



(51) International Patent Classification:

A23L 3/015 (2006.01)

A23L 3/54 (2006.01)

A23L 3/32 (2006.01)

(21) International Application Number:

PCT/US2019/041102

(22) International Filing Date:

10 July 2019 (10.07.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/697,502

13 July 2018 (13.07.2018)

US

62/798,748

30 January 2019 (30.01.2019)

US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: NONTHERMAL INACTIVATION OF MICROORGANISMS USING COMBINATIONS OF SHEAR AND ELECTRIC FIELDS

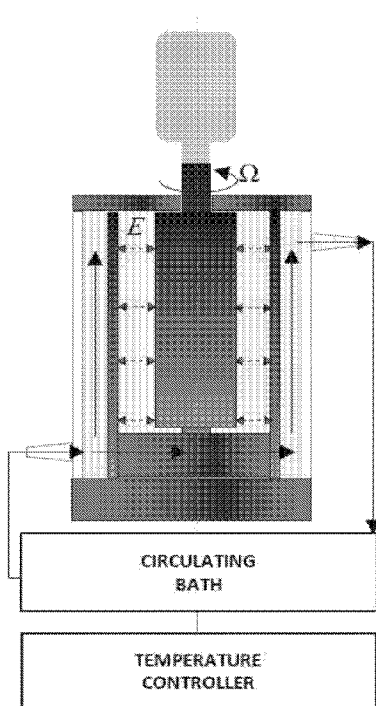


FIG. 1A

(57) Abstract: Disclosed herein are methods for inactivating microorganisms in fluids, such as fruit and vegetable juices, that involve fluid shearing in combination with moderate electric fields (typically less than about 1000V/cm and of arbitrary waveform) while cooling to maintain a constant temperature. The combination of these treatments has been shown to increase inactivation of *Escherichia coli* K12 in buffer solutions as well as apple juice and apple-kale juice. The rate of inactivation increases with shear, electric field strength, duty cycle and temperature.

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
- *of inventorship (Rule 4.17(iv))*

Published:

- *with international search report (Art. 21(3))*

NONTHERMAL INACTIVATION OF MICROORGANISMS USING COMBINATIONS OF SHEAR AND ELECTRIC FIELDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application No. 62/697,502,
5 filed July 13, 2018, and U.S. Provisional Application No. 62/798,748, filed January
30, 2019, which are hereby incorporated herein by reference in their entireties.

BACKGROUND

Due to risk of illness through consumption of inadequately processed fruits
and vegetables juice products, US FDA requires all manufacturers to subject juice
10 products to achieve a 5-log reduction in number of the most resistant pathogens.
Currently, the most reliable methods of inactivating pathogenic bacteria from foods
and biological fluids typically involve heat. Industry (particularly food and beverage
industries) are interested in producing high quality fruit and vegetable juices, which
contain antioxidants and bioactive compounds, which are degraded by heat. Current
15 non-thermal processing methods (e.g. high pressure, pulsed electric fields) are
expensive and allow only a limited throughput. Improved, inexpensive and scalable
methods of inactivation would be desirable. Alternatives to conventional processes
that do not cause detrimental changes in the organoleptic and nutritional properties
of the product are therefore needed.

20 It is well known that high intensity pulsed electric fields at greater than about
10,000 V/cm can inactivate vegetative bacterial cells. See U.S. Patent 5,690,978.
Typically such fields result in intense ohmic heating, which can only be controlled by
restricting electric field exposure to short pulses of a few microseconds. The theory
behind this thinking is that the electric field across cell membranes needs to reach 1
25 V in order to cause cells to be permeabilized and result in leakage of intercellular
constituents and cell death. Further, the theory posits that very high field strengths
are needed to irreversibly permeabilization to occur.

According to the disclosed methods, it is possible to permeabilize cells at far
lower electric field strengths, typically below 1000 V/cm operated continuously in
30 alternating current mode. While it is possible to operate such fields in pulsing mode,
it is significantly more advantageous to operate with the electric field on in continuous
alternating mode. It is also seen that such fields exhibit marked synergies with
mechanical shearing at relatively low shear rates. Further, according to the disclosed
methods, such treatments enable permeabilization of cells allowing ingress of
35 bacteriocins, which render the said bacteriocins effective even against bacteria that

they do not normally inhibit. It is also seen that such permeabilization is maintained well after the duration of the electric field and shear treatment, so that bacteriocins may gain ingress to the interior of bacteria even after the treatment.

SUMMARY

Disclosed herein are methods for inactivating microorganisms in fluids, such as fruit and vegetable juices, that involve fluid shearing in combination with moderate electric fields (typically less than about 1000V/cm and of arbitrary waveform) while cooling to maintain a constant temperature. The combination of these treatments has been shown to increase inactivation of *Escherichia coli* K12 in buffer solutions as well as apple juice. The rate of inactivation increases with shear, electric field strength and temperature.

As disclosed herein, the presence of shear can also result in a reduction in the rate of loss of antioxidant capacity. Thus it is possible to better retain bioactive components within processed products.

Finally, the process is scalable using a shearing assembly with rotor and stator, and a pair of suitably placed electrodes (which might coincide with the rotor or stator in some embodiments). Since it does not require specialized high pressure equipment or high voltages, it is expected to have advantages over high pressure and pulsed electric field processing for these applications.

Moreover, the disclosed methods can be used to disinfect fluids other than fruit and vegetable juices, especially in cases where high temperatures are not feasible or desirable.

Therefore, disclosed herein is a method for inactivating microorganisms in a fluid that involves treating the fluid with a combination of mechanical shear and a moderate electric field. In some embodiments the fluid is a fruit or vegetable juice.

In some embodiments, the mechanical shear has a shear rate of at least, 150 s⁻¹, 200 s⁻¹, 250 s⁻¹, 300 s⁻¹, 350 s⁻¹, 400 s⁻¹, 450 s⁻¹, 500 s⁻¹, 550 s⁻¹, 600 s⁻¹, 650 s⁻¹, 700 s⁻¹, 750 s⁻¹, 800 s⁻¹, 850 s⁻¹, 900 s⁻¹, 950 s⁻¹, 1,000 s⁻¹, 1,050 s⁻¹, 1,100 s⁻¹, 1,150 s⁻¹, 1,200 s⁻¹, 1,250 s⁻¹, 1,300 s⁻¹, 1,350 s⁻¹, 1,400 s⁻¹, 1,450 s⁻¹, 1,500 s⁻¹, 1,550 s⁻¹, 1,600 s⁻¹, 1,650 s⁻¹, 1,700 s⁻¹, 1,750 s⁻¹, 1,800 s⁻¹, 1,950 s⁻¹, 2,000 s⁻¹, 2,050 s⁻¹, 2,100 s⁻¹, 2,150 s⁻¹, 2,200 s⁻¹, 2,250 s⁻¹, 2,300 s⁻¹, 2,350 s⁻¹, 2,400 s⁻¹, 2,450 s⁻¹, 2,500 s⁻¹, 2,550 s⁻¹, 2,600 s⁻¹, 2,650 s⁻¹, 2,700 s⁻¹, 2,750 s⁻¹, 2,800 s⁻¹, 2,950 s⁻¹, or 3,000 s⁻¹.

In some embodiments, the electric field is less than about 1000 V/cm. In some embodiments, the electric field is less than about 200 V/cm. In some embodiments, the electric field is about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65,

70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 V/cm. In some embodiments, the electric field has an arbitrary wave form.

In some embodiments, the fluid is treated at a temperature less than 100 °C, 90 °C, 80 °C, 70 °C, 60 °C, 50 °C, 40 °C, or 30 °C. In some embodiments, the fluid is treated at room temperature. In some embodiments, the fluid is cooled while treated. In some embodiments, the fluid is treated at atmospheric pressure.

In some embodiments, the mechanical shear, electric field, or a combination thereof, may be applied for a time of from less than 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 30, 40 min to the fluid.

In some embodiments, the microorganism is a Gram-positive bacteria, a Gram-negative bacteria, or a combination thereof.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIGs. 1A and 1B are cross-sectional view (Fig. 1A) and a perspective view (Fig. 1B) of an experimental apparatus for creating shear stress (SS) and moderate electrical fields (MEF, square wave at 60 Hz) on microorganisms.

FIG. 2 is an example flow cytometry plot of bacteria double stained for cell damage and cell viability.

FIGs. 3A to 3F are survival curves for Gram-negative (*E. coli* K12, Figs. 3A, 3C, 3E) and Gram-positive (*L. innocua*, Figs. 3B, 3D, 3F) bacteria inactivated by SS (464.6, 1666.8, 2879 s⁻¹) and MEF at 27 °C with a duty cycle of 0.50 (Figs. 3A, 3B), 0.75 (Figs. 3C, 3D), or 0.99 (Figs. 3E, 3F) in clear apple juice.

FIGs. 4A and 4B are survival curves for Gram-negative (*E. coli* K12, Fig. 4A) and Gram-positive (*L. innocua*, Fig. 4B) bacteria inactivated by SS at 2879 s⁻¹ and MEF under duty cycle of 0.99, with and without nisin (NS, 100 IU/ml) in apple:kale blend juice at 27 °C.

FIGs. 5A and 5B are cytograms showing cell viability for Gram-negative (*E. coli* K12, Fig. 5A) and Gram-positive (*L. innocua*, Fig. 5B) bacteria inactivated by SS+MEF with and without nisin (NS) treatment in apple:kale blend juice at 27 °C.

FIGs. 6A and 6B are graphs showing cF (carboxyfluorescein) extrusion activity (%) of Gram-negative (*E. coli* K12, Fig. 6A) and Gram-positive (*L. innocua*, Fig. 6B) bacteria inactivated by SS+MF or SS+MEF+NS in apple:kale blend juice at 27 °C.

FIGs. 7A and 7B are schematics illustrating synergistic mechanisms of SS+MEF+NS on the inactivation of Gram-negative (Fig. 7A) and Gram-positive (Fig. 7B) bacteria.

FIGs. 8A to 8D are survival curves for *E. coli* K12 inactivated with 151.5 s⁻¹ (Fig. 8A), 454.6 s⁻¹ (Fig. 8B), 1666.8 s⁻¹ (Fig. 8C), or 2879 s⁻¹ (Fig. 8D) shear and 60 Hz sine waveform electric field of 0 V/cm, 20 V/cm, 35 V/cm, 60 V/cm, or 120 V/cm in sterile 1:1 (v/v) deionized water (DIW):buffered peptone water (BPW) at 40 °C.

FIGs. 9A and 9B are bar graphs showing log CFU/ml as a function of time for *E. coli* K12 in sterile 1:1 (v/v) mixture of deionized water (DIW):buffered peptone water (BPW) (Fig. 9A) and apple juice (Fig. 9B) at room temperature treated with 60 Hz square waveform electric field at a 0.50 duty cycle and 9500 rpm shear stress using MCA or PCA.

FIG. 10 is a bar graph showing antioxidant reduction rate (%) after inactivation with MEF or SS+MEF in commercial apple juice at 40°C and 50°C.

FIG. 11 A to 11C show *E. coli* K12 treated at 40°C with a shear rate of 152 s⁻¹ (Fig. 12A), 455 s⁻¹ (Fig. 12B), 1667 s⁻¹ (Fig. 12C), or 2879 s⁻¹ (Fig. 12D) and 60 Hz sine waveform electric field of 0 V/cm, 20 V/cm, 60 V/cm, or 120 V/cm in apple juice.

FIGs. 12A and 12B show *E. coli* K12 treated at 50°C with a shear rate of 151.5 s⁻¹ (Fig. 12A) or 2879 s⁻¹ (Fig. 12B) and 60 Hz sine waveform electric field of 0 V/cm, 60 V/cm, or 120 V/cm in apple juice.

FIGs. 13A and 13B show sustained damage and inactivation of *E. coli* K12 as a function of shear history (dimensionless, Figs. 13A, 13C) and total energy expended (J/m³, Figs. 13B, 13D) under SS at (a) 40°C (Figs. 13A, 13B) and 50°C (Figs. 13C, 13D) in apple juice.

FIG. 14A to 14D are cytogram plots showing cell viability for *E. coli* K12 (Figs. 14A, 14C) and *Listeria innocua* (Figs. 14B, 14D) in apple juice at 27°C treated with NS, SS+MEF, or NS post-treatment after SS+MEF.

FIG. 15A to 15C are sample response surface with contour plots presenting the log cycle reduction of *E. coli* K12 at 40 °C for SS and MEF, at a treatment time of 23.9 min (Fig. 15A); SS and treatment time at a field strength of 52.2 V/cm (Fig. 15B); and MEF and treatment time at a shear rate of 2650.5 s⁻¹ (Fig. 15C) (Desirable target was set to 5-log cycle; applicable criteria area for pasteurization (shaded)).

FIG. 16 is a sample response surface with contour plots presenting the effects of MEF and treatment time on the log cycle inactivation of *E. coli* K12 under a shear rate of 2879 s⁻¹ at 50 °C (Desirable target was set to 5-log cycle; applicable criteria area for pasteurization (shaded)).

DETAILED DESCRIPTION

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure.

Further, the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or

spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of chemistry, biology, and the like, which are within the skill of the art.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to perform the methods and use the probes disclosed and claimed herein. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C, and pressure is at or near atmospheric. Standard temperature and pressure are defined as 20 °C and 1 atmosphere.

Before the embodiments of the present disclosure are described in detail, it is to be understood that, unless otherwise indicated, the present disclosure is not limited to particular materials, reagents, reaction materials, manufacturing processes, or the like, as such can vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It is also possible in the present disclosure that steps can be executed in different sequence where this is logically possible.

It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

Disclosed herein are non-thermal methods for inactivating microorganisms in fluids that involve fluid shearing in combination with moderate electric fields while cooling to maintain a constant temperature. The rate of inactivation increases with shear, electric field strength, duty cycle (fraction of time that electric field is on) and temperature. As disclosed herein, the presence of shear can also result in a reduction in the rate of loss of antioxidant capacity. Thus it is possible to better retain bioactive components within processed products.

Finally, the process is scalable using a shearing assembly with rotor and stator, and a pair of suitably placed electrodes (which might coincide with the rotor or stator in some embodiments). Since it does not require specialized high pressure equipment or high voltages, it is expected to have advantages over high pressure and pulsed electric field processing for these applications.

Therefore, disclosed herein is a method for inactivating microorganisms in a fluid that involves treating the fluid with a combination of mechanical shear and a moderate electric field.

Fluids

5 The disclosed methods can be used to disinfect any fluids where high temperatures are not feasible or desirable. In some embodiments the fluid is a fruit or vegetable juice. In addition, the methods described herein can be used to preserve water or other beverages (drinkable liquids) or other liquids that may be used for enteral or parenteral introduction to humans and animals. In effect, any liquid
10 medium, for any use, that supports bacterial growth, and is sensitive to heat treatment may be disinfected by this method.

Mechanical Shear

Fluid undergoes shear when one area of fluid travels with a different velocity relative to an adjacent area. A high-shear mixer uses a rotating impeller or high-
15 speed rotor, or a series of such impellers or inline rotors, usually powered by an electric motor, to "work" the fluid, creating flow and shear. The tip velocity, or speed of the fluid at the outside diameter of the rotor, will be higher than the velocity at the center of the rotor, and it is this velocity difference that creates shear.

A stationary component may be used in combination with the rotor, and is
20 referred to as the stator. The stator creates a close-clearance gap between the rotor and itself and forms an extremely high-shear zone for the material as it exits the rotor. The rotor and stator combined together are often referred to as the mixing head, or generator. A large high-shear rotor–stator mixer may contain a number of generators.

25 In some embodiments, mechanical shear is produced using a Taylor-Couette Device. Taylor-Couette flow is the flow of a viscous fluid sheared in the gap between two rotating coaxial cylinders. The basic apparatus consists of two coaxial cylinders. The gap between them is filled with fluid and the inner cylinder is made to rotate by a motor. Fluid elements follow circular paths and the flow field has no axial or
30 azimuthal dependence. However, as the rotation rate of the inner cylinder is increased the flow suddenly undergoes an instability and a cellular pattern of toroidal vortices called Taylor rolls emerges. As the rotation is increased further, the Taylor rolls themselves become unstable giving way to a progressively more complicated flow states, eventually leading to turbulence.

35 In some embodiments, the mechanical shear has a shear rate of at least, 150 s^{-1} , 200 s^{-1} , 250 s^{-1} , 300 s^{-1} , 350 s^{-1} , 400 s^{-1} , 450 s^{-1} , 500 s^{-1} , 550 s^{-1} , 600 s^{-1} ,

650 s⁻¹, 700 s⁻¹, 750 s⁻¹, 800 s⁻¹, 850 s⁻¹, 900 s⁻¹, 950 s⁻¹, 1,000 s⁻¹, 1,050 s⁻¹, 1,100 s⁻¹, 1,150 s⁻¹, 1,200 s⁻¹, 1,250 s⁻¹, 1,300 s⁻¹, 1,350 s⁻¹, 1,400 s⁻¹, 1,450 s⁻¹, 1,500 s⁻¹, 1,550 s⁻¹, 1,600 s⁻¹, 1,650 s⁻¹, 1,700 s⁻¹, 1,750 s⁻¹, 1,800 s⁻¹, 1,950 s⁻¹, 2,000 s⁻¹, 2,050 s⁻¹, 2,100 s⁻¹, 2,150 s⁻¹, 2,200 s⁻¹, 2,250 s⁻¹, 2,300 s⁻¹, 2,350 s⁻¹, 2,400 s⁻¹, 2,450 s⁻¹, 2,500 s⁻¹, 2,550 s⁻¹, 2,600 s⁻¹, 2,650 s⁻¹, 2,700 s⁻¹, 2,750 s⁻¹, 2,800 s⁻¹, 2,950 s⁻¹, or 3,000 s⁻¹. The use of the rotating shear may be generated, for example, by using a motor, connected to concentric rotating cylinder systems. The methods described herein can create the shear rate with a defined intensity at the middle of the annulus between the inner and the outer cylinders by rotating inner, outer, or both (co-rotating or counter-rotating). The shear rate was generated in the present embodiment by an angular rotation of the motor with an intensity as measured by the RPM (revolutions per minute) by tachometer. Shear rate values may be calculated by the RPM and radii of inner and outer cylinders. A person of ordinary skill in the art will recognize that the RPM referred to herein is the speed of the rotating body as measured at the center of rotor.

Alternatively, it is possible to create a high shear environment by pumping fluid between parallel plates at a high rate, and using the parallel plates as electrodes. Other embodiments are also possible including electric fields parallel to, perpendicular to the primary flow (or shearing) plane; or any combination thereof. The description of any such embodiments is not intended to be restrictive, rather any means of shearing combined with a moderate electric field is intended to be covered by this invention.

Moderate Electric Field

In some embodiments, the electric field is applied between the rotor and the stator. Therefore, in some embodiments, one electrode is located on the rotor, and one electrode is located on the stator. In other embodiments, both electrodes are placed on the stator (e.g. opposite ends of the stator). In some embodiments, shear is created by pumping fluid between static parallel plates, which may serve wholly or partially as electrodes.

In some embodiments, the electric field is less than about 1000 V/cm. In some embodiments, the electric field is less than about 200 V/cm. In some embodiments, the electric field is about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 V/cm.

In some embodiments, the electric field has an arbitrary wave form. As described elsewhere, the moderate electric field may be provided as a squared

waveform. Common waveforms for electrical currents include the square wave, the sine wave, the ramp, the Sawtooth wave, and the triangular wave. In a squared waveform, the amplitude of the wave alternates at a steady frequency between fixed minimum and maximum values, with the same duration at minimum and maximum.

5 In addition to having a waveform, moderate electric field can be provided with a duty cycle of about 0 to about 1. In some embodiments, the squared waveform can have a frequency of about 0 to about 100 kHz. In some embodiments the frequency is about 60 Hz or above.

Temperature and Pressure

10 In some embodiments, the fluid is treated at a temperature less than 100 °C, 90 °C, 80 °C, 70°C, 60 °C, 50 °C, 40 °C, or 30 °C. In some embodiments, the fluid is treated at a temperature of about 10 to 100 °C, including about 20 to 60 °C. In some aspects, the fluid may be treated to a temperature in the range of about 25 °C to about 50 °C. In some embodiments, the fluid is treated at room temperature. In some
15 embodiments, the fluid is cooled while treated. In some embodiments the apparatus comprises elements to reduce the temperature in the chamber to a desired temperature, such as in the range of about 20 °C to about 60 °C. In some embodiments, the fluid is treated at atmospheric pressure. In some embodiments, the pressure may be less than 50 MPa, for example about 1 atm (101.3 kPa).

