganism in the sample. The trapped microorganism was washed with 2 ml of 0.33% Triton™ X-100 in lytic-10 broth followed by a wash with 9 ml of Phoenix® ID

broth. The preparation procedure was repeated using a negative blood culture sample ("NBC") as a control, *i.e.*, a sample known to be negative for microorganisms.

Preparation of Column with Pre-Coat Layer

[0072] Duplicate suspensions (1 ml each) at a concentration of 50% were made with each of Sigmacell® Cellulose 50μm and Sigmacell® Cellulose 20μm. Each suspension was applied to a 5 ml disposable plastic column containing a 30 μm pore size polyethylene frit disc. The liquid was withdrawn from the bottom of the column forming a layer of cellulose on the column frit. The cellulose layer was washed with 10 ml of Dulbecco's phosphate buffered saline (DPBS) to stabilize and equilibrate the particle layer. The DPBS was withdrawn from the bottom of the column to form a cellulose pre-coat layer.

Isolation, Identification and AST Testing of Viable Microorganism using Phoenix® ID/AST

[0073] The cellulose-trapped bacteria was removed from the column by adding 6 ml Phoenix® ID broth to the column to create a suspension, vortexing the sample, allowing the mixture to settle for 40 minutes, then performing a slow centrifugation of the supernatant at 750 rpm for 5 minutes. A portion of the supernatant containing the resuspended bacteria was used directly, or diluted to 0.5 McFarland, for identification and AST using the BD Phoenix® ID/AST system. The same method was applied to the columns containing the NBC samples.

Results

[0074] The results are summarized in Table 2 below. For each of the PBC samples a positive Phoenix® ID and AST was obtained. As expected, each of the NBC samples was negative for Phoenix® ID and AST. These results demonstrate that cellulose fibers can be used as a sorbent material for isolating and recovering viable microorganism from complex

samples, such as a PBC sample. Specifically, microorganism recovered from the cellulose fibers can be applied directly to Phoenix ID/AST analyses, or diluted to approximately 0.5 McFarland, and still obtain positive Phoenix® ID and AST, both analyses which require viable microorganism for accurate results.

Table 2: Isolation and recovery of viable microorganism using cellulose sorbent material.

PBC	Cellulose	PHX sample	McF	PHX ID	PHX AST
E. faecalis 29212 (G+ panel)	50 µm	no dilution	1.36	E. faecalis	Ok
		diluted	0.51	E. faecalis	Ok
	20 µm	no dilution	2.16	E. faecalis	Ok
		diluted	0.55	E. faecalis	Ok
NBC	50 µm	no dilution	0.15	No ID	No AST
		diluted	0	No ID	No AST
	20 µm	no dilution	0.22	No ID	No AST
		diluted	0	No ID	No AST

[0075] Example 7: Comparison of Sample Mixed with Sorbent Material Prior to Adding to the Column versus Applying Sample to the Column Directly

Preparation of PBC Samples

[0076] A BACTEC plus aerobic/F blood culture bottle was inoculated with 10-100 cfu of *S. aureus* (ATCC 29213) and 10 ml of blood and allowed to incubate until a positive signal was formed in a BACTEC FX instrument. A portion of the PBC sample (20 ml) was withdrawn from the PBC bottle and mixed with 20 ml of lytic reagent containing 0.66% Triton™ X-100 in Bactec Lytic 10 media. After mixing for 10 minutes, the lysed PBC sample was divided into two equal portions. One of the 20 ml portions of the lysed PBC sample was applied directly onto

the column prepared below containing a Celite® pre-coat layer. The second 20 ml portion of the lysed PBC sample was mixed with 4.5 ml of a 50% suspension of Hyflo Super Cel® particles (Sigma Aldrich catalog number 56678) before applying to a second column without a Celite® pre-coat layer.

Preparation of Columns

[0077] A disposable plastic column (20 ml size) with a 20 µm polyethylene frit was pre-coated using 4.5 ml 50% suspension of Hyflo Super Cel® particles (Sigma Aldrich catalog number 56678) using the same method as described in Example 1 to form a Celite® pre-coat layer. The column containing the Hyflo Super Cel® pre-coat layer was used for capturing the lysed PBC sample that was not pre-mixed with sorbent material. A vacuum manifold was used to initiate column flow.