Antimicrobial Agents

20 In some embodiments, the fluid is treated with a bacteriocin or other natural antimicrobial agent. In some embodiments, any compound or agent with antimicrobial activity may be used in the disclosed methods. Bacteriocins are proteinaceous or peptidic toxins produced by bacteria to inhibit the growth of similar
25 or closely related bacterial strain(s). There are several large categories of bacteriocin which are only phenomenologically related. These include the bacteriocins from gram-positive bacteria, the colicins, the microcins, and the bacteriocins from Archaea.

30 The class I bacteriocins are small peptide inhibitors and include nisin and other lantibiotics.

The class II bacteriocins are small (<10 kDa) heat-stable proteins. This class is subdivided into five subclasses. One example of Class IIa bacteriocin is pediocin PA-1. The class IIb bacteriocins (two-peptide bacteriocins) require two different peptides for activity. One such an example is lactococcin G, which permeabilizes cell
35 membranes for monovalent sodium and potassium cations, but not for divalent cations. Class IIc encompasses cyclic peptides, in which the N-terminal and C-

terminal regions are covalently linked. Enterocin AS-48 is the prototype of this group. Class IId cover single-peptide bacteriocins, which are not post-translationally modified and do not show the pediocin-like signature. The best example of this group is the highly stable aureocin A53.

5 Class III bacteriocins are large, heat-labile (>10 kDa) protein bacteriocins. This class is subdivided in two subclasses: subclass IIIa (bacteriolysins) and subclass IIIb. Subclass IIIa comprises those peptides that kill bacterial cells by cell wall degradation, thus causing cell lysis. The best studied bacteriolysin is lysostaphin, a 27 kDa peptide that hydrolyzes the cell walls of several
10 Staphylococcus species, principally *S. aureus*. Subclass IIIb, in contrast, comprises those peptides that do not cause cell lysis, killing the target cells by disrupting plasma membrane potential.

Class IV bacteriocins are defined as complex bacteriocins containing lipid or carbohydrate moieties. Confirmation by experimental data was established with the
15 characterization of sublancin and glycocin F (GccF) by two independent groups.

In some embodiments, the bacteriocin is nisin. In some embodiments, the bacteriocin may include nisin, nisin A, nisin U, nisin Z, lactacin, lactococcin, leucocin, reuterin, variacin, bovicin HC5, Gasserin A and T, variacin, pediocin PA1, carnobacteriocins, aureocin A70 or enterocins, or synthesized and isolated from
20 biologically pure culture of the bacteriocin-producing strains.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Example 1: Exploiting the Combined Effect of Shear Stress, Moderate Electric Field and Nisin as Non-thermal Techniques for the Inactivation of *Escherichia Coli* K12 and *Listeria Innocua* in Fruit and Vegetable juices.

30 In the present study, hybrid combination technologies using rotational shear stress (SS, 2879 s⁻¹), moderate electric field (MEF, square-waveform at 60 Hz; Duty cycle: 0.50-0.99 (=120-168 V/cm)) and nisin (NS, 0-100 IU/ml) were applied alone or in combination to study their effect on inactivation of Gram-negative (*Escherichia coli* K12) and Gram-positive (*Listeria innocua*) bacteria in apple and apple:kale blend
35 juices with minimum thermal effect (at 27 ± 0.5°C). This new concept of combination processing was tested using a lab-scale device, consisting of a graphite-electrode

concentric cylinder system, the inner cylinder being rotated by a variable-speed motor.

Antibacterial susceptibility was evaluated using plate-counting method concomitantly with flow cytometric analysis with double-staining (PI-cFDA). Selected quality attributes (pH, color, antioxidant activity and chlorophyll contents) were also evaluated pre- and post-processing.

The maximum inactivation level with more than 5-log cycle reductions of both *E. coli* K12 and *L. innocua* were found at combined treatment of 2879 s⁻¹ and 168 V/m with nisin treatment of 100 IU/ml (within 5 min). The flow cytometry results showed the mechanism of cellular damage differed according to the bacterial species. Considering that heat treatment may destroy some heat-sensitive qualities of products, this study shows the synergistic effects of SS combined with MEF and nisin in a short treatment time without intense heat generation. This provides evidence for such combined treatment in the fruit-vegetable juice and related beverage industries.

Materials and Methods

Apparatus

- SS+MEF device (Inner rotational cylinder/Outer stationary electrodes)
- Power supply & integrated-gate bipolar transistor (IGBT)
- Variable speed DC motor for angular rotation
- Circulating cooling jacket

Treatment conditions

- Testing samples
 - Unpasteurized apple juice
 - Apple:kale blend juice (1:1)
 - Temperature: 27 ± 0.5°C
- SS: 464.6, 1666.8, 2879 s⁻¹
- NS: 0 – 100 IU/ml
- MEF: Square wave at 60 Hz
 - Duty cycle 0.50, 0.75, 0.99 (120, 145, 168 V/cm, respectively)

Microbiological analysis

- *Escherichia coli* K12, plated on MacConkey agar(MCA)
- *Listeria innocua* ATCC 33090, plated on Trypticase Soy Agar with 0.6% Yeast Extract plus 5% NaCl (TSAYE+NaCl)
- Flow cytometric analysis (FCM) with double staining
- Cell damage and cell viability assay

- PI-cFDA: esterase activity

Quality analysis

- pH, color and antioxidant capacity
- measurement of pre- and post-processed juice samples
- Radical-scavenging activity method

$$\text{Scavenging Effect \%} = \frac{A_0 - (A - A_b)}{A_0} \times 100\%$$

- Chlorophyll changes in apple:kale blend juice

Results

FIGs. 3A to 3F are survival curves for Gram-negative (*E. coli* K12, Figs. 3A, 3C, 3E) and Gram-positive (*L. innocua*, Figs. 3B, 3D, 3F) bacteria inactivated by SS (464.6, 1666.8, 2879 s⁻¹) and MEF at 27 °C with a duty cycle of 0.50 (Figs. 3A, 3B), 0.75 (Figs. 3C, 3D), or 0.99 (Figs. 3E, 3F) in clear apple juice.

FIGs. 4A and 4B are survival curves for Gram-negative (*E. coli* K12, Fig. 4A) and Gram-positive (*L. innocua*, Fig. 4B) bacteria inactivated by SS at 2879 s⁻¹ and MEF under duty cycle of 0.99, with and without nisin (NS, 100 IU/ml) in apple:kale blend juice at 27 °C.

FIGs. 5A and 5B are cytograms showing cell viability for Gram-negative (*E. coli* K12, Fig. 5A) and Gram-positive (*L. innocua*, Fig. 5B) bacteria inactivated by SS+MEF with and without nisin treatment in apple:kale blend juice at 27 °C.

FIGs. 6A and 6B are graphs showing cF extrusion activity (%) of Gram-negative (*E. coli* K12, Fig. 6A) and Gram-positive (*L. innocua*, Fig. 6B) bacteria inactivated by SS+MF or SS+MEF+NS in apple:kale blend juice at 27 °C.

This shows the degree of injury on the cellular pump activity.

Table 1 shows pH, color and DPPH antioxidant activities of untreated and treated apple juice samples under combined treatment of SS+MEF and conventional heating (at 90 °C for a holding time of 30 s).

Table 2 shows pH, color, DPPH antioxidant activities, chlorophyll contents of untreated and treated apple:kale blend juice samples combined treatment of SS+MEF+NS and conventional heating (at 90 °C for a holding time of 30 s).

Conclusions

The application of a combination approach to preserve fruit juice using SS+MEF with nisin increased the inactivation of *E. coli* K12 and *L. innocua*. These results demonstrate that appropriate combination of nisin and SS+MEF are capable of satisfying the FDA requirement of a greater than 5-log reduction of target

microorganisms, as an alternative method for juice pasteurization, while potentially avoiding the quality deterioration.

FIGs. 7A and 7B are schematics illustrating synergistic mechanisms of SS+MEF+NS on the inactivation of Gram-negative (Figs. 7A) and Gram-positive (Fig. 7B) bacteria.

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Table 1.							
Parameters		pH	Color				DPPH (%)
			L^*	a^*	b^*	ΔE	
Without NS	Negative Control	3.5 ± 0.01^a	91.41 ± 0.15^a	-1.67 ± 0.09^a	24.43 ± 0.16^a	-	55.54 ± 0.11^a
	SS+MEF	3.5 ± 0.01^a	91.35 ± 0.17^{ab}	-1.64 ± 0.11^{ab}	23.35 ± 0.11^b	1.08 ± 0.10^a	55.30 ± 0.05^a
	Positive Control	3.5 ± 0.01^a	91.19 ± 0.21^b	-1.61 ± 0.07^b	23.16 ± 0.10^{bc}	1.29 ± 0.08^b	55.38 ± 0.20^a
	Conventional (90°C, 30 s)	3.48 ± 0.01^b	90.33 ± 0.63^c	-1.59 ± 0.12^b	22.83 ± 0.40^c	1.93 ± 0.12^c	51.19 ± 0.08^b
With NS	Negative Control	3.49 ± 0.01^A	92.59 ± 0.18^A	-1.22 ± 0.11^A	24.02 ± 0.10^A	-	54.19 ± 0.07^A
	SS+MEF+NS	3.48 ± 0.01^B	92.54 ± 0.03^A	-1.18 ± 0.06^B	23.80 ± 0.05^B	0.23 ± 0.09^A	54.17 ± 0.05^A
	Positive Control	3.48 ± 0.01^B	91.84 ± 0.10^B	-1.20 ± 0.07^A	23.79 ± 0.07^B	0.78 ± 0.09^B	54.10 ± 0.10^A
	Conventional (90°C, 30 s)	3.47 ± 0.02^C	91.38 ± 0.75^C	-1.15 ± 0.09^C	23.32 ± 0.15^C	1.40 ± 0.12^C	51.98 ± 0.25^B
In each row, dissimilar small letters in each column indicate significant difference at 0.05 levels. a^* = redness/greenness b^* = yellowness/blueness ΔE = net color difference							

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Parameters		pH	Color				DPPH (%)	Chlorophyll	
			<i>L</i> [*]	<i>a</i> [*]	<i>b</i> [*]	ΔE		Chl <i>a</i>	Chl <i>b</i>
SS + MEF	Negative Control	4.11 ± 0.01 ^a	36.29 ± 0.15 ^a	-7.53 ± 0.10 ^a	15.21 ± 0.04 ^a	-	52.77 ± 0.25 ^a	2.38 ± 0.04 ^a	0.90 ± 0.02 ^a
	0	4.11 ± 0.01 ^a	36.11 ± 0.11 ^a	-7.53 ± 0.05 ^a	15.18 ± 0.08 ^a	0.03 ± 0.04 ^a	52.81 ± 0.12 ^a	2.38 ± 0.01 ^a	0.90 ± 0.01 ^a
	2.5	4.11 ± 0.01 ^a	36.08 ± 0.19 ^b	-7.53 ± 0.09 ^a	14.33 ± 0.23 ^b	0.90 ± 0.06 ^b	52.80 ± 0.17 ^a	2.37 ± 0.14 ^{ab}	0.89 ± 0.03 ^{ab}
	5	4.11 ± 0.02 ^a	35.55 ± 0.07 ^b	-7.52 ± 0.11 ^a	13.90 ± 0.19 ^{bc}	1.22 ± 0.06 ^c	52.85 ± 0.24 ^a	2.37 ± 0.02 ^b	0.88 ± 0.04 ^{ab}
	7.5	4.12 ± 0.01 ^a	35.14 ± 0.16 ^c	-7.51 ± 0.08 ^{ab}	13.74 ± 0.11 ^{cd}	1.87 ± 0.05 ^d	53.01 ± 0.08 ^a	2.35 ± 0.07 ^b	0.88 ± 0.01 ^{ab}
	10	4.07 ± 0.01 ^a	34.95 ± 0.31 ^c	-7.38 ± 0.17 ^c	13.48 ± 0.39 ^{de}	2.19 ± 0.08 ^d	52.80 ± 0.05 ^a	2.30 ± 0.05 ^c	0.87 ± 0.02 ^b
	Positive Control	4.02 ± 0.02 ^a	35.22 ± 0.22 ^c	-7.33 ± 0.09 ^c	13.41 ± 0.24 ^a	1.69 ± 0.12 ^d	52.81 ± 0.34 ^a	2.36 ± 0.04 ^{bc}	0.87 ± 0.03 ^b
SS + MEF + NS	Conventional	4.02 ± 0.02 ^b	33.94 ± 0.32 ^d	-4.67 ± 0.64 ^a	11.33 ± 0.30 ^f	5.37 ± 0.35 ^g	43.24 ± 1.05 ^b	2.15 ± 0.30 ^d	0.81 ± 0.05 ^c
	Negative Control	4.02 ± 0.01 ^A	35.41 ± 0.14 ^A	-6.70 ± 0.12 ^A	12.72 ± 0.07 ^A	-	53.52 ± 0.08 ^A	2.36 ± 0.02 ^A	0.96 ± 0.02 ^A
	0	4.02 ± 0.01 ^A	35.32 ± 0.25 ^A	-6.69 ± 0.11 ^A	12.69 ± 0.27 ^{AB}	0.09 ± 0.08 ^A	53.53 ± 0.14 ^A	2.35 ± 0.01 ^A	0.95 ± 0.03 ^A
	2.5	4.02 ± 0.01 ^A	34.81 ± 0.13 ^B	-6.67 ± 0.06 ^A	12.55 ± 0.15 ^B	0.62 ± 0.06 ^B	53.50 ± 0.18 ^A	2.33 ± 0.04 ^B	0.97 ± 0.04 ^A
	5	4.02 ± 0.01 ^A	34.60 ± 0.30 ^{AB}	-6.67 ± 0.17 ^A	12.05 ± 0.20 ^C	1.05 ± 0.09 ^C	53.60 ± 0.04 ^B	2.34 ± 0.05 ^{AB}	0.95 ± 0.05 ^{AB}
	7.5	4.02 ± 0.02 ^A	34.26 ± 0.05 ^C	-6.35 ± 0.06 ^B	11.88 ± 0.05 ^C	1.47 ± 0.05 ^D	53.55 ± 0.04 ^{AB}	2.29 ± 0.02 ^B	0.93 ± 0.03 ^B
	10	4.01 ± 0.02 ^A	33.98 ± 0.04 ^D	-6.27 ± 0.05 ^C	11.85 ± 0.06 ^C	1.73 ± 0.04 ^E	53.59 ± 0.05 ^{AB}	2.26 ± 0.05 ^{BC}	0.91 ± 0.01 ^C
	Positive Control	4.02 ± 0.01 ^A	34.09 ± 0.21 ^{BC}	-6.34 ± 0.09 ^{BC}	11.78 ± 0.40 ^D	1.61 ± 0.08 ^F	53.43 ± 0.22 ^B	2.29 ± 0.03 ^B	0.90 ± 0.05 ^{CD}
	Conventional	3.96 ± 0.04 ^B	33.55 ± 0.19 ^E	-4.60 ± 0.68 ^E	10.48 ± 0.45 ^E	3.59 ± 0.15 ^I	43.86 ± 1.35 ^C	2.06 ± 0.14 ^C	0.76 ± 0.10 ^E

In same column, dissimilar small letters in each row indicate significant difference at 0.05 levels (a-f: SS+MEF and A-F: SS+MEF+NS).

Example 2: Combination Shear-Electric Field Treatment for Inactivation of Vegetative Microorganisms in Liquids, Liquid Foods and Beverages.

The effectiveness of a combined shear stress and moderate electrical field technique on the inactivation of *E. coli* K12 in buffered peptone water (BPW) and apple juice has been investigated. Shear stress offers large localized forces to shear the cell surface resulting in possible physical damage to the cells. With help from the electric field, due to enhanced electroporation, a greater rate of microbial inactivation than conventional pasteurization method may be expected. 50 ml samples were treated at different temperatures (40 °C and 50 °C) and for different times (up to 60 min) by combined treatment, compared with water bath method and individual treatments (shear rate: 454.6, 1666.8, and 2879 s⁻¹); electric field strength: 60 and 120 V/cm). Bacterial reduction resulting from shear and MEF combination was significantly different ($p < 0.05$) from that resulting from conventional heating and individual treatments at 40°C and 50°C. The highest lethal rate (5.70 ± 0.85 log reduction in 7.5 min) of *E. coli* K12 was obtained for samples treated with the shear of 2879 s⁻¹ and MEF of 120 V/cm combination at 50 °C, which may be due to the effects of shear assisted by electric field effectively damaging the cell membrane, eventually leading to cell disruption at sublethal temperatures. Therefore, higher shear combined with higher MEF strength treatment has the great potential to shorten the processing time and improve the inactivation of microorganisms in juice products, ensuring food safety and retaining food quality.

Materials and Methods

Apparatus

- Shear configuration
 - Inner cylinder: rotary ($r=R_i$)
 - Outer cylinder: stationary ($r=R_o$)
- Moderate electric field (MEF) system
 - Power and integrated-gate bipolar transistor (IGBT)
 - Graphite electrode: inner and outer cylinder
 - On/off duty cycle: 0.5
 - Sine waveform (60 Hz)
 - Square waveform (60 Hz; On/off duty cycle: 0.50)

Testing sample (40ml)

- Sterile 1:1 (v/v) DIW:buffered peptone water (BPW)
- Pasteurized apple juice

Microbial analysis

- *E. coli* K12 using non-selective and selective plate agar (plate count agar (PCA) and MacConkey agar (MCA), respectively)

Testing conditions (treated up to 60 min)

- Temperature range: 40 °C and 50 °C
- Electric field strength: 0V/cm, 20V/cm, 35V/cm, 60V/cm, 120 V/cm
- Shear rate: 151.5 s⁻¹, 454.6 s⁻¹, 1666.8 s⁻¹, 2879 s⁻¹
- Working frequency: 0.06 kHz and 20 kHz

pH and antioxidant capacity measurement for apple juice

- Modified radical-scavenging activity method

$$\text{Scavenging Effect \%} = \frac{A_0 - (A - A_b)}{A_0} \times 100\%$$

Statistical analysis

- Analysis of variance (ANOVA, p < 0.05) and t-test (p<0.05) analyses were used.

Results

As illustrated in FIGs. 8A-8D, mechanical shear alone has very little effect on *Escherichia coli* K12. However, as field strength increases, the inactivation rate increases markedly. In addition, higher shear rates result in greater cooling, allowing higher field strengths to be implemented.

The process vessels can be scaled to sufficient size so that throughputs of pilot and plant scale are feasible (indeed, this approach is in current commercial practice with HPP). Electrodes of sufficient capacitance (e.g. platinized titanium, dimensionally stable anodes, pyrolytic graphite, among others) will suppress electrolytic gas production. Indeed, it is possible to operate the electric fields at high intensity pulse mode (PEF), which is typically effective against vegetative microorganisms, but may cause protein coagulation in sensitive products such as liquid egg.

In addition, we have processed a sample (apple) juice, and found that it is possible to inactivate microorganisms to below detection limit at temperatures as low as 27 °C within a process time of 40 minutes (a 5 log reduction was achieved in about 30 minutes) by changing electric field processing parameters (e.g. waveform, electric field strength, duty cycle or frequency).. We have also monitored the radical scavenging activity of samples processed using mechanical shear + MEF and MEF alone, and found no difference in activity at 40 °C.

Finally, the influence of mechanical shear +MEF was also tested in combination with a bacteriocin (nisin). Under normal conditions, nisin is not effective against gram negative bacteria, such as *E. coli* K12. However, when used in combination with mechanical shear+ MEF, a significant inhibitory effect is observed. This suggests that there may be possible synergies with mechanical shear + MEF together with natural antimicrobials.

Conclusion

The higher electric field strength by MEF with higher shear rate presented on enhancing levels of inactivation of *E. coli* K12 at both 40 °C and 50 °C.

Low frequency (60 Hz) moderate electric field effectively inactivated targeting bacteria.

Example 3: Combined effect of shear stress and moderate electric field on the inactivation of *Escherichia coli* K12 in apple juice

Materials and methods

Experimental apparatus

A rotating cylinder electrode system was set up as shown in Fig. 1. The device consisted of concentric electrode cylinders and a cooling jacket, connected to a water bath used for temperature control (Fig. 1(S)). The device could hold a sample volume up to 50 ml. Both concentric electrode cylinders were made of graphite (Ohio Carbon Blank Inc., Willoughby, OH): a stationary outer cylinder ($R_o = 22.8$ mm, $L = 90$ mm) and a rotating inner cylinder ($R_i = 12.7$ mm, $H = 77$ mm) (Fig. 1(B)) resulting in an annular gap of 10.1 mm. Details of apparatus geometry and operating conditions are given in Table 3. The entire food-contact part of the apparatus (Fig. 1(B)) was sterilized by autoclaving at 110 °C for 15 min before use for experiments.

Table 3	
Parameter	Value
R_i	12.7 mm
R_o	22.8 mm
d	10.1 mm
ε	1.799
H	77 mm
η	0.131

A motor was connected to the rotating inner electrode by a plastic sleeve to provide electrical insulation between them. A motor brush was used to apply electrical voltage to the rotating electrode. A copper contact soldered to electrical wire was connected to the outer stationary electrode to apply voltage and complete the

circuit with the inner rotating electrode. The angular rotation of the motor was controlled by a variable speed DC motor.

The cooling jacket was connected to a refrigerated circulating bath (RTE10 Bath/Circulator) to control the temperature. The sample temperature was monitored by a T-type thermocouple probe (T-type, SCPSS-062G-6, Omega Engineering, Inc., Stamford, CT) covered with heat-shrink tubing embedded into the cooling jacket. A data acquisition unit (DAQ, Agilent 39704A, Agilent Technologies, Inc., Palo Alto, CA) was used to monitor and collect the applied voltage and current, and temperature of the sample and cooling water in the cooling bath. Data were scanned and transmitted at an interval of 1 s.

SS and MEF treatments

For individual and combination treatments, 35 ml of apple juice sample inoculated with *Escherichia coli* K12 (preparation, inoculation, and enumeration procedures described below in sections 2.3-2.5) was placed into the sample bath and tested at 40 and 50 °C. For MEF treatments, samples were tested using sinusoidal wave alternating current at 60 Hz over a range of field strengths from 0 to 120 V/cm.

For mechanical fluid shear, consider a concentric cylindrical system where an inner cylinder of radius is rotating at a constant speed Ω (rad/s) in an outer cylinder or cup of radius. The shear rate (γ) for the Newtonian fluid can be calculated from the following equation (Nguyen Q.D., et al. Rheol. Acta 1987 26:508–515):

$$\gamma = \frac{2\Omega}{\left(1 - \frac{1}{\varepsilon^2}\right)} = \frac{4\pi NR_o^2}{60(R_o^2 - R_i^2)}$$

where N is rotation speed of the inner cylinder (rpm), operating between 500 and 9500 rpm in present studies (500, 1500, 5500, and 9500 rpm were calculated to shear rates of 151.5, 454.6, 1666.8, and 2879 s⁻¹, respectively).

The treatment under 0 V/cm with the slowest angular rotation at 151.5 s⁻¹ was assumed to be equivalent to conventional treatment with gentle stirring. Constant temperature was maintained by circulating cooling water, with agitation being increased by the rotating inner electrode.

Bacterial cell preparation

The bacterial strain used for the experiments: *Escherichia coli* K12 (chosen because it is a nonpathogenic surrogate of *Escherichia coli* O157:H7) was obtained. The culture was kept in a frozen condition at -80 °C. In preparation for experiments, a loop of frozen culture was inoculated in tryptic soy broth (Difco, Becton-Dickinson, Sparks, MD) and incubated overnight at 37 °C. This was followed by another transfer into fresh tryptic soy broth for a second overnight incubation. After adding 0.4 ml of

overnight culture to 50 ml of apple juice samples, the initial concentration was roughly 10^7 colony-forming unit (CFU)/ml. This value was chosen to ensure that survivors would remain even after a pasteurization process (5-log reduction of target pathogens, per FDA targets).

5 *Sample preparation*

Commercial pasteurized clear apple juice (Mott's®, unsweetened, vitamin C added) was purchased from a local market. Juice bottles were kept at 4 °C and remained closed until their experimental use.

Microbial enumeration

10 Treated and untreated (control) samples (1 ml) were collected and kept in ice. Later, they were serially diluted in 0.1 % wt/vol peptone water (Difco, Becton-Dickinson, Sparks, MD) and surface plated onto MacConkey agar (Difco, Becton-Dickinson, Sparks, MD). Inoculated Petri dishes were incubated at 37 °C for 24–48 h. After colony counting, data were analyzed and expressed as log CFU/ml.

15 *Determination of pH and color*

The pH of apple juice samples was measured using a digital pH meter (Delta 320 pH meter, Mettler Toledo Instruments Co., Ltd., Shanghai, China). Fifteen ml of sample was placed in a beaker and stirred continuously with a magnetic stirrer and pH was measured at 20 ± 0.5 °C. The pH meter was calibrated with commercial
20 buffer solutions of pH 7.0 and 4.0.

Color of apple juice samples was measured using a ColorQuest XE colorimeter (HunterLab, Hunter Associates Laboratories Inc., Reston, VA, USA). The equipment was set for total transmittance with specular included and D65/10° was used for the measurements. Samples were placed into a plastic cell (path length 2
25 mm) and put on reflectance port. The instrument was calibrated by using an instrument standard white tile. Color values were expressed as CIE $L^*a^*b^*$ system and all measurements were taken in triplicate. The net color difference (ΔE) was calculated as:

$$\Delta E = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

30 where the subscripts 1 and 2 refer to color components before and after treatment, respectively.

DPPH free radical scavenging activity

Radical scavenging activity of apple juice against stable DPPH· was determined according to the method proposed by previous studies (Islam M.S., et al.
35 Innov. Food Sci. Emerg. Technol. 2016 34:344–351) with necessary modifications, where the stable free radical DPPH· is reduced to the corresponding hydrazine by

reaction with hydrogen donors. A 50 µl apple juice aliquot was mixed with 1.5 ml of an ethanol solution of DPPH (100 µM). The samples were kept in the dark for 30 min at room temperature before measurement of the decrease in the absorption at 517 nm using a UV–visible spectrophotometer (Genesys 20 UV-VIS spectrophotometer, Thermo Scientific, NY). The same procedure was conducted for control; however, ethanol was used instead of the sample solution. DPPH free radical scavenging activity was calculated by using the following equation:

$$\text{Scavenging Effect (\%)} = \frac{A_0 - (A - A_b)}{A_0} \times 100\%$$

where A_0 is the absorbance of DPPH solution without sample, A is the absorbance of the test sample mixed with DPPH, and A_b is the absorbance of the sample without DPPH solution. Results were expressed as percentage of inhibition of the DPPH radical.

Response surface methodology and optimization

The combined effects of SS and MEF on the reduction of *E. coli* K12 were evaluated to obtain the optimized response within the region of the three-dimensional observation space using Response Surface Methodology (RSM). The RSM was conducted to assess the effect of shear rate and electric field strength on inactivation of *E. coli* in apple juice at 40 °C. Three factors: shear rate (X_1), electric field (X_2) and treatment time (X_3) with central composite design. Secondly, another RSM was employed to investigate under constant shear rate (2879 s⁻¹) at higher temperature (at 50 °C). In this case, two factors, electric field (X_2) and treatment time (X_3) were studied with the same experimental design model. The variables were coded according to the following equation:

$$\chi_i = \frac{\chi_i - \chi_0}{\Delta X}$$

Table 4 shows the corresponding response RSM parameters for each experiment. Experimental data were analyzed using a response surface regression fitted to a second-order polynomial equation model:

$$Y = B_0 + \sum_{i=1}^n B_i \chi_i + \sum_{i < j}^n B_{ij} \chi_i \chi_j + \sum_{j=1}^n B_{jj} \chi_j^2$$

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Table 4. Response surface methodology parameters								
Factor	Symbol		40 °C			50 °C		
			Levels ^a			Levels ^b		
	Coded	Encoded	-1	0	1	-1	0	1
Shear rate (s ⁻¹)	χ_1	X_1	454.6	1666.8	2879	N/A: Fixed at 2879		
Electric field strength (V/cm)	χ_2	X_2	20	40	60	0	60	120
Treatment time (min)	χ_3	X_3	10	20	30	2.5	5	7.5
^a $\chi_1 = (X_1 - 1666.8)/1212.2$; $\chi_2 = (X_2 - 40)/20$; $\chi_3 = (X_3 - 20)/10$ ^b $\chi_2 = (X_2 - 60)/60$; $\chi_3 = (X_3 - 5)/2.5$								

JMP Pro version 13.1 (SAS Institute Inc., Cary, NC) was used to obtain a three dimensional plot from the response surface analysis and determine the optimization by superposition of the contour profiles. Specifically, based on the FDA requirement for pertinent pathogens, a 5-log cycle reduction was used as a target level of inactivation by combined SS and MEF via setting the desirability to max at this level ($-5 \text{ Log } (N/N_0)$). Then, processing optimization considering reduction in the intensive use of one preservation treatment (shear rate, electric field strength, and treatment time) that may produce the lowest impact on food quality was analyzed. The model was obtained by regression analysis and analysis of variance (ANOVA).

Statistical analysis

There were at least three independent replicates, with two samples per replicate, for each treatment. Average microbial reductions, expressed in logarithmic values, were used in the statistical analyses. In this study, data are expressed as means $\pm$ SD. Differences between groups were analyzed using an ANOVA of SAS software 9.3 (SAS Institute, Cary, NC). A p-value < 0.05 was considered statistically significant.

Results and Discussion

Inactivation of E. coli K12 by individual treatment of SS and MEF and combination of SS and MEF (SS + MEF) at 40 and 50 °C

As shown in Figs. 11A-11D and 12A-12B, individual treatment by shear stress only (expressed as 0 V/cm) produced up to 1.27 ± 0.26 and 2.36 ± 0.30 log reductions of *E. coli* K12 at 40 and 50 °C, respectively (at their respective maximum treatment times of 30 and 10 min). In comparison between results from 151.5 s^{-1} at 40 °C for 10 min (Fig. 11A) and those of 50 °C (Fig. 12A), improvement in inactivation of *E. coli* K12 was thought to be derived from the thermal effect (up to approximately 1.31 log greater reduction). At 40 °C, except for treatment at 2879 s^{-1} , it appears that there were no significant ($p > 0.05$) differences between the inactivation of *E. coli* K12 exposed to shear stress only and conventional heat treatment (data from the lowest shear rate under 0 V/cm). At both temperature conditions (40 °C and 50 °C), only the results from the shear rate of 2879 s^{-1} showed a biphasic inactivation curve characterized by a slow lag phase followed by a log linear inactivation phase. Previous studies have similarly reported biphasic inactivation patterns of *E. coli* O157:H7 strains in ultrasound-treated growth media and fruit juices (Gabriel A.A., et al. Food Control 2015 50:722–728). This behavior has been explained by the accumulation of damages that lowers the resistance of the cells, resulting in progressively shorter time to inactivate the same portion of the population (Peleg M.,

Adv. Quant. Microbiol. Foods Biosyst. 2006 1–48). From these results, it could be thought that it is necessary to exceed a minimum shear, between 1666.8 and 2879 s⁻¹, in order to change the *E. coli* inactivation kinetics significantly and, therefore, shorten the treatment times, which is mainly linked to the duration of the lag-phase.

Several studies in different contexts indicate that the shear rate and exposure time have positive correlations with cell lethality. The energy dissipation rate generated by rotation speed on *Saccharomyces cerevisiae* suspensions was studied (Yusaf T., Biochem. Eng. J. 2013 79:7–14). These results showed a non-linear reduction of yeast cells as shearing time increased (reaching less than 2% of cell viability after 17 min treatment at 35 °C). In relation to bioreactor cultivation of plant cell, the effects of shear on *Perilla frutescens* viability under different shear rates and times was reported; the lowest cell viability (close to 60%) was found with 866.4 s⁻¹ after 20 min shearing time (Zhong J.J., et al. Iotechnol. Bioeng. 1994 44:649–654). In similar shear effects studies on culture viability, 1193 s⁻¹ and a 12-hr shearing time resulted in a decrease by 95% of *Nicotiana tabacum* cells (Hooker B.S., et al. Enzym. Microb. Technol. 1989 11:484–490). The above studies involve either yeast or eukaryotic plant cells, which may be more sensitive to external lethal agents than bacteria.

The inactivation of *E. coli* by MEF treatments at the lowest shear rate at 40 and 50 °C (Figs. 11A and 12A except for 0 V/cm data) resulted, (after an initial shoulder region at 40 °C), in linear inactivation curves with greater, more rapid log reduction in viable cells as the electric field strength increased. Previous studies relating to ohmic heating also found greater non-thermal inactivation effects as electric field strength increased (Park I.-K., et al. Appl. Environ. Microbiol. 2013 79:7122–7129) and nonthermal inactivation effects were also found against bacterial spores (Murashita S., et al. J. Food Prot. 2016 80:164–168). Notably, those studies were conducted at higher temperatures than this work. In the current study and published studies (Machado L.F., et al. J. Food Eng. 2010 96:520–527), in comparison to conventional heating at lower temperatures (25–50 °C), MEF had additional effects beyond thermal injury. Nonetheless, while applying the highest electric field strength available, it was still not possible to achieve a 5-log cycle inactivation of *E. coli* K12 for 30 min and 10 min individual MEF treatment at 40 and 50 °C, respectively.

At both 40 and 50 °C (Fig. 11B-11D and Fig 12B), the survivor curves under SS combined with zero and low electric field strength (20 V/cm) MEF treatment

showed an initial lag phase (indicated by a shoulder in the curve), followed by a log-linear section. On the other hand, at the higher shear rates, with high electric field strengths (40–120 V/cm), two or multiple log-linear inactivation stages without lag (shoulder) phases were observed. The absence of a lag-phase could indicate that there was no limiting step in the conditions applied to affect the mechanisms of action of electric field under the rotating shear, resulting in faster inactivation rate with short treatment time. Figs. 12A and 12B presents the inactivation curve of *E. coli* K12 subjected to SS + MEF at higher temperature (50 °C). Compared to the same electric field strength and highest shear rate conditions (60 V/cm and 120 V/cm under 2879 s⁻¹) at 40 °C, treatment at 50 °C allowed faster inactivation under both conditions (approximately 50% and 62.5% reduction in treatment time to reach 5-log reduction respectively). Based on survivor curve shapes from Figs. 11A-11D and 12A-12B, the application of heat to the SS + MEF treatments results in the elimination of the lag phase, which is responsible for most of the process time reduction. The greatest inactivation of 5-log reduction cycles was achieved by SS + MEF at 50 °C and under a constant electric field strength of 120 V/cm and a shear rate of 2879 s⁻¹ for 7.5 min (5.62 ± 0.44 log reduction).

Above all, these studies revealed that fluid-mechanical shear stress can achieve enhanced microbial inactivation under the same MEF treatment at both test temperatures. The applied stresses, including cell interaction with surface, high turbulence, shear, and other related impact forces on the cell boundary, can cause cell deformation and partially damage the cell membrane. In studies on inactivation of *S. cerevisiae*, flow over rotating cylinders may enhance cell disruption by enhanced localized shear (Yusaf T., Biochem. Eng. J. 2013 79:7–14). In addition, the higher shear rates would also correspond to higher agitation and cooling effects, allowing higher field strengths to be applied under a given temperature. In this sense, these results are in agreement with observations in the context of PEF treatment, with continuous flow PEF showing higher levels of inactivation of pathogens (Gavahian M., et al., Extraction from food and natural products by moderate electric field: mechanisms, benefits, and potential industrial applications, In: Comprehensive Reviews in Food Science and Food Safety, 2018; Qin B.-L., et al. IEEE Trans. Ind. Appl. 1998 34:43–50; Sastry, S.K. . J. Food Sci. 2016 81:E1431-E1446) than batch systems.

For better mechanistic understanding of the role of shear stress alone, the results from 0 V/cm on microbial inactivation were analyzed in two ways. Firstly, the role of shear history (defined by the dimensionless product of shear rate ($\dot{\gamma}$) and

treatment time (t) was evaluated by plotting Log (N/N₀) versus the shear history (γt). Second, the role of cumulative energy dissipation (Dunlop E.H., et al. *hem. Eng. Sci.* 1994 49:2263–2276; Kieran P.M., et al. In: *Influence of Stress on Cell Growth and Product Formation*, 2000, Springer, 139–177), determined for annular flow as described below, was investigated. Briefly, the energy dissipation rate (ε) was calculated using the following equation (Bradshaw P., *An Introduction to Turbulence and its Measurement: Thermodynamics and Fluid Mechanics Series*, 2013, Elsevier; Yusaf T., *Biochem. Eng. J.* 2013 79:7–14):

$$\varepsilon = \frac{P}{\rho V} = \frac{u_{\tau}^3}{Ky}$$

where friction velocity (u_τ) can be described through dimensional analysis as:

$$u_{\tau} = \frac{0.7U_{\infty}}{\frac{1}{K}(\log y + C)}$$

where C is 2.05 as assumed for a smooth solid surface, K = 0.41, and y = 0.1 × d; and U_∞ is the linear velocity (m/s) at working rotational speed.

Figs. 13A-13D are plots of the above analyses both 40 °C (Figs. 13A, 13B) and 50 °C (Figs. 13C, 13D). Fig. 13A shows that at 40 °C, regardless of whether shear history (γt) or cumulative energy dissipation is used, Log (N/N₀) shows a similar pattern. It is notable that even at the lowest shear rates, regardless of whether t or cumulative energy dissipation are used, some inactivation occurs. However, at the highest shear rates, the inactivation ratio, while greater, requires considerably more energy input (or shear history). Fig. 13B shows similar plots at 50 °C, where it becomes apparent that the inactivation at the lowest shear history/energy dissipation is significant, and comparable to that at the highest shear rates. In addition, the plots at 50 °C are qualitatively different from those at 40 °C, suggesting a significant thermal effect. It is possible that the thermal dependence of membrane fluidity of *E. coli* plays a role. Since membranes are composed of structured phospholipid assemblies, dynamic and structural characteristics of membranes can be changed by changing the working temperature (Beney L., et al. *Appl. Microbiol. Biotechnol.* 2001 57:34–42). Likewise, there was a negative linearity between membrane fluidity and temperature between 0 and 50 °C using fluorescent dyes that undergo torsional relaxation only in a lipid phase (Nedwell D.B., et al. *FEMS Microbiol. Ecol.* 1999 30:101–111). Regardless, the mechanical effects alone are small in comparison to combined shear-electric field effects.

Regression model for inactivation of E. coli K12 and localization of optimum condition

The combined effects of SS and MEF on the inactivation of *E. coli* K12 were analyzed using RSM. The response (Y) was measured in terms of log-cycle (Log (N/N₀)). The design matrix of the variables in coded units is summarized in Table 5. Multiple regression analysis of the experimental data gave the following second-order polynomial equation as follows:

Y(Log reduction at 40°C)

$$= 2.5859 + 0.0006\chi_1 - 0.1814\chi_2 + 0.1167\chi_3 - 7.2819 \times 10^{-6}\chi_1\chi_2 \\ - 2.7755 \times 10^{-5}\chi_1\chi_3 - 0.0046\chi_2\chi_3 - 8.6557 \times 10^{-8}\chi_1^2 + 0.0027\chi_2^2 \\ - 0.0016\chi_3^2$$

Y(Log reduction at 40°C)

$$= 1.7637 + 0.0025\chi_2 - 0.7447\chi_3 - 0.0056\chi_2\chi_3 - 1.2759 \times 10^{-5}\chi_2^2 \\ + 0.0492\chi_3^2$$

Table 5 shows the effects of independent variables on the inactivation of *E. coli* K12. The results indicated the effects of shear rate, electric field strength as well as the treatment time, significantly affected the *E. coli* lethality ($p < 0.05$). Particularly, interaction of the electric field strength-treatment time resulted the variable with the highest effect on *E. coli* inactivation at both 40 and 50 °C.