[0078] A second column was prepared without a sorbent material pre-coat layer by washing a column with DPBS. The second column without a pre-coat layer was used to capture lysed PBC sample that was pre-mixed with Hyflo Super Cel® sorbent material. A vacuum manifold was used to initiate column flow.

Comparison of Capture Performance

[0079] Sample capture on the two columns was initiated simultaneously by applying the two columns to vacuum manifold and allowing the sample to flow through the column. If the total sample volume flowed through the column, the column was washed with 4.5 ml of Phoenix® ID broth followed by a wash with 4.5 ml of water. The wash cycle with BD Phoenix ID broth and water was repeated one time.

Results

[0080] The total volume (20 ml) of sample that was pre-mixed with sorbent material passed through the column within 5 minutes of applying the vacuum. This column was subsequently washed as described above after which all

residual PBC sample was removed as indicated by the washing away of all of the red color from red blood cells in the sample.

[0081] In contrast, very little volume of the PBC sample that was not pre-mixed with sorbent material prior to applying to the column passed through the column after 5 minutes of applying the vacuum. Capture time was extended to 30 minutes after which approximately 1 ml of the sample volume had passed through the column.

[0082] These results clearly indicate the advantage provided by pre-mixing complex samples, such as a lysed PBC sample, with sorbent material prior to applying the sample to a column.

[0083] Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the appended claims.

CLAIMS

1. A method for preparing a complex sample suspected to contain at least one microorganism therein for further analysis, the method comprising:

(a) obtaining a complex sample suspected to contain at least one microorganism;

(b) mixing the sample with a sorbent material to create a suspension;

(c) applying the suspension to a column;

(d) allowing the at least one microorganism in the sample to be captured by the sorbent material;

(e) separating the sorbent material with captured microorganisms from other sample constituents by retaining the sorbent material in the column and removing other sample constituents from the column; and

(f) separating the captured microorganism from the sorbent material.

2. The method of claim 1 further comprising the step of applying a sorbent material pre-coat layer to the column prior to applying the suspension to the column.

3. The method of claim 1, wherein the complex sample is a positive blood culture sample.

4. The method of claim 1, wherein the sorbent material is selected to preserve microorganism viability.

5. The method of claim 4, wherein at least a portion of the captured microorganism remains viable.

6. The method of claim 4, wherein the sorbent material comprises diatomaceous earth particles.

7. The method of claim 1, wherein the other sample constituents are removed from the column by washing the sorbent material with captured microorganism with a wash buffer.

8. The method of claim 1 further comprising the step of adding a suspension buffer to the separated sorbent material with captured microorganisms prior to separating the captured microorganism from the sorbent material.

9. The method of claim 8, wherein the captured microorganism is separated from the sorbent material by vortexing the sorbent material.

10. A method for preparing a sample determined to contain at least one microorganism therein for further analysis, the method comprising:

(a) obtaining a sample having been determined to contain at least one microorganism;

(b) mixing the sample with a sorbent material resulting in a suspension wherein at least a portion of the microorganism in the suspension remains viable;

(c) applying the suspension to a column;

(d) allowing the at least one microorganism in the sample to be captured by the sorbent material;

(e) separating the sorbent material with captured microorganisms from other sample constituents by retaining the sorbent material in the column and removing the other sample constituents from the column; and

(f) separating the captured microorganism from the sorbent material wherein at least a portion of the captured microorganisms remains viable.

11. The method of claim 10 further comprising the step of applying a sorbent pre-coat layer to the column prior to applying the suspension to the column.

12. The method of claim 10, wherein the sorbent material contains diatomaceous earth particles.

13. The method of claim 10, wherein the other sample constituents are removed from the column by washing the sorbent material with captured microorganism thereon with a wash buffer.

14. The method of claim 10 further comprising the step of adding a suspension buffer to the separated sorbent material with captured microorganisms prior to separating the captured microorganism from the sorbent material.

15. The method of claim 14, wherein the captured microorganism is separated from the sorbent material by vortexing the sorbent material.