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Table 5. Effects of electric field strength, shear rate, and treatment time on <i>E. coli</i> K12 inactivation (Log (N/N ₀)) under SS + MEF at 40 and 50 °C.				
SS + MEF		Independent variables	Dependent variables	
			Log (N/N ₀)	
			Log worth	p-value
Temperature	40 ° C	Electric field strength (V/cm)*Treatment time (min)	2.295	0.005
		Shear rate (s ⁻¹)*Treatment time (min)	0.672	0.213
		Electric field strength (V/cm)	0.555	0.279
		Treatment time (min)	0.353	0.444
		Shear rate (s ⁻¹)*Electric field strength (V/cm)	0.301	0.500
		Shear rate (s ⁻¹)	0.234	0.584
	50 ° C	Electric field strength (V/cm)*Treatment time (min)	2.208	0.006
		Treatment time (min)	1.266	0.054
		Electric field strength (V/cm)	0.048	0.896

The effects of the variables were visualized using a three-dimensional response surface and contour plots in Figs. 15A to 15C and 16, which are graphical representations of equations (8) and (9), respectively. Fig. 15A shows the effect of electric field strength and shear rate on the inactivation of *E. coli* K12 while keeping the treatment time to 23.9 min. The reduction of *E. coli* K12 increased with increasing treatment intensities. Similar patterns were observed in the effect of treatment time against shear rate (under a field strength of 52.2 V/cm) and electric field strength (2650.5 s⁻¹) (Figs. 15B 15C, respectively). The corresponding contour plots shows the log-cycle reduction of *E. coli* K12 in apple juice, which can be used at different controllable ranges of key variables for the prediction of inactivation level. From the results, the sensitivity of *E. coli* K12 in apple juice to increasing electric field strength was higher than the sensitivity to the shear rate increase. It was observed that log reductions changed significantly ($p < 0.05$) with the interactions between electric field strength and treatment time (Fig. 16), indicating that the electric field strength and treatment time played an important effect on the inactivation of *E. coli* K12 in apple juice at 50 °C. Through the shear rate-electric field strength-time plots, a significant synergistic effect is observed among the three parameters. Among possible combination conditions, the optimum values for 5-log cycle reduction of *E. coli* K12 in apple juice were estimated to 2650.5 s⁻¹, 52.2 V/cm 23.9 min at 40 °C. With a shear rate of 2650.5 s⁻¹ at 50 °C, the optimum values were estimated to 108.9 V/cm and 6.8 min. The shortest treatment time under maximum applicable treatment conditions for fulfilling 5-log cycle reduction of target microorganisms was estimated to 23.1 and 6.2 min at 40 and 50 °C, respectively.

Quality attribute tests under SS, MEF and SS + MEF

Changes in quality assessment factors (pH, color, and antioxidant capacity) for SS + MEF treated apple juice samples compared with those of SS, MEF and untreated (control) samples are presented in Table 6. Treatment conditions were determined by the best inactivation results obtained from the previous section (23.1 min at 40 °C and 6.2 min at 50 °C under electric field strength of 120 V/cm and shear rate of 2879 s⁻¹). Note that in Table 6 additional quality data from 120 V/cm, 2879 s⁻¹ for 40 °C after 23.1 min were also included.

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Table 6. pH, color, and DPPH antioxidant activities of untreated and treated samples under individual and combined treatment of SS and MEF.						
Parameter		Treatment				
		4 °C	40 °C for 23.1 min			50 °C for 6.2 min
		Control	SS at 2879 s ⁻¹	SS + MEF at 60 V/cm; 2879 s ⁻¹	SS + MEF at 120V/cm; 2879 s ⁻¹	SS + MEF at 120V/cm; 2879 s ⁻¹
pH		3.50 ± 0.01 ^a	3.48 ± 0.02 ^a	3.46 ± 0.01 ^b	3.48 ± 0.02 ^a	3.49 ± 0.01 ^a
Color	L*	91.17 ± 0.14 ^a	90.96 ± 0.22 ^a	90.67 ± 0.48 ^a	90.08 ± 0.32 ^a	90.76 ± 0.26 ^a
	a*	-1.62 ± 0.01 ^a	-1.59 ± 0.08 ^a	-1.52 ± 0.07 ^b	-1.59 ± 0.02 ^a	-1.61 ± 0.02 ^a
	b*	24.31 ± 0.03 ^a	22.42 ± 0.75 ^b	21.24 ± 0.66 ^b	22.45 ± 0.80 ^c	23.60 ± 0.29 ^c
	ΔE	—	1.90 ± 0.41 ^a	3.11 ± 0.08 ^b	2.15 ± 0.36 ^a	0.82 ± 0.43 ^c
DPPH (%)		93.93 ± 0.83 ^a	89.97 ± 0.89 ^b	87.44 ± 0.31 ^c	90.97 ± 1.19 ^{bd}	92.56 ± 1.37 ^{ad}
a, b, c, d -Within each row, values followed by the same letters indicate no significant difference (p > 0.05).						

Although pH of apple juice samples showed some changes after treatment, the values for pH of treated juice samples still lay within the range of desirable acidity for apple juice (within approximately 0.05). Results obtained are in agreement with previous studies that reported no significant alteration in pH of apple juice even after high temperature treatment and/or similar treatment time (Abid M., et al. Ultrason. Sonochem. 2014 21:984–990; Aguilar-Rosas S., et al. J. Food Eng. 2007 83:41–46; Liao H., et al. Ultrason. Sonochem. 2018 42:244–249).

Color attributes of samples subjected to the SS + MEF treatment at 50 °C was not significantly affected: a total color difference (ΔE) below 1.5 was recorded. Though there were no significant differences between and values of treated and untreated samples, the b^* value decreased significantly as compared with untreated juice ($p < 0.05$). The ΔE values of all treated juices at 40 °C were greater than 1.5, indicating that the three treatments result in a slightly noticeable color difference. Mostly, noticeable color changes were detected as a result of the significant ($p < 0.05$) decrease observed in a^* and b^* values. These changes in color values of all treated apple juice might be due to combined effects of long treatment time at a certain temperature. Other studies (Liao H., et al. Ultrason. Sonochem. 2018 42:244–249), suggested that the difference in b^* value might mainly result from the degradation of vitamin C followed by browning of juice. Although the instant studies did not include vitamin C, somewhat analogous results were found through significant decreases in antioxidant activities of SS, MEF, and SS + MEF treated juice at 40 °C as compared to untreated juice ($p < 0.05$).

The highest percentage retention of DPPH was 92.6% for SS + MEF for the 50 °C treated sample, which showed no significant difference compared to the control, $93.93 \pm 0.83\%$. Thus, one of the major merits of SS + MEF for pasteurization is that the microbial inactivation can be achieved at lower temperature than conventional pasteurization, thereby avoiding the loss of heat sensitive physicochemical properties and nutrients.

From this work, it is clear that electric fields at relatively low field strengths may influence microbial inactivation, although over a longer time scale than at higher field strengths. It is commonly held in the PEF literature that a threshold field strength of 10 kV/cm is required to achieve electroporation of bacterial cell membranes. Clearly, the result of this work demonstrates that such high field strengths are not necessary for inactivation of vegetative bacterial cells.

Conclusion

The present work demonstrates the potential of simultaneous combination of mechanical shear flow between concentric rotating cylinders and MEF to enable microbial inactivation at moderate temperatures. The instant results show that these combinations act synergistically in inactivating *E. coli* K12 in apple juice.

Within the range of conditions of this work, the best processing conditions for apple juice are a shear rate of 2879 s^{-1} and 120 V/cm at $50\text{ }^{\circ}\text{C}$ for 6.2 min , which shows 5-log cycle reduction of *E. coli* K12 at the shortest time. In light of the results gathered in this study, the best inactivation could be achieved by combining the highest shear rate and high electric field and temperatures for short treatment time. One might expect a beneficial effect of SS + MEF on inactivation of different bacterial species, including Gram-positive bacteria and spores through synergistic effect of combining SS + MEF but this synergy remains to be established. Additionally, important microbiological issues that should be investigated include the microbial inactivation kinetics and mechanisms under which the synergistic action takes place, as well as the effect of combination treatment on bacterial injury and subsequent recovery.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method for inactivating microorganisms in a fluid, comprising treating the fluid with a combination of mechanical shear and a moderate electric field.
2. The method of claim 1, wherein the fluid is a fruit or vegetable juice.
3. The method of claim 1 or 2, wherein the mechanical shear has a shear rate of at least 150 s⁻¹, 200 s⁻¹, 250 s⁻¹, 300 s⁻¹, 350 s⁻¹, 400 s⁻¹, 450 s⁻¹, 500 s⁻¹, 550 s⁻¹, 600 s⁻¹, 650 s⁻¹, 700 s⁻¹, 750 s⁻¹, 800 s⁻¹, 850 s⁻¹, 900 s⁻¹, 950 s⁻¹, 1,000 s⁻¹, 1,050 s⁻¹, 1,100 s⁻¹, 1,150 s⁻¹, 1,200 s⁻¹, 1,250 s⁻¹, 1,300 s⁻¹, 1,350 s⁻¹, 1,400 s⁻¹, 1,450 s⁻¹, 1,500 s⁻¹, 1,550 s⁻¹, 1,600 s⁻¹, 1,650 s⁻¹, 1,700 s⁻¹, 1,750 s⁻¹, 1,800 s⁻¹, 1,950 s⁻¹, 2,000 s⁻¹, 2,050 s⁻¹, 2,100 s⁻¹, 2,150 s⁻¹, 2,200 s⁻¹, 2,250 s⁻¹, 2,300 s⁻¹, 2,350 s⁻¹, 2,400 s⁻¹, 2,450 s⁻¹, 2,500 s⁻¹, 2,550 s⁻¹, 2,600 s⁻¹, 2,650 s⁻¹, 2,700 s⁻¹, 2,750 s⁻¹, 2,800 s⁻¹, 2,950 s⁻¹, or 3,000 s⁻¹.
4. The method of any one of claims 1 to 3, wherein the electric field is less than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, or 1000V/cm
5. The method of any one of claims 1 to 4, wherein the fluid is treated at a temperature less than 80 °C.
6. The method of claim 5, wherein the fluid is treated at room temperature.
7. The method of any one of claims 1 to 6, wherein the fluid is treated at atmospheric pressure.
8. The method of any one of claims 1 to 7, wherein the microorganism is a Gram-positive bacteria, a Gram-negative bacteria, or a combination thereof.
9. The method of any one of claims 1 to 8, wherein the fluid is treated with a bacteriocin.
10. The method of any one of claims 1 to 9, wherein the fluid is treated with a bacteriocin prior to application of shear stress and moderate electric field.
11. The method of any one of claims 1 to 9, wherein the fluid is treated with a bacteriocin after the application of shear stress and moderate electric field.
12. The method of any one of claims 1 to 9, wherein the bacteriocin used is nisin.

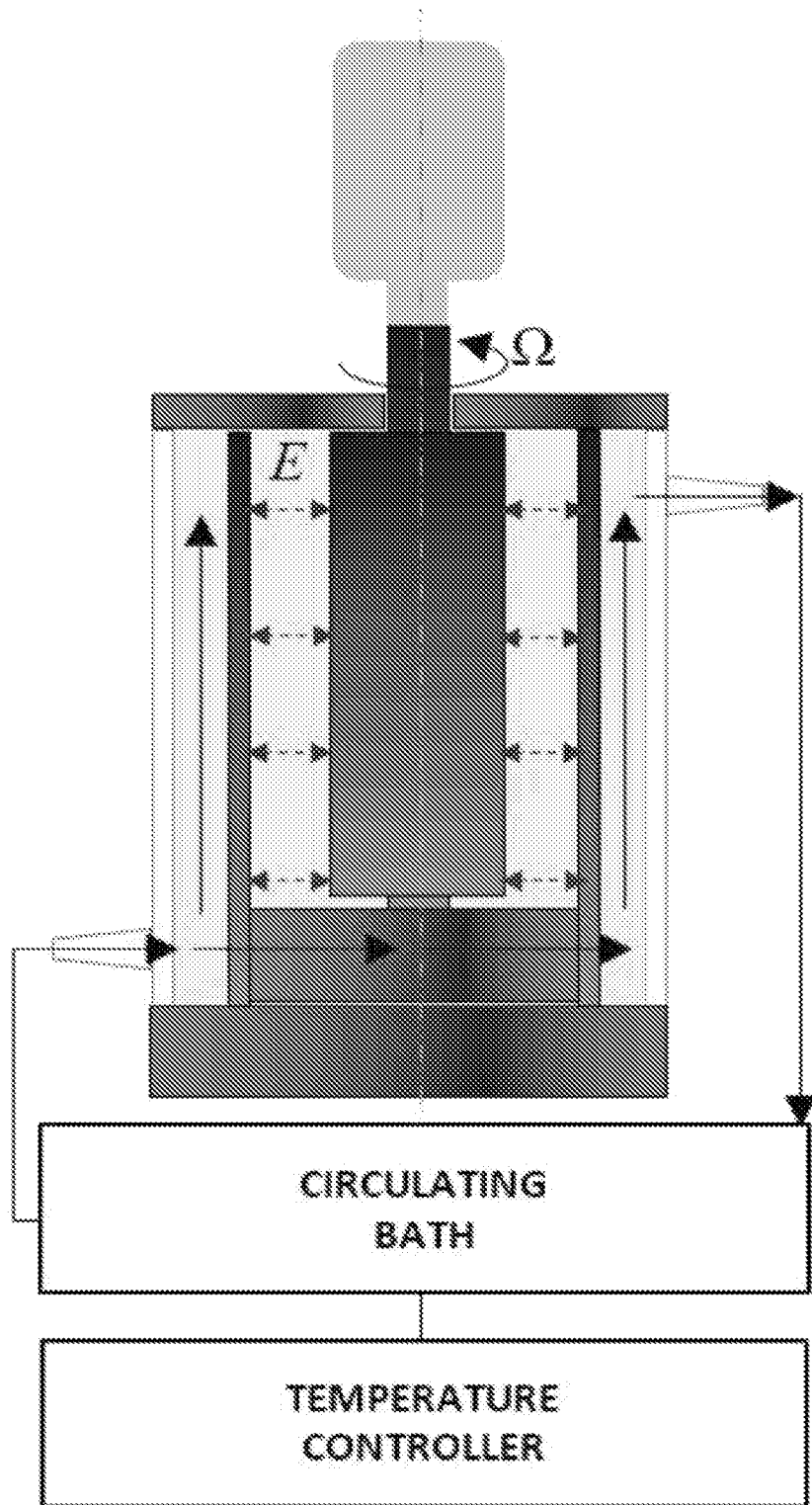


FIG. 1A

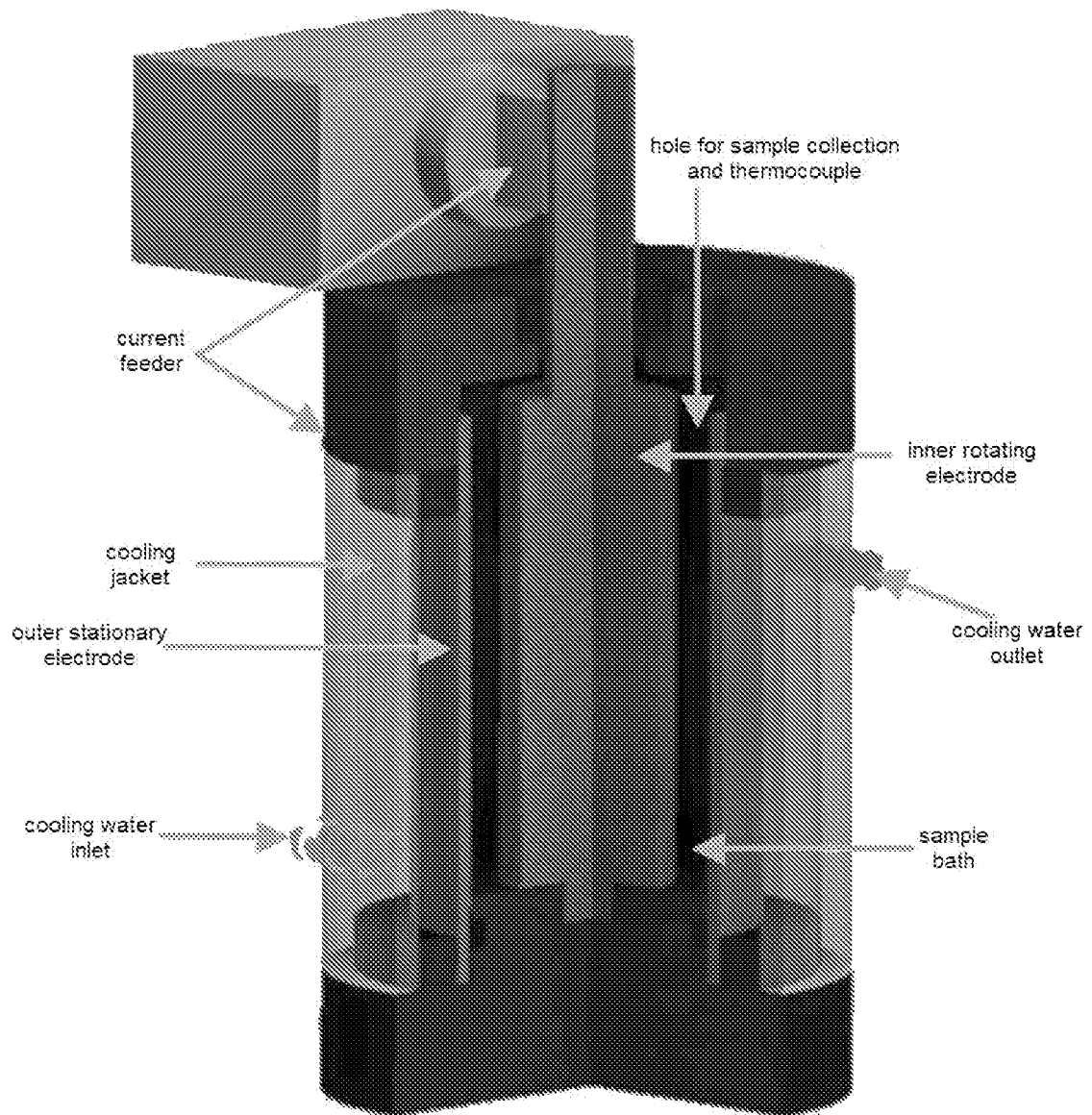


FIG. 1B

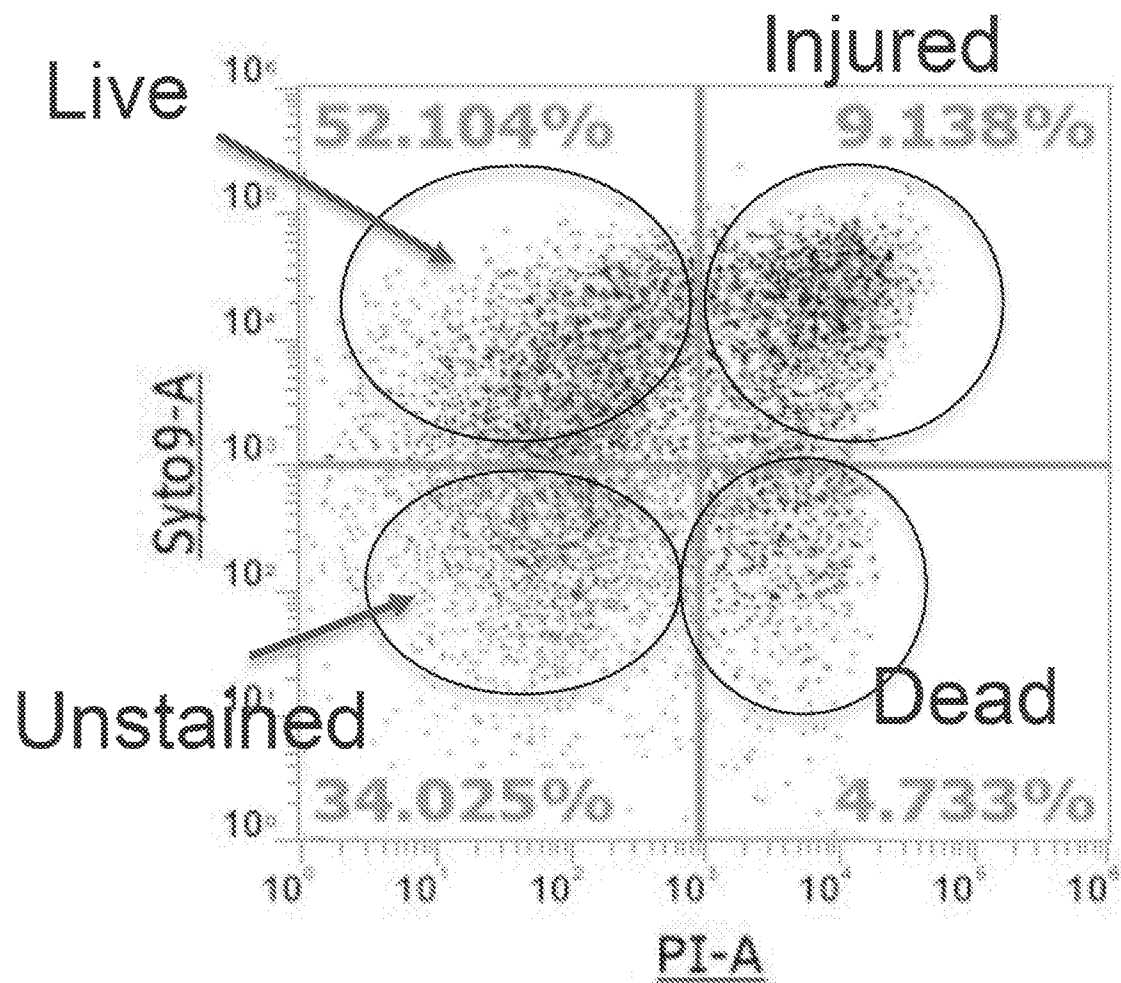


FIG. 2

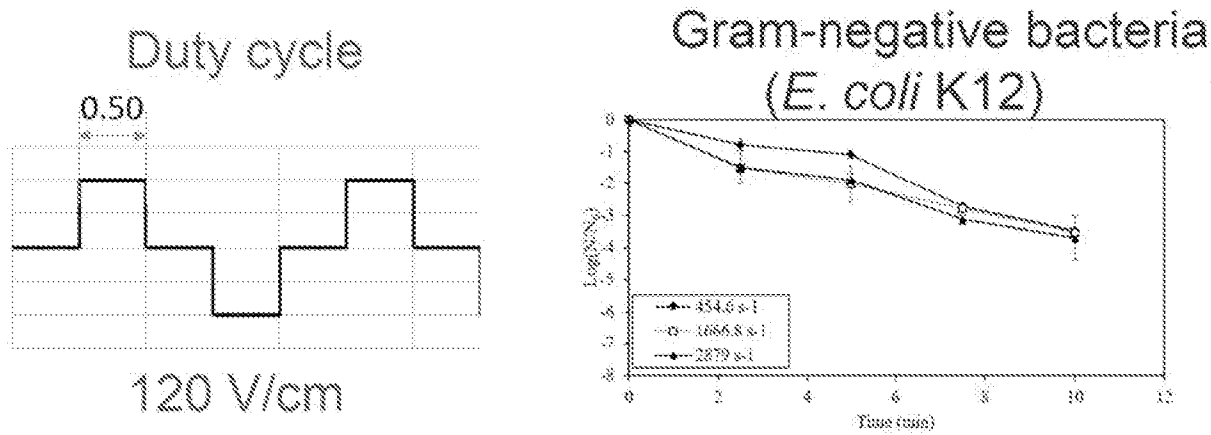


FIG. 3A

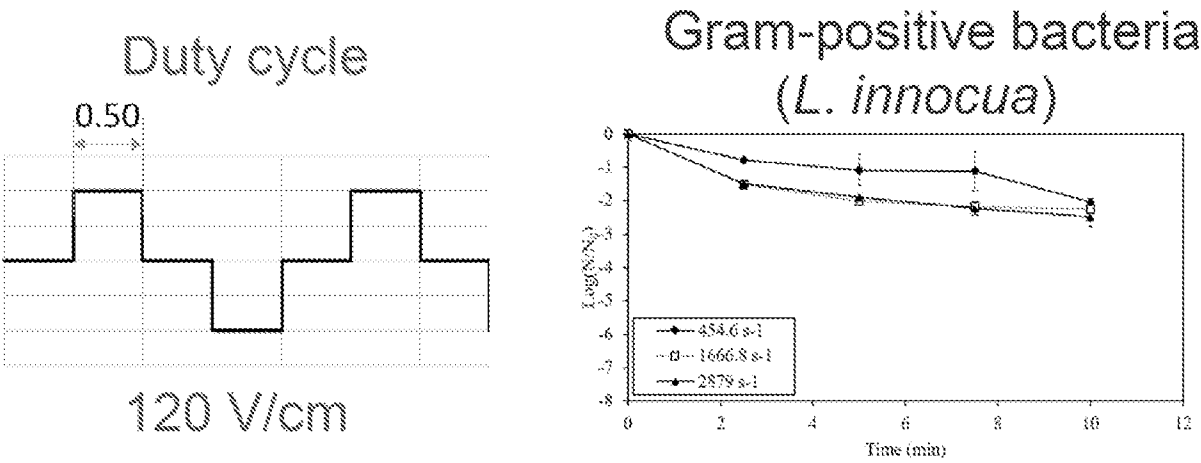


FIG. 3B

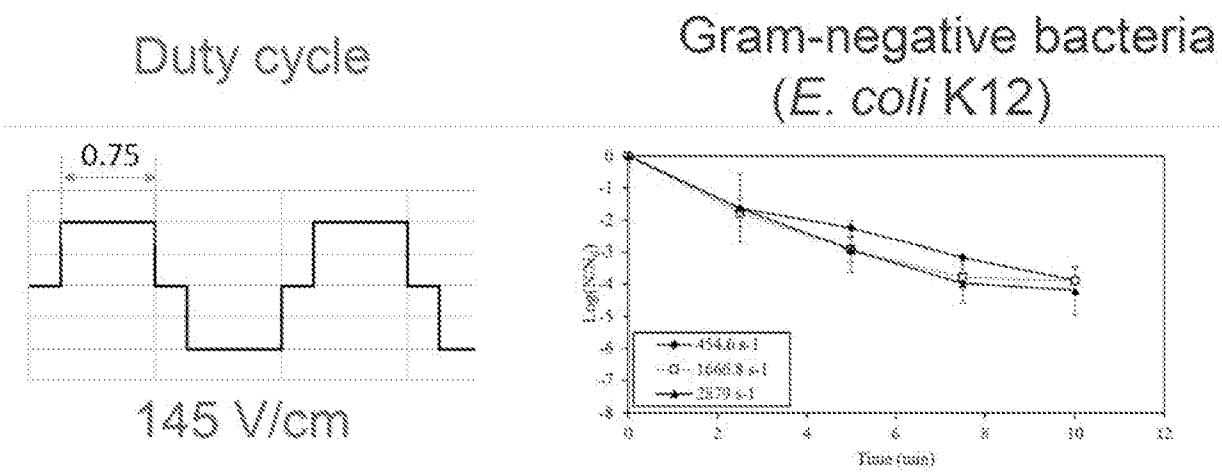


FIG. 3C

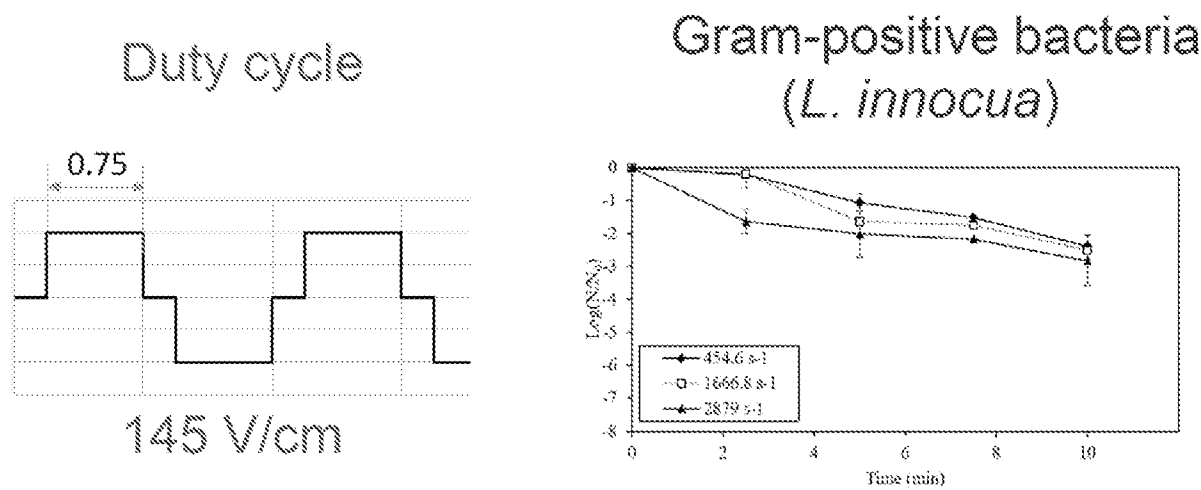


FIG. 3D

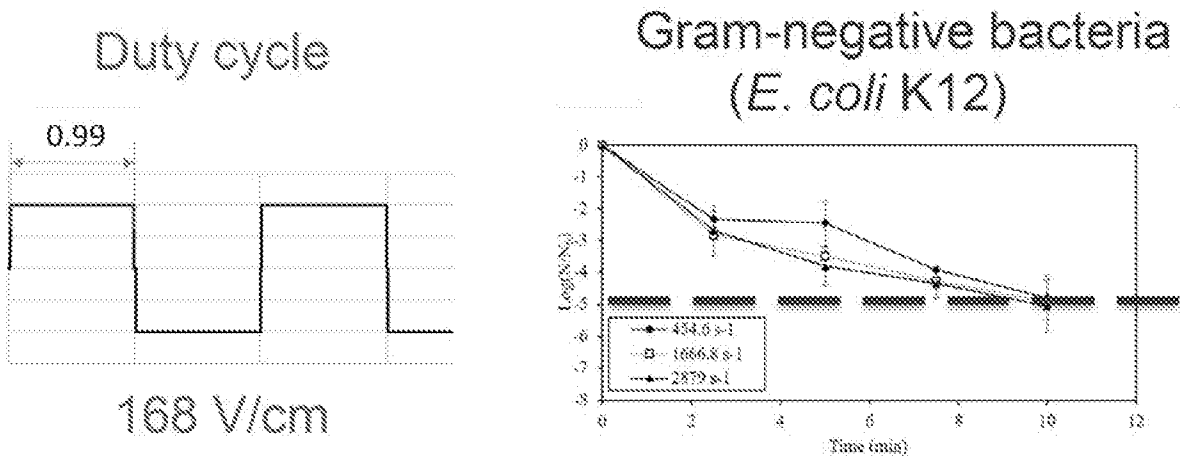


FIG. 3E

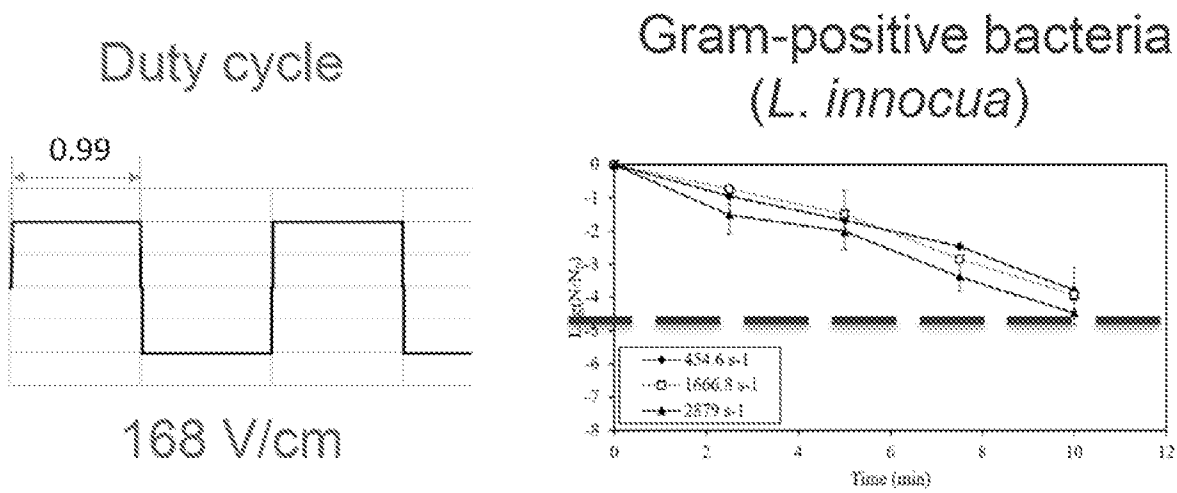


FIG. 3F

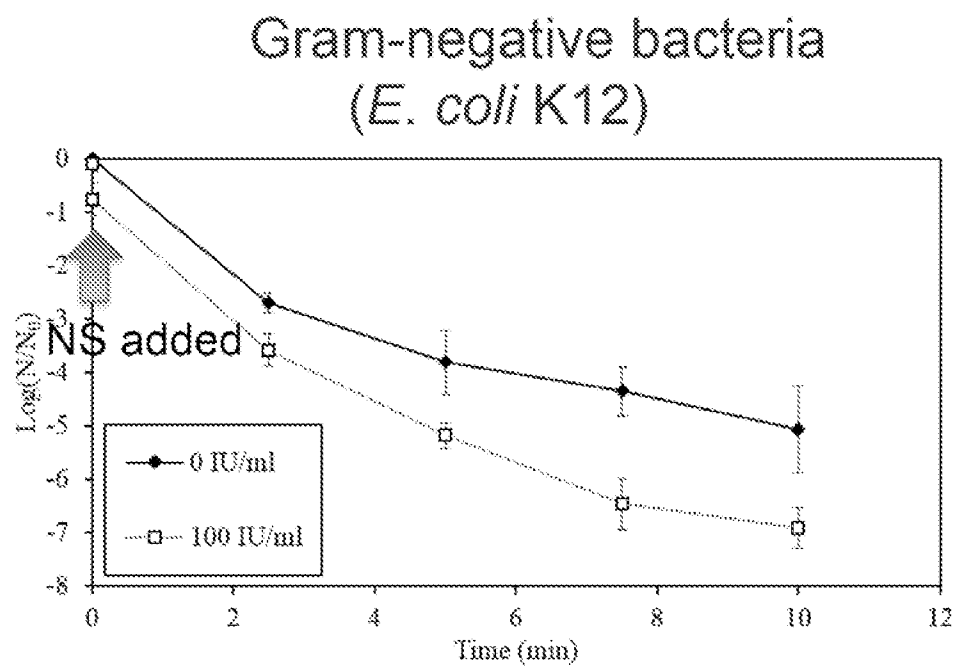


FIG. 4A

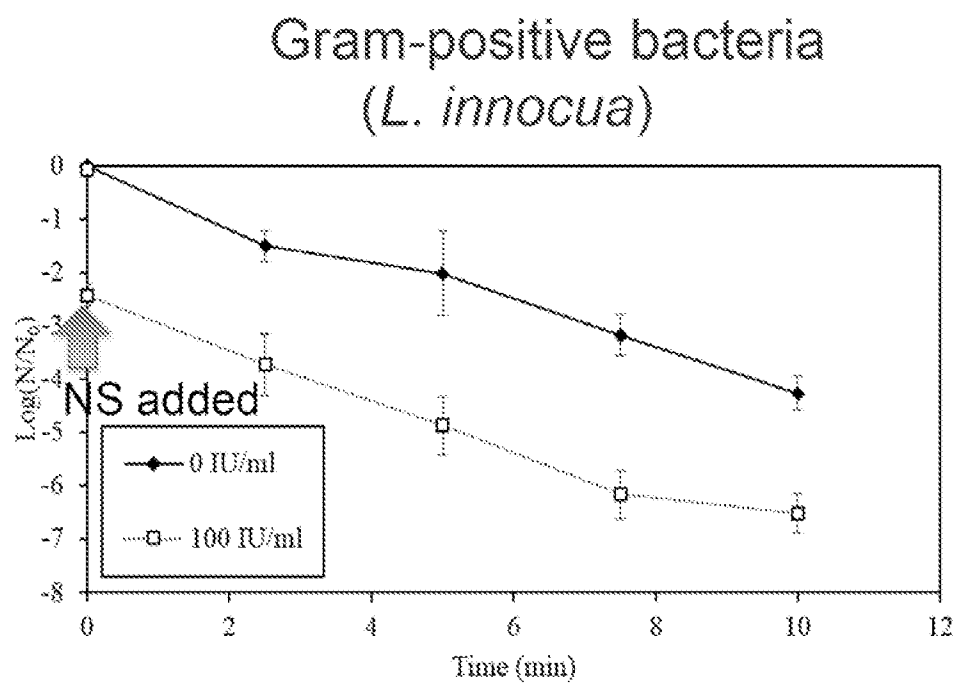


FIG. 4B

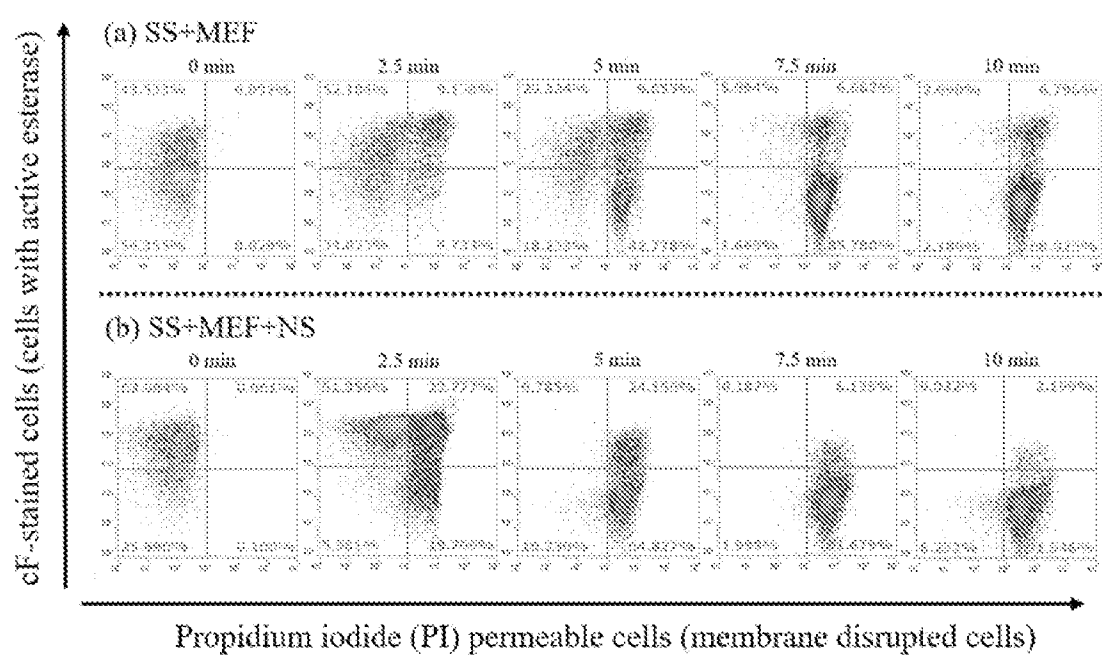


FIG. 5A

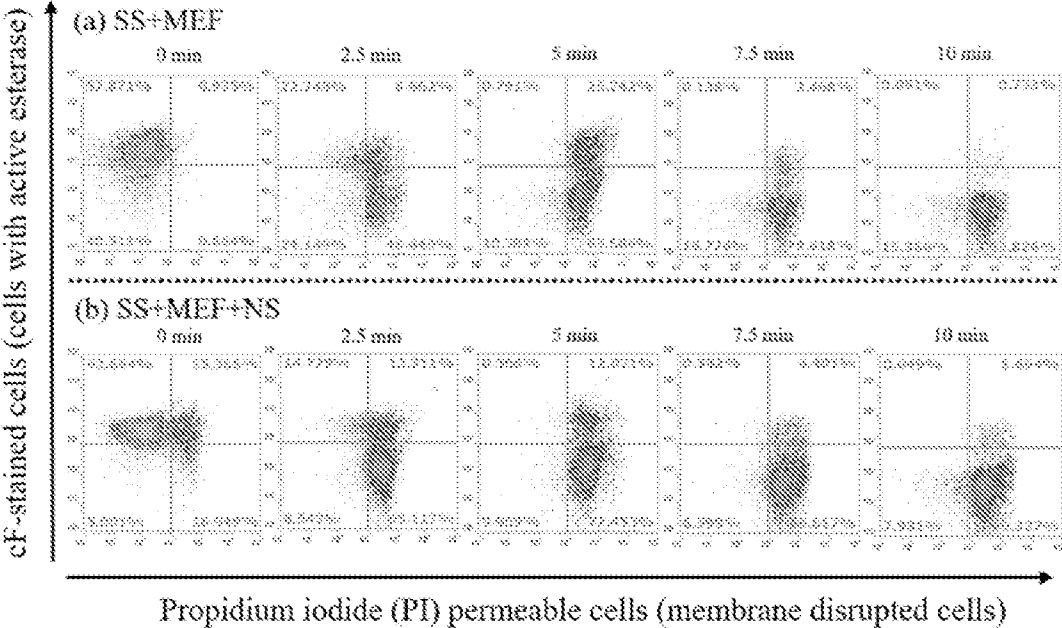


FIG. 5B

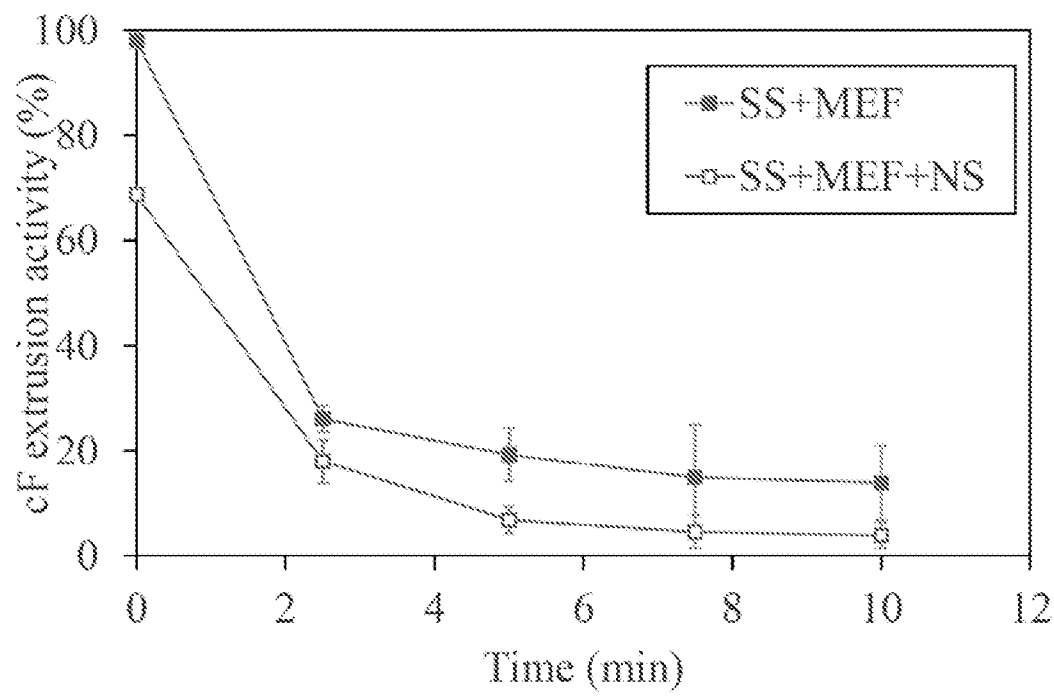


FIG. 6A

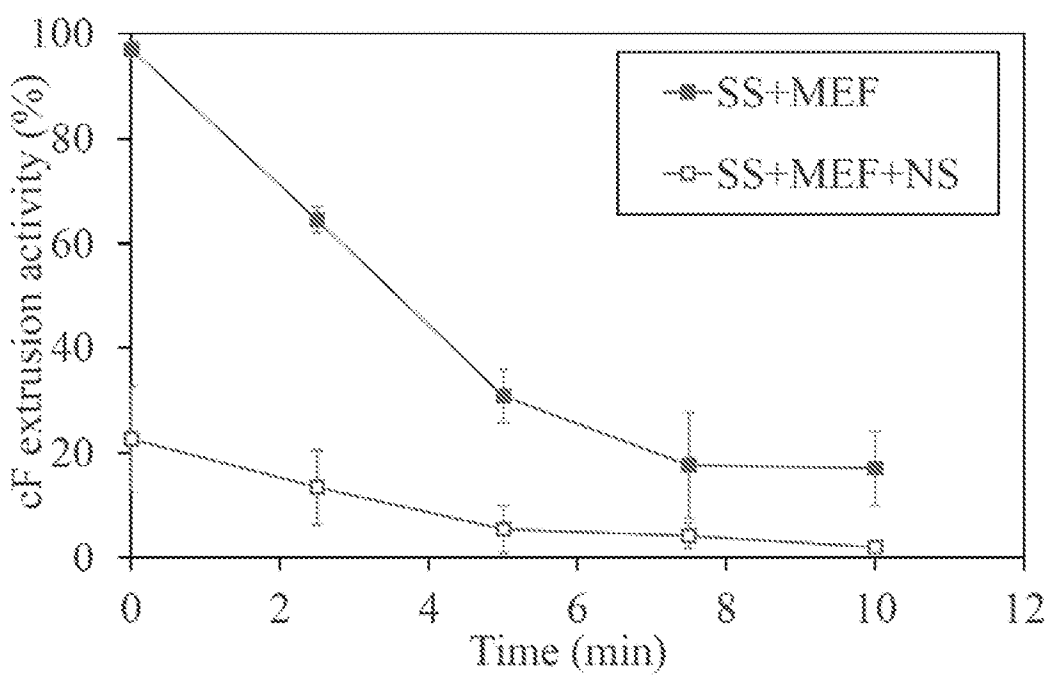


FIG. 6B

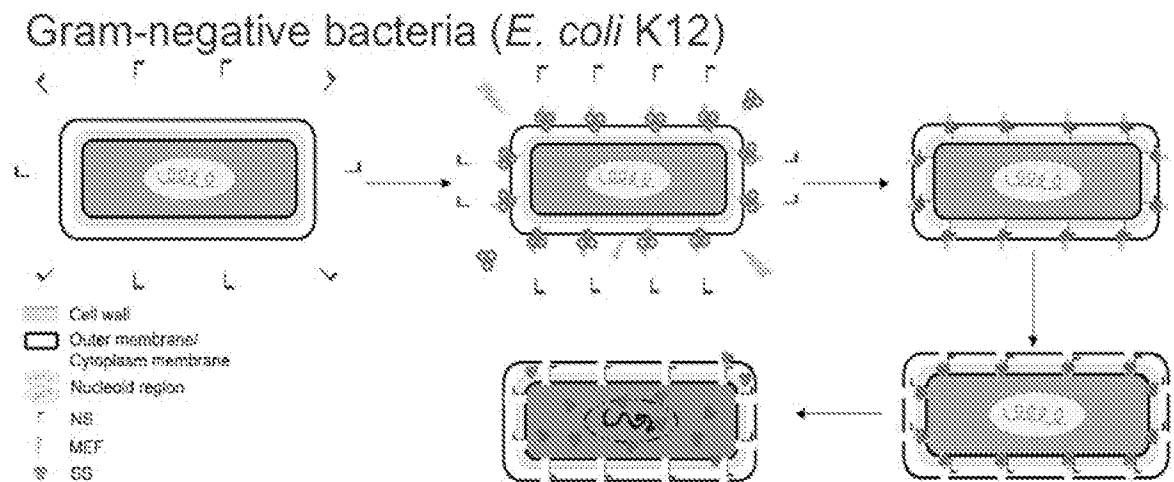


FIG. 7A

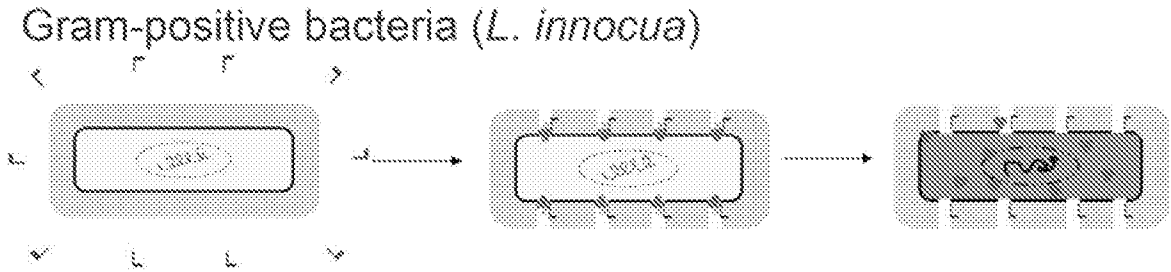


FIG. 7B

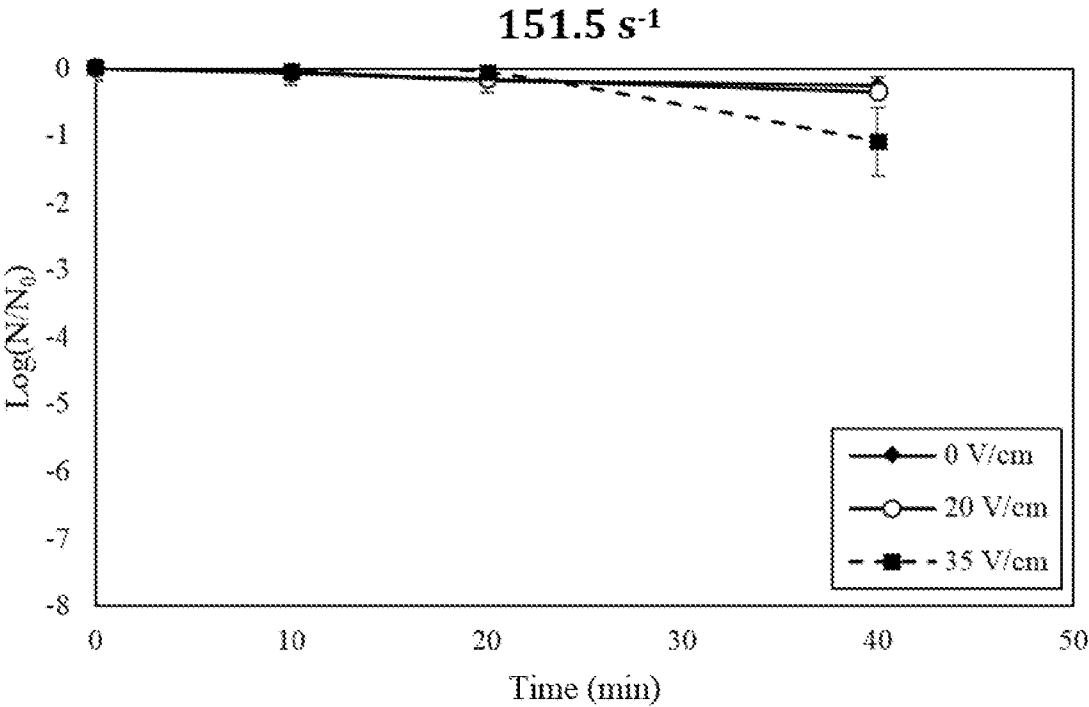


FIG. 8A

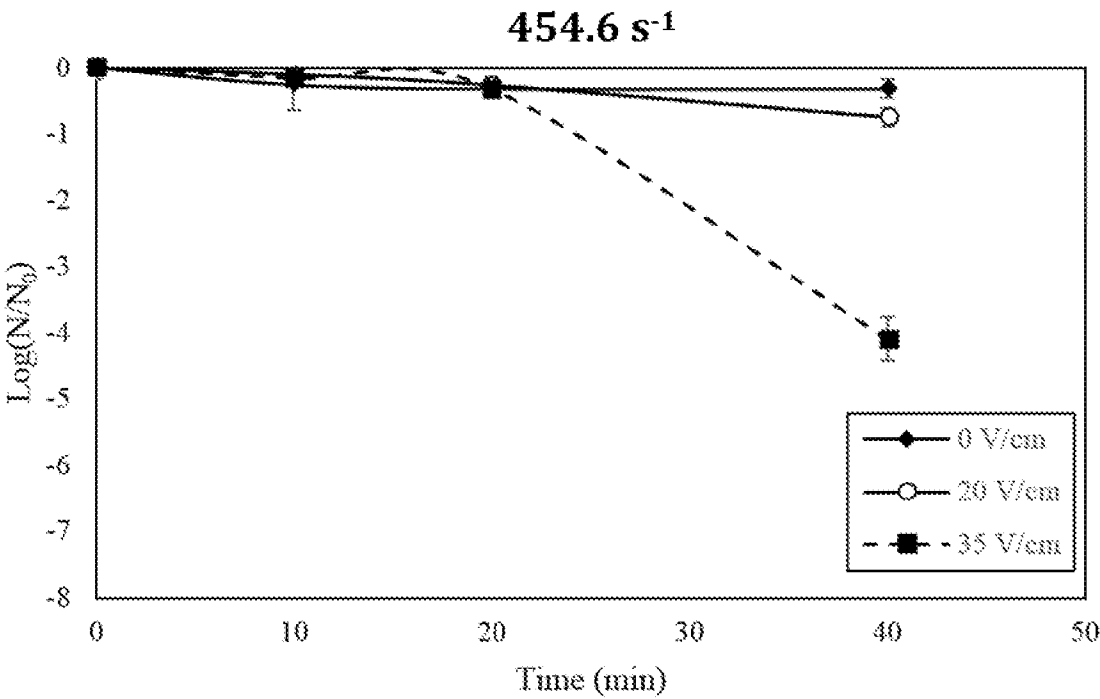