16. A method for preparing a sample determined to contain at least one microorganism therein for further analysis, the method comprising:

(a) obtaining a sample having been determined to contain at least one microorganism;

(b) mixing the sample with a sorbent material to create a suspension;

(c) applying the suspension to a column;

(d) allowing the at least one microorganism in the sample to be captured by the sorbent material;

(e) separating the sorbent material with captured microorganisms from other sample constituents by retaining the sorbent material in the column and removing the other sample constituents from the column;

(f) extracting the microorganism from the sorbent material in the column by:

(i) washing the microorganism-captured sorbent material with an organic solvent;

(ii) subjecting the microorganism in the sample to an on-column extraction using an aqueous solution of an acid; and

(iii) collecting the flow-through from the extraction; and

(g) analyzing the microorganisms in the flowthrough from the extraction.

17. The method of claim 16 further comprising the step of applying a sorbent pre-coat layer to the column prior to applying the suspension to the column.

18. The method of claim 16, wherein the removing of the remaining components in the sample from the column comprises washing the captured microorganism with a wash buffer.

19. The method of claim 16, wherein the extracting step further comprises a second extraction using an acid combined with a solvent.

20. The method of claim 16, wherein the acid is formic acid.

21. The method of claim 16, wherein the acid is 70% solution of formic acid in water.

22. The method of claim 16, wherein the acid is formic acid and the solvent is acetonitrile.

23. The method of claim 22, wherein the formic acid is a 70% solution of formic acid in water combined with an approximately equal part of acetonitrile.

Obtain PBC sample and mix with sorbent
100
Optionally, Prepare column with sorbent pre-coat layer
105
Apply sample/sorbent mixture to a column with optional sorbent pre-coat layer and initiate column flow
110
Allow matrix bed to form with sorbent and capture microorganism
115
Wash sorbent containing captured microorganism
120
Create suspension of sorbent-captured microorganism
125
Transfer suspension
130
Vortex suspension in ID broth
135
Settled sorbent
140
Perform Phoenix ID/AST and/or MALDI
150
Viable microorganism in supernatant
145