FIG. 8B

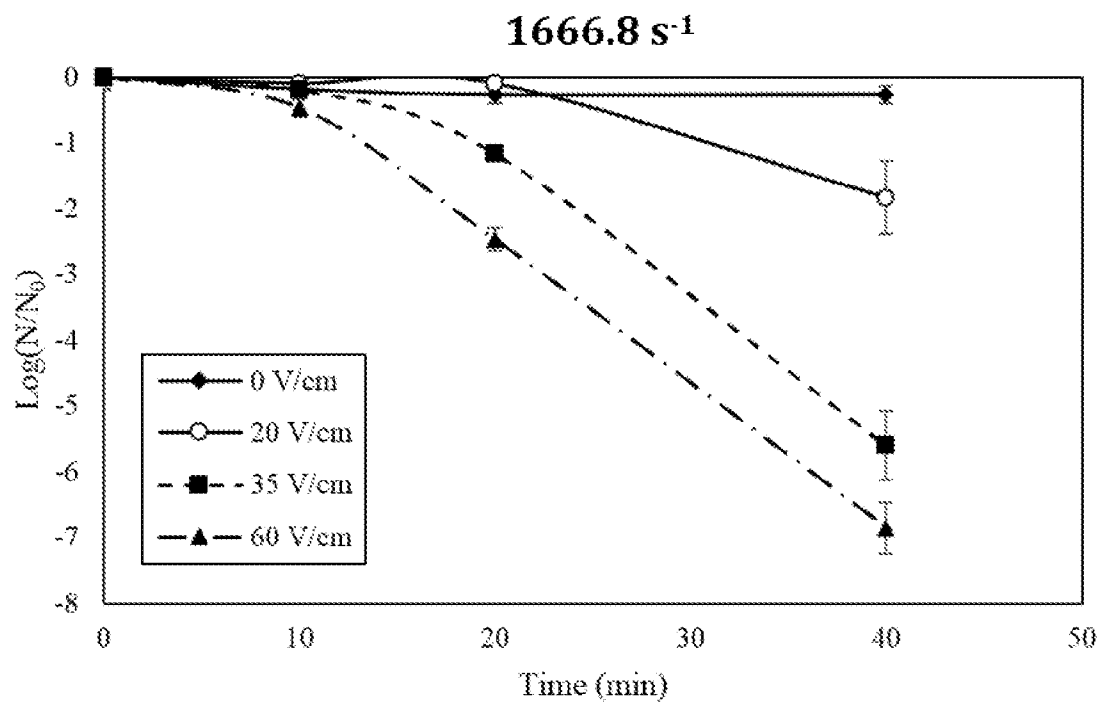


FIG. 8C

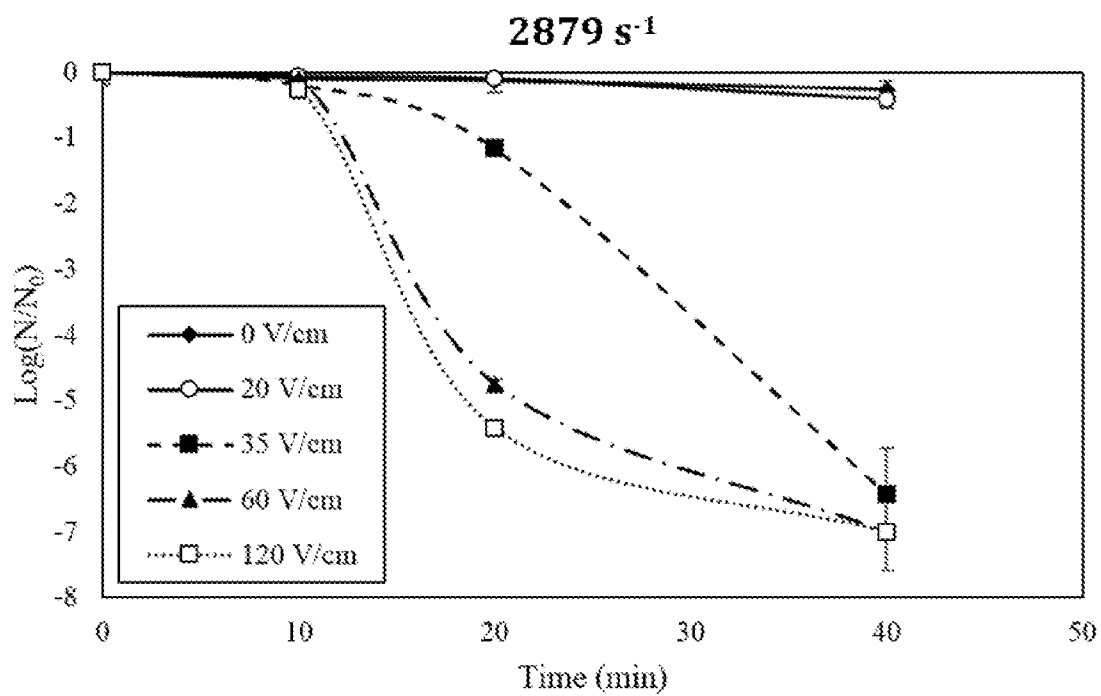


FIG. 8D

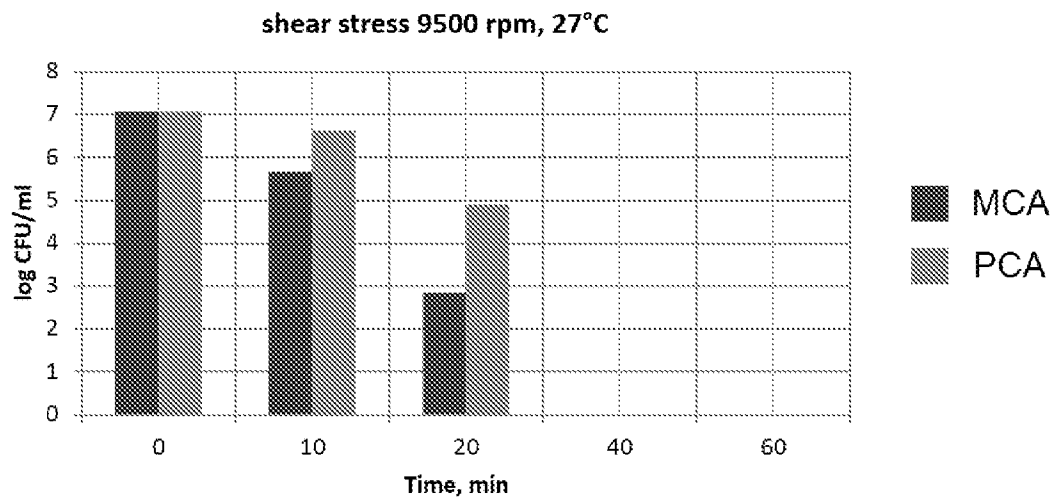


FIG. 9A

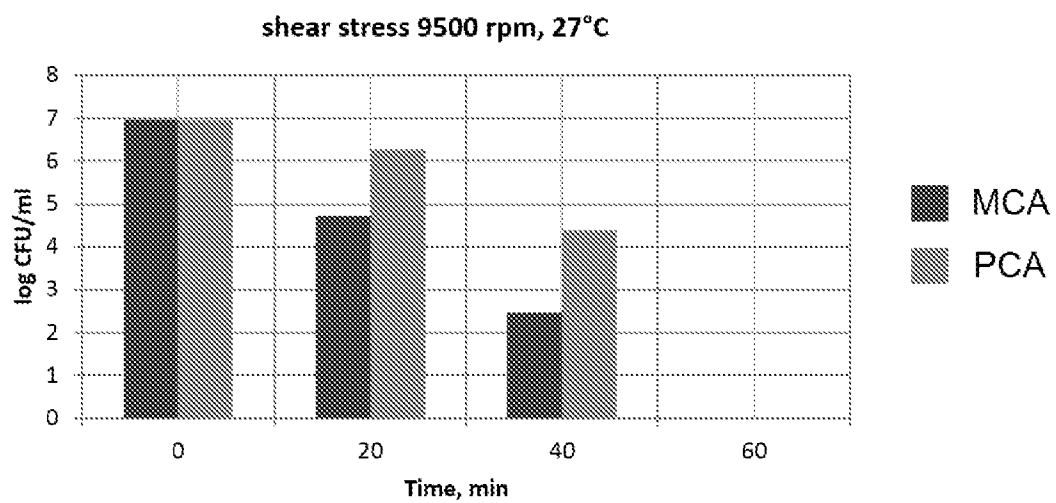


FIG. 9B

Antioxidant reduction rate

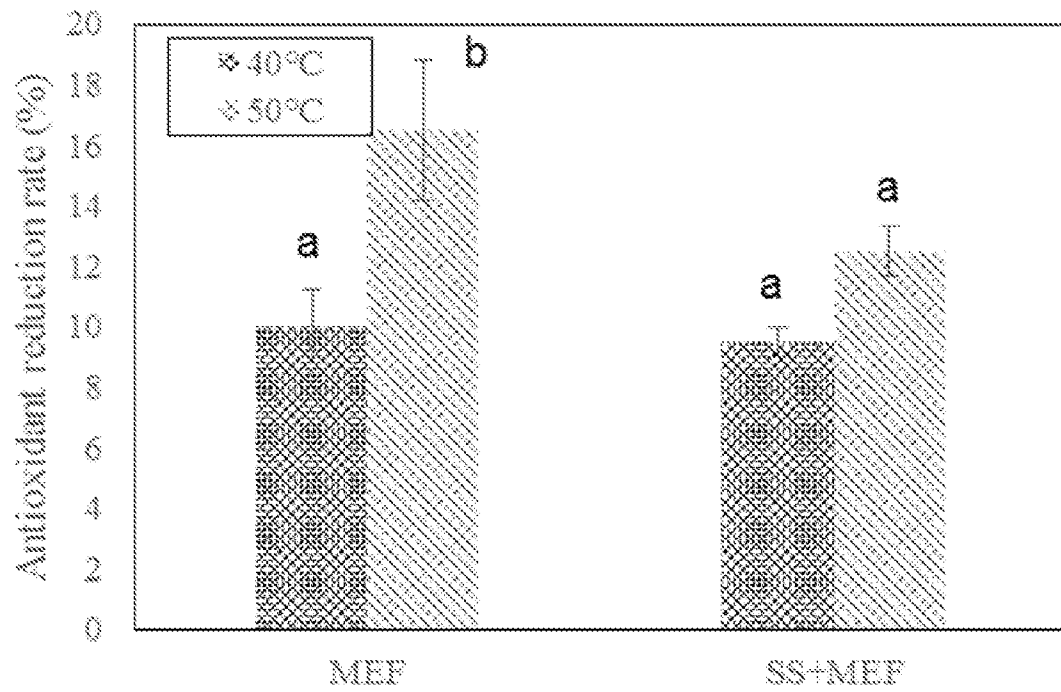


FIG. 10

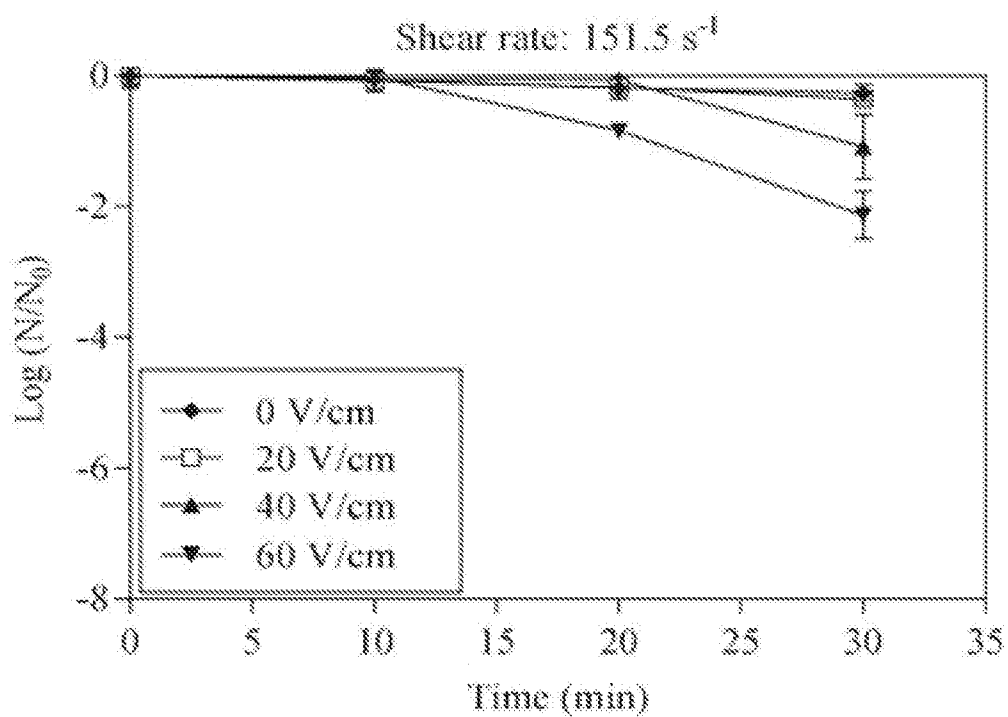


FIG. 11A

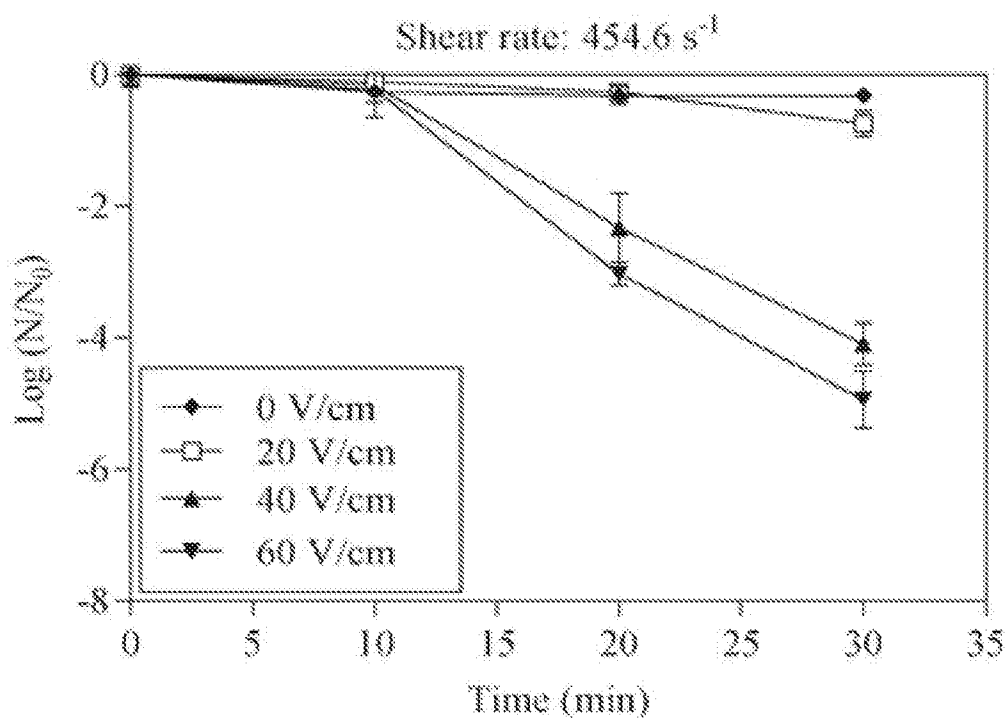


FIG. 11B

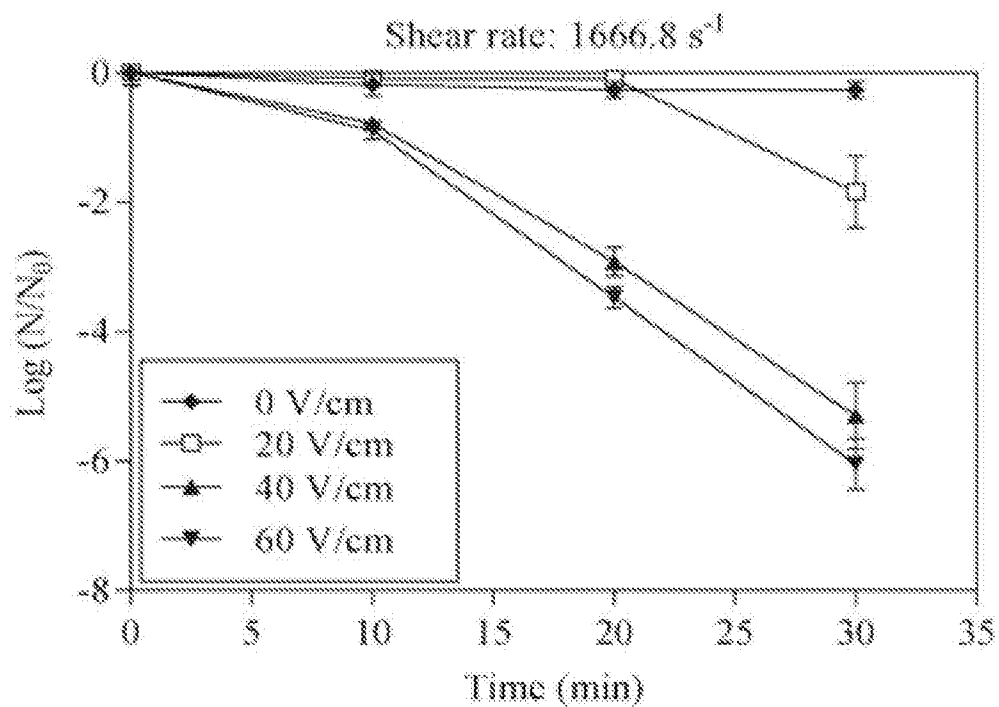


FIG. 11C

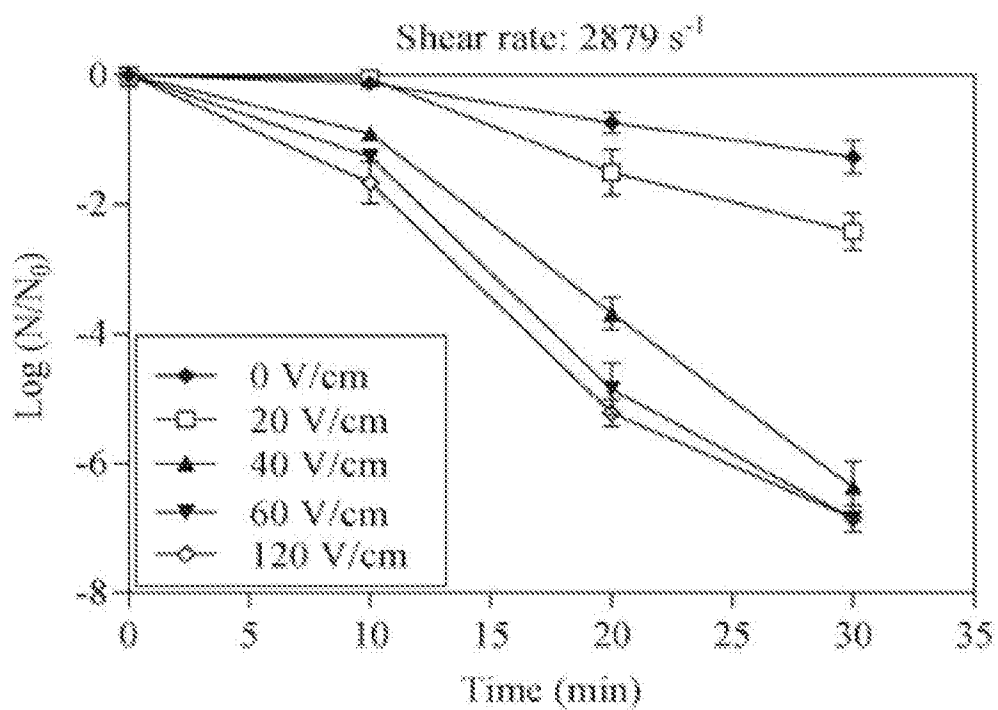


FIG. 11D

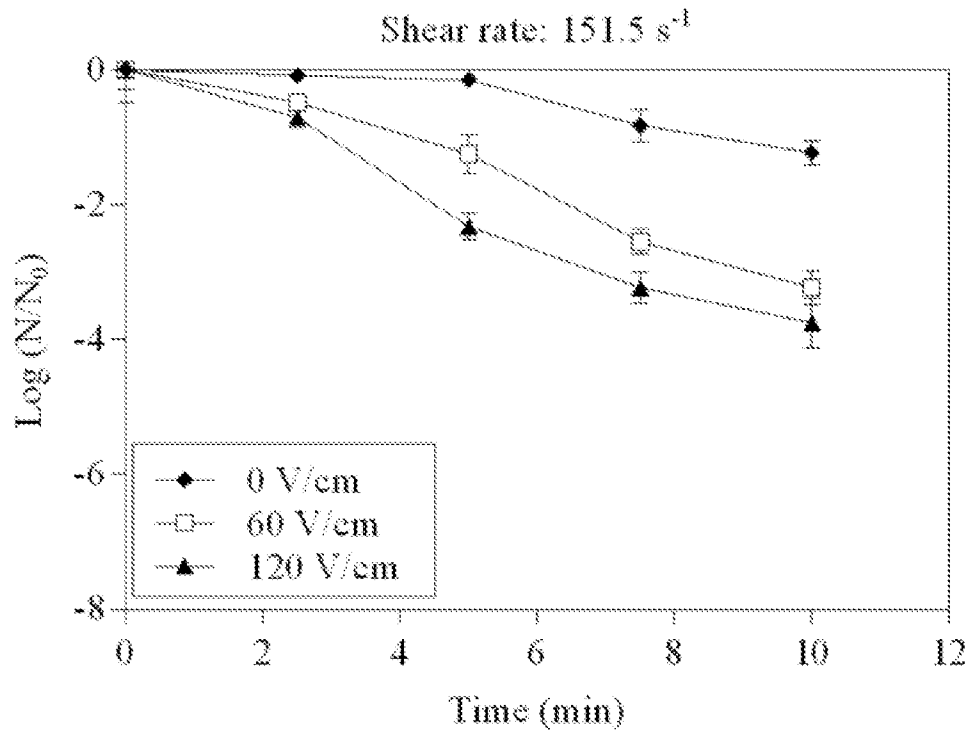


FIG. 12A

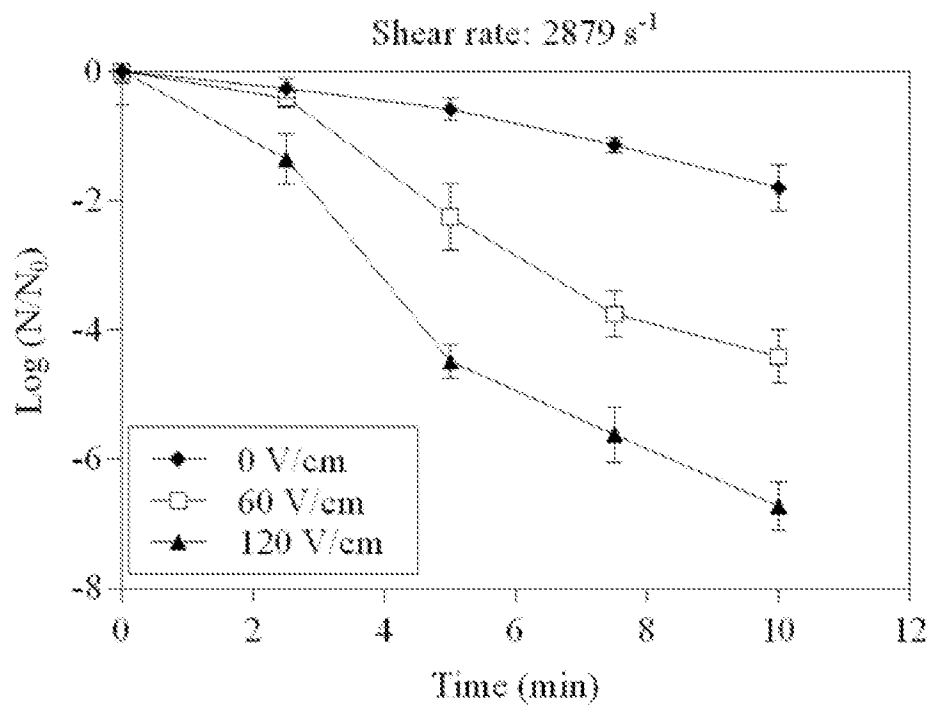


FIG. 12B

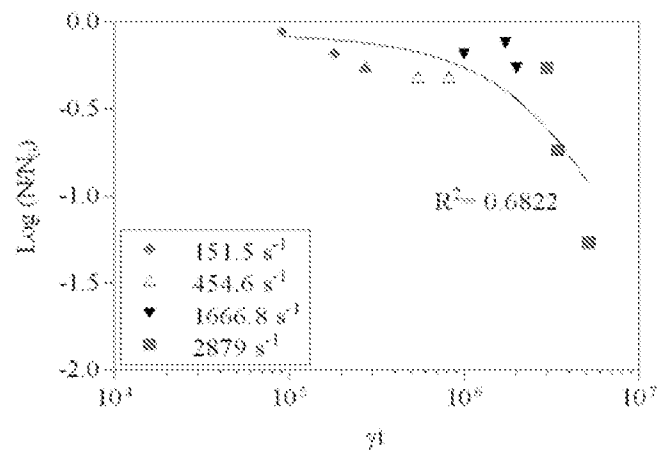


FIG. 13A

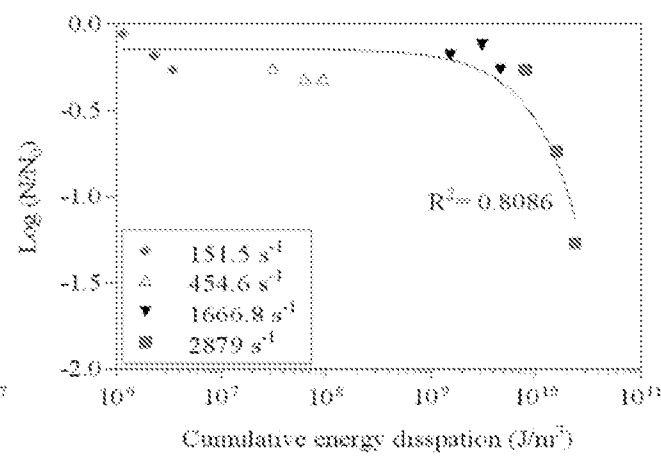


FIG. 13B

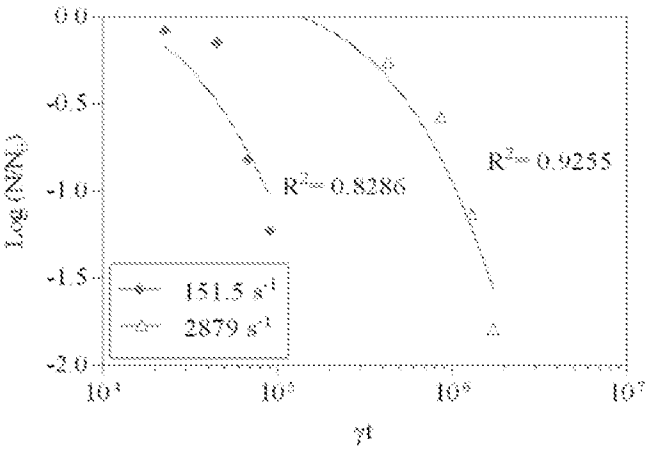


FIG. 13C

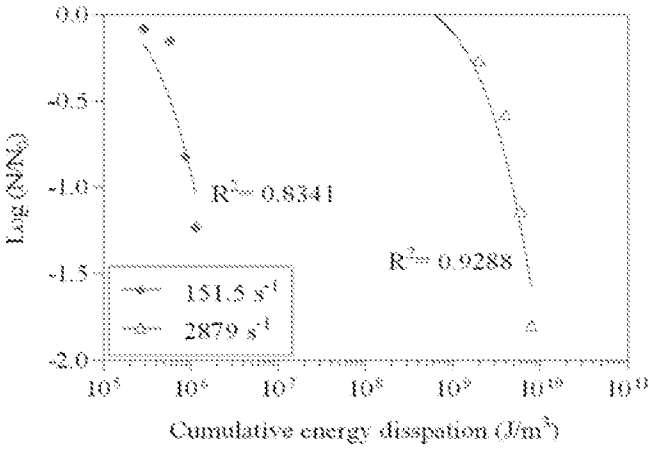


FIG. 13D

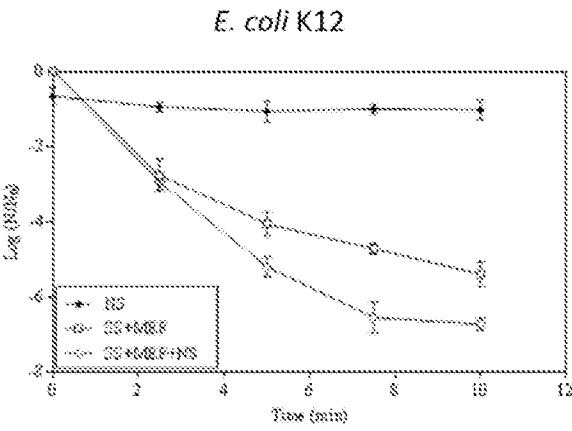


FIG. 14A

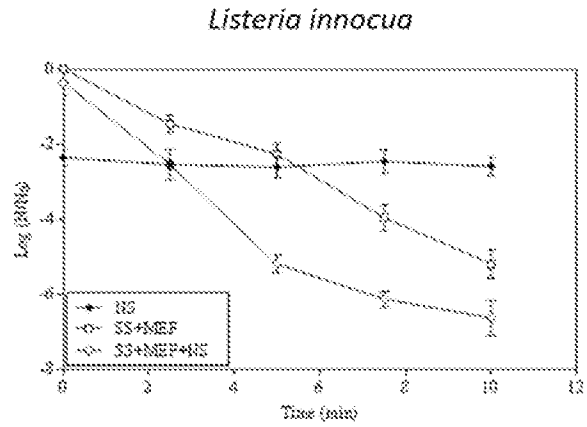


FIG. 14B