FIG. 1

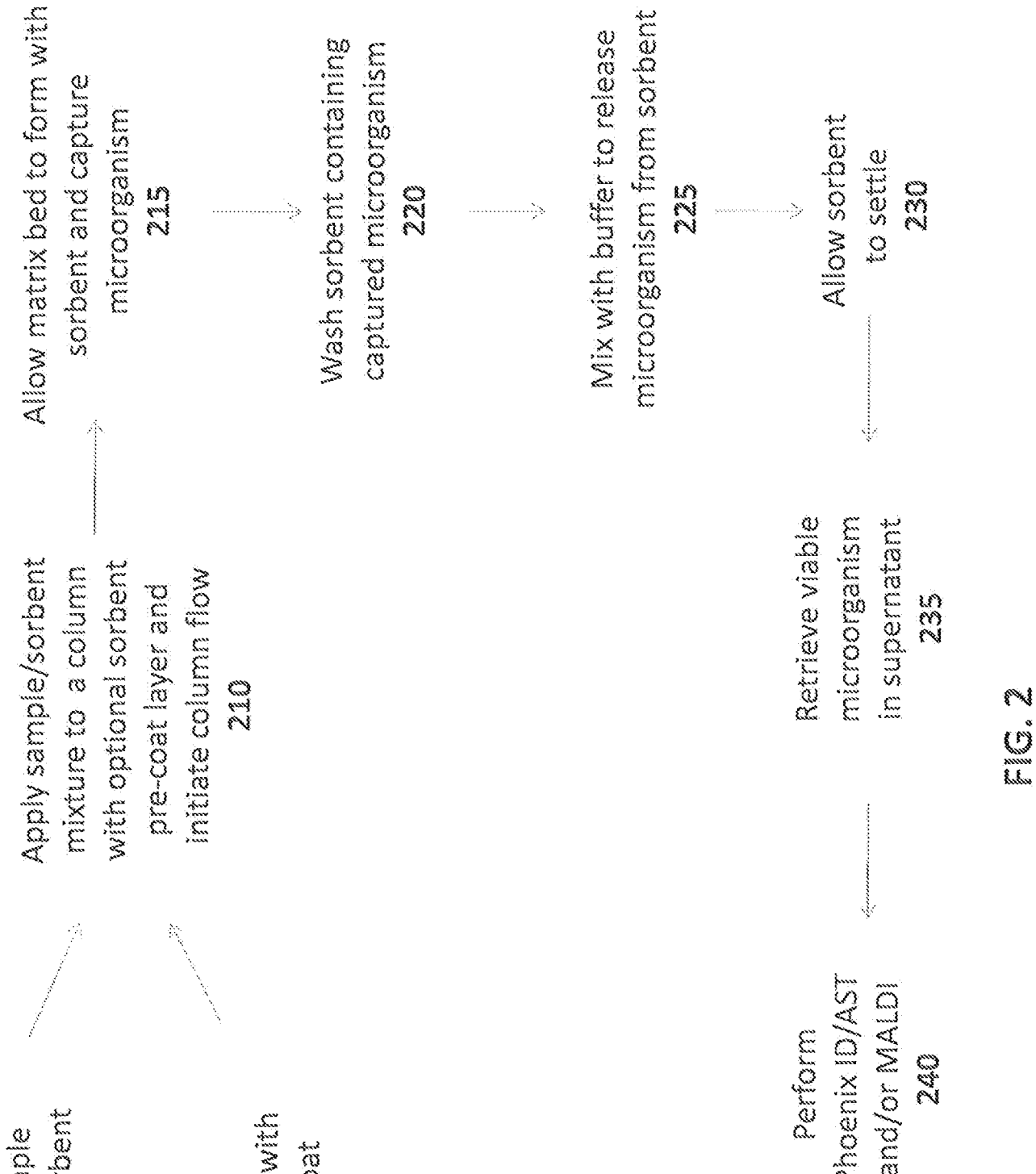
Obtain PBC sample and mix with sorbent
200
Optionally, Prepare column with sorbent pre-coat layer
205
Apply sample/sorbent mixture to a column with optional sorbent pre-coat layer and initiate column flow
210
Allow matrix bed to form with sorbent and capture microorganism
215
Wash sorbent containing captured microorganism
220
Mix with buffer to release microorganism from sorbent
225
Allow sorbent to settle
230
Retrieve viable microorganism in supernatant
235
Perform Phoenix ID/AST and/or MALDI
240

FIG. 2

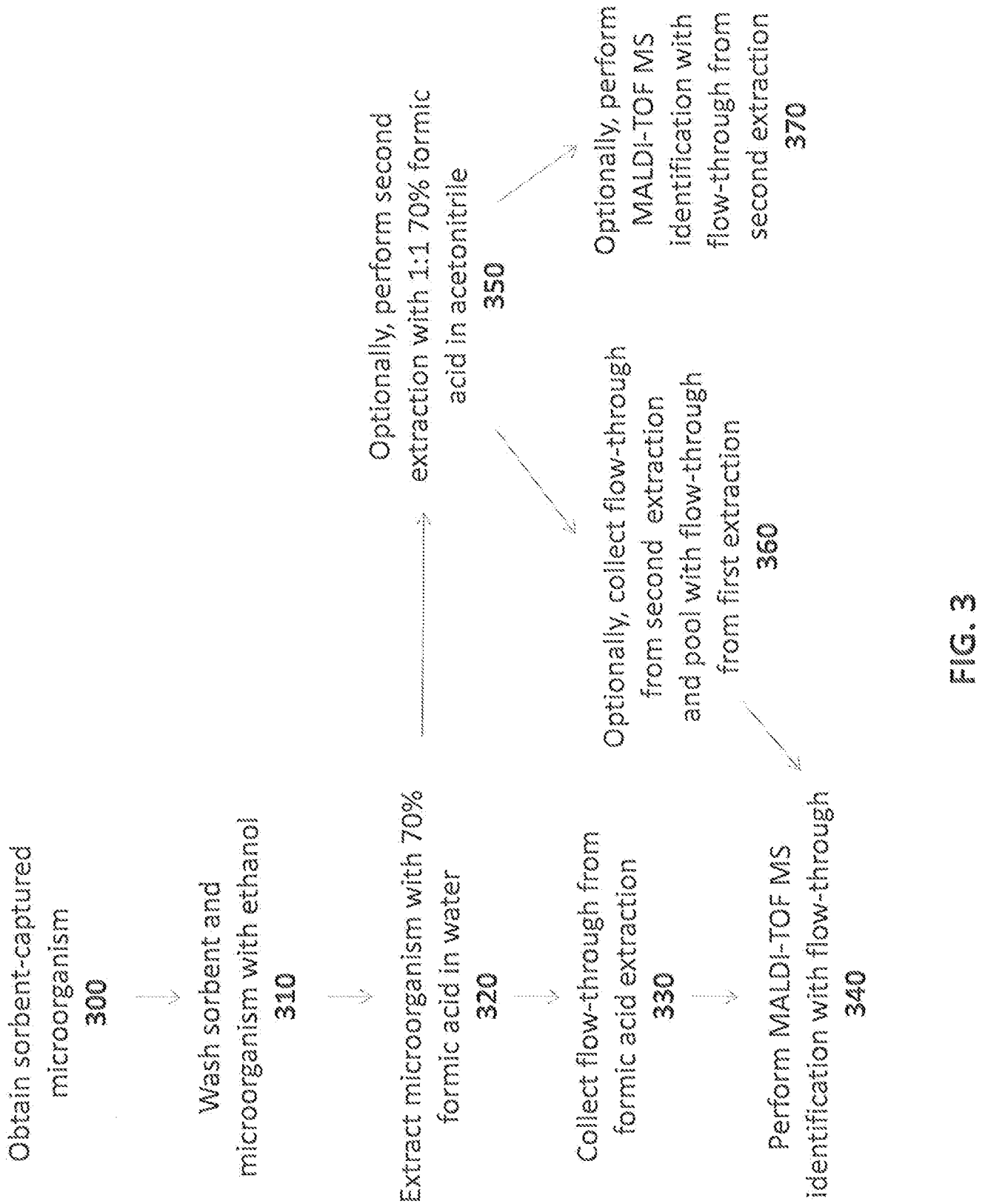
Obtain sorbent-captured microorganism
300
Wash sorbent and microorganism with ethanol
310
Extract microorganism with 70% formic acid in water
320
Collect flow-through from formic acid extraction
330
Perform MALDI-TOF MS identification with flow-through
340
Optionally, perform second extraction with 1:1 70% formic acid in acetonitrile
350
Optionally, collect flow-through from second extraction and pool with flow-through from first extraction
360
Optionally, perform MALDI-TOF MS identification with flow-through from second extraction
370

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/051454

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/24(2006.01)i, C12Q 1/04(2006.01)i, C12Q 1/02(2006.01)i

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)
C12Q 1/24; G01N 1/40; C12Q 1/04; C12P 19/34; G01N 33/53; B01J 20/285; C12Q 1/68; B01J 20/16; C12M 1/34; C12Q 1/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: microorganism, isolation, sorbent(diatomaceous earth), positive blood culture

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011-079038 A1 (3M INNOVATIVE PROPERTIES COMPANY et al.) 30 June 2011 See abstract; claims 1-18; and pages 1-22.	1-23
A	US 2012-0009569 A1 (KSHIRSAGAR et al.) 12 January 2012 See the whole document.	1-23
A	US 2011-0081676 A1 (SENGUPTA et al.) 07 April 2011 See the whole document.	1-23
A	US 2005-0037408 A1 (CHRISTENSEN et al.) 17 February 2005 See the whole document.	1-23
A	CHAPIN et al., 'Direct susceptibility testing of positive blood cultures by using Sensititre broth microdilution plates' Journal of Clinical Microbiology, Vol.41, No.10, pp.4751-4754 (2003) See the whole document.	1-23
A	JULAK, 'Chromatographic analysis in bacteriologic diagnostics of blood cultures, exudates, and bronchoalveolar lavages' Prague Medical Report, Vol.106, No.2, pp.175-194 (2005) See the whole document.	1-23

☐ 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 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
07 October 2013 (07.10.2013)	**08 October 2013 (08.10.2013)**

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/051454

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 2516984 A4	12/06/2013
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		EP 2414809 A1	08/02/2012
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		WO 2010-114725 A1	07/10/2010
US 2011-0081676 A1	07/04/2011	None	
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