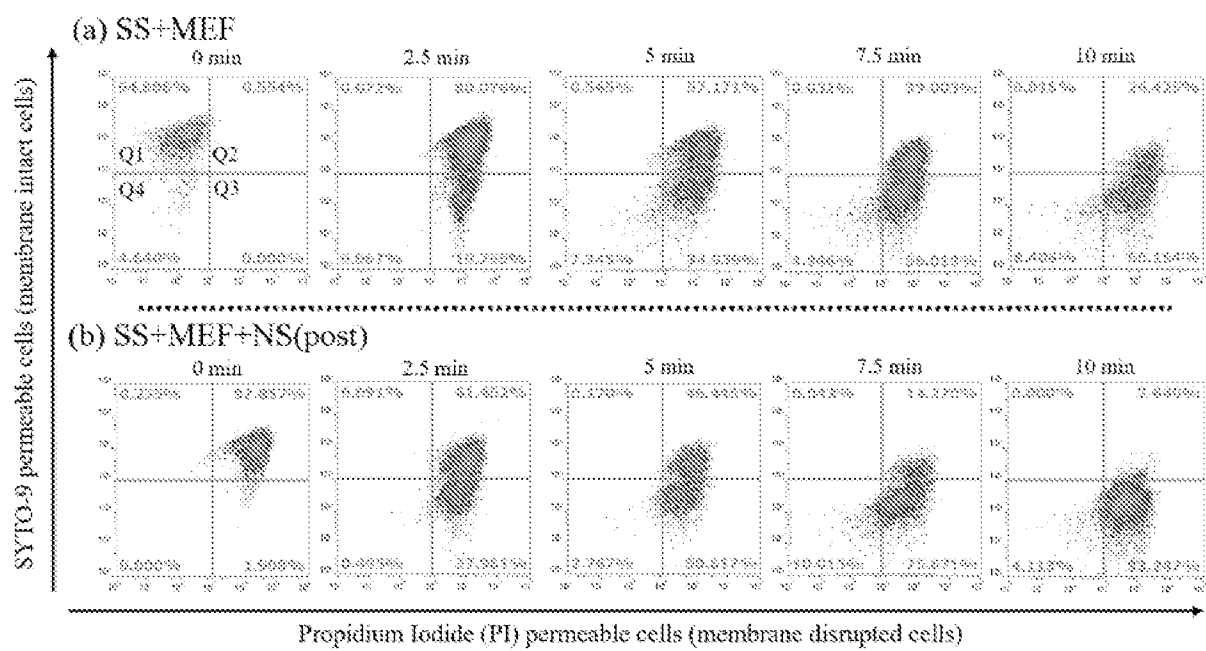


FIG. 14C

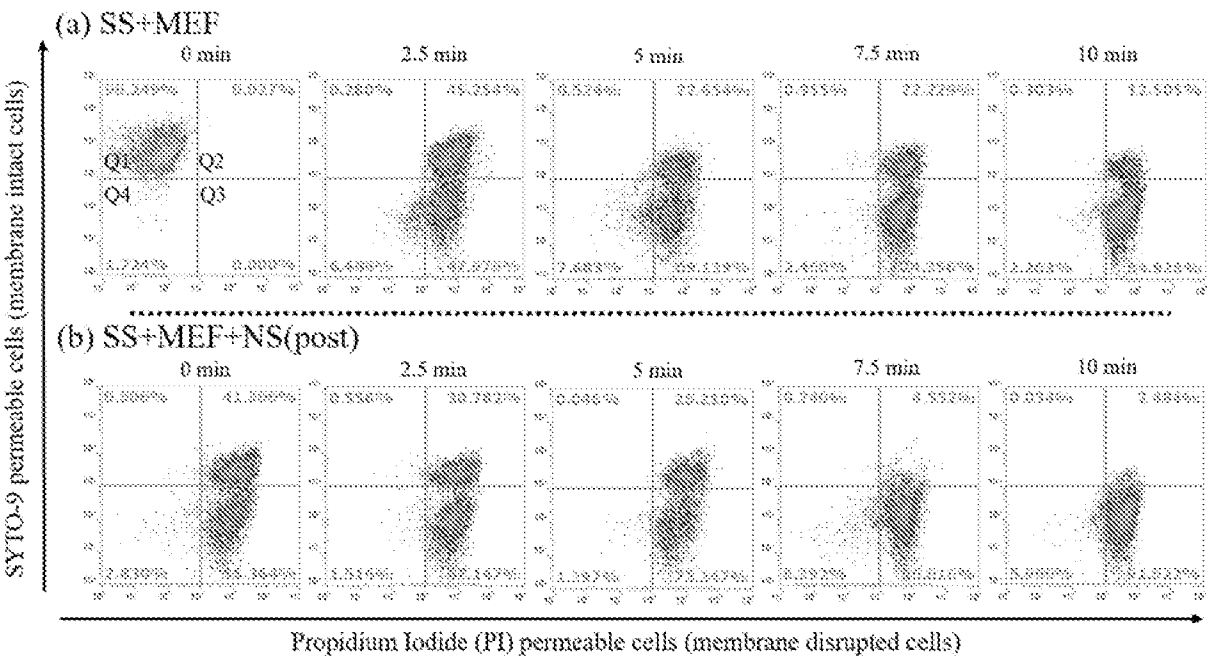


FIG. 14D

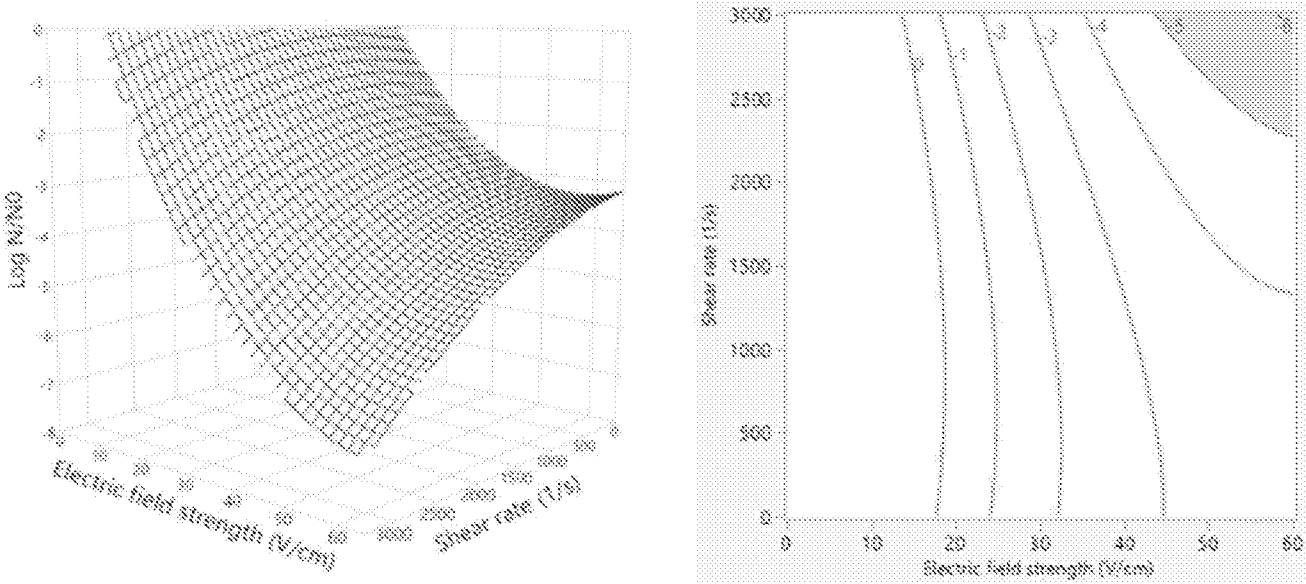


FIG. 15A

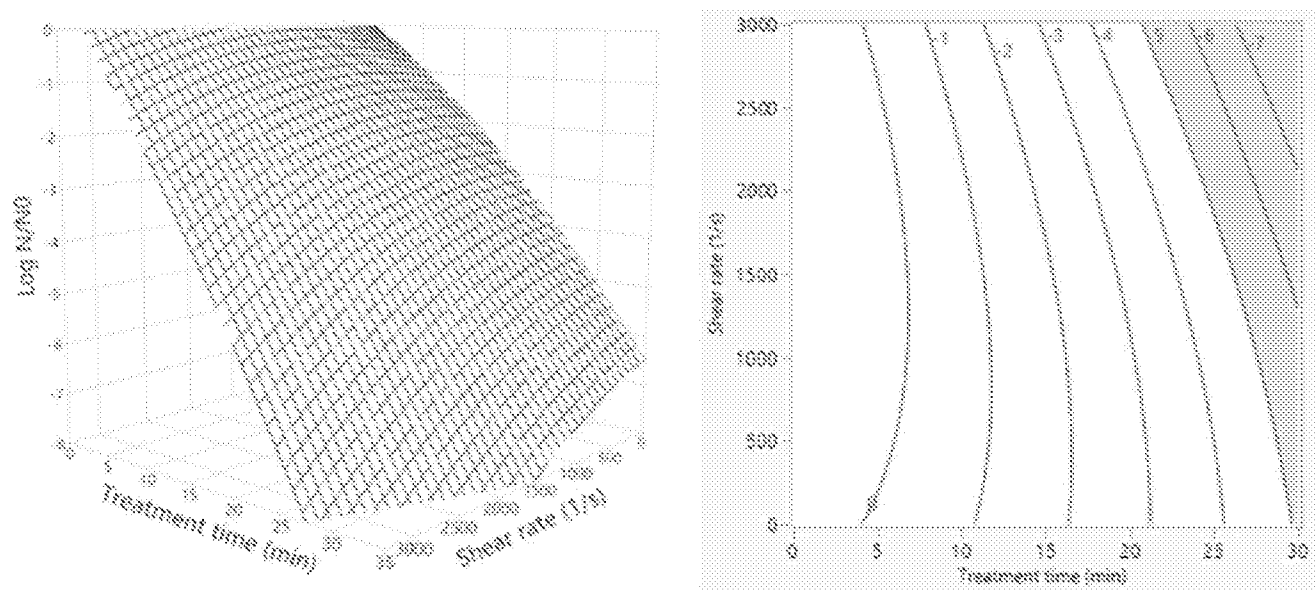


FIG. 15B

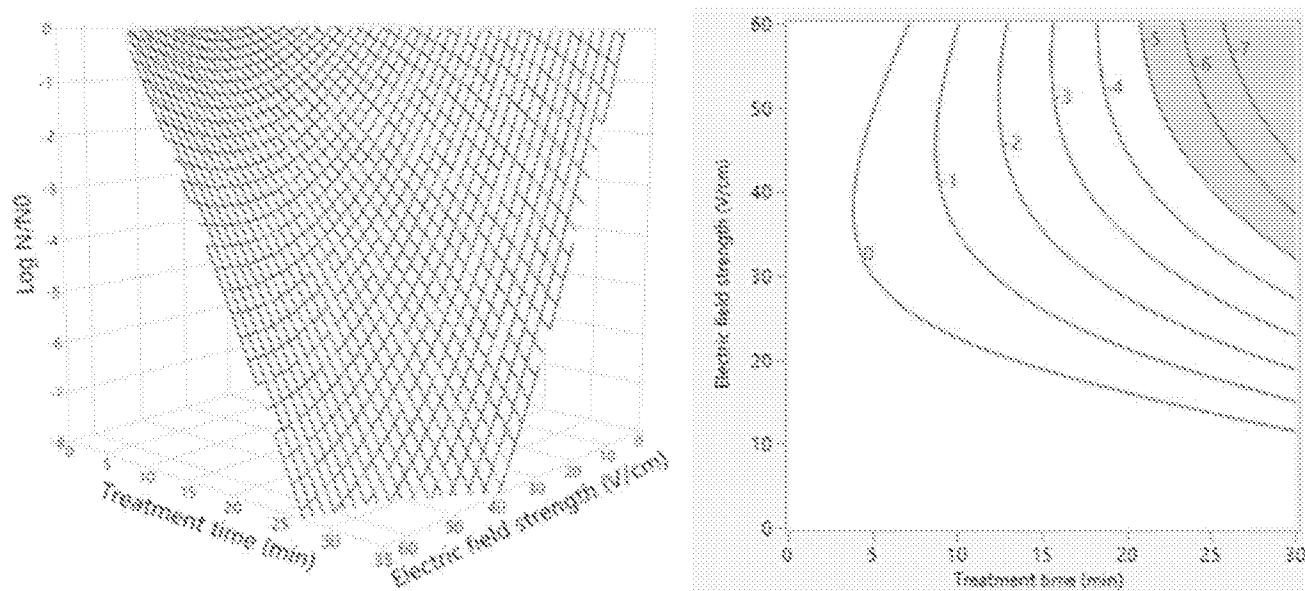


FIG. 15C

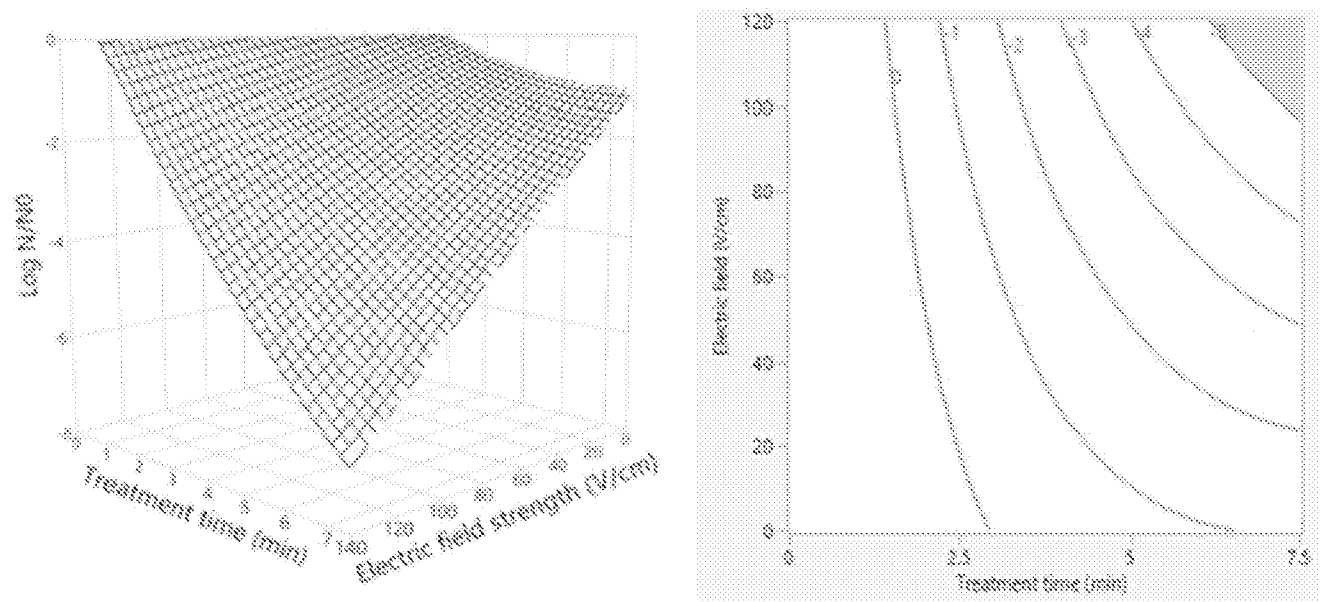


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/41102

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A23L 3/015, 3/32, 3/54 (2019.01)

CPC - A23L 3/015, 3/32, 3/54

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 ---	US 2016/0298104 A1 (DECANT TREATMENT SYESTEMS, LLC) 13 October 2016; paragraphs [0024], [0048], [0053]-[0054]	1 ---
Y	US 6,331,321 B1 (ROBBINS, JA) 18 December 2001; column 3, lines 4-19 and 43-58	2, 3/1-2
Y	(BULUT, S et al.) Effects of Combined Shear and Thermal Forces on Destruction of Microbacterium lacticum. Applied and Environmental Microbiology. October 1999, Vol. 65, No. 10, pp. 4464-4469; abstract; page 4468, first column, fourth paragraph	2, 3/2
Y	(BULUT, S et al.) Effects of Combined Shear and Thermal Forces on Destruction of Microbacterium lacticum. Applied and Environmental Microbiology. October 1999, Vol. 65, No. 10, pp. 4464-4469; abstract; page 4468, first column, fourth paragraph	3/1-2
A	(MACHADO, LF et al.) Moderate electric fields can inactivate Escherichia coli at room temperature. Journal of Food Engineering. 2009, Vol. 96, pp. 520-527; enitre document	1-2, 3/1-2

☐ 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

"D" document cited by the applicant in the international application

"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

27 August 2019 (27.08.2019)

Date of mailing of the international search report

17 SEP 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

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/41102

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.: 4-12
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.