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Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.

METHOD FOR THE BIOLOGICAL CONTROL OF PSEUDOMONAS

5 The present invention relates to a novel method for the biological control of the presence of *Pseudomonas*, and the proliferation thereof.

10 *Pseudomonas* is a Gram-negative bacterium belonging to the family *Pseudomonadaceae*. In humans, this bacterium is responsible for various skin, visceral and pulmonary infections, in particular cystic fibroses (4, 19). These bacteria are capable of resisting numerous antiseptics and antibiotics (2) (7), which doubtless partly explains their increasingly frequent presence in hospitals, where they can be isolated from the moist environment (sinks, U-bends, vases, towels and washing objects, containers containing water, etc.). Some species also have a pathogenic power toward plants (8), nematodes (10) and amoebae (1, 11, 15 16). Thus, the monitoring and the control of this bacterium constitute an increasingly important preoccupation.

Generally, it is known that, in the environment, *Pseudomonas* has a ubiquitous distribution (5), since this bacterium has been isolated from the soil, from sewage 20 or from industrial wastewater, and biofilms, characteristics that it shares with free-living amoebae. Several potentially pathogenic bacteria (*Legionella pneumophila*, *Mycobacterium spp.* and *Escherichia coli* O157:H7) have developed mechanisms for surviving and replicating inside free-living amoebae (15). Furthermore, it has been demonstrated that bacteria, including *Pseudomonas*, can develop various 25 strategies allowing them to evade predation by free-living amoebae (12, 13, 18). In particular, biofilm formation by *Pseudomonas aeruginosa* is one of the mechanisms which allows the bacterium to effectively evade predation by free-living amoebae such as *Acanthamoeba polyphaga* (18). Although it is known that certain free-living amoebae such as *Acanthamoeba* are capable of developing a chemotactic response toward *Pseudomonas* and of feeding on these bacteria (17, 30 18), it is also been demonstrated that *Pseudomonas aeruginosa* rapidly inhibits the growth of these amoebae and induces their encystment and their death by secreting toxins (11-13, 17, 18). The toxic effect of *Pseudomonas* has also been demonstrated on ciliated protozoa (9).

35 It therefore clearly appears that free-living protozoa and amoebae constitute an important element of the ecology of *Pseudomonas*. Furthermore, the capacity of *Pseudomonas* to infect and survive intracellularly in protozoa is a powerful

indicator that these protozoa are factors which promote the resistance of *Pseudomonas* to the biocidal treatments currently used, as indicated by Michel et al. (14).

5 In this context, the inventors have demonstrated, totally unexpectedly, that the amoebic genus *Willaertia magna* eradicates *Pseudomonas* bacteria. This biocidal effect is added to by the already demonstrated capacity of *Willaertia magna* for predation toward other amoebic agents that may serve as a vector for *Pseudomonas* (3).

10

A subject of the present invention is therefore first of all a method for controlling the proliferation of *Pseudomonas*, which uses protozoa of the *Willaertia magna* genus. The methods in accordance with the invention do not include the treatment methods applied to the human or animal body. In the method according to the
15 invention, it is most commonly a gas or liquid stream which is treated with protozoa of the *Willaertia* genus and in particular the *Willaertia magna* species.

The method according to the invention can in particular be used in the disinfection of sanitation water or industrial water distribution networks, cooling circuits for
20 industrial plants, or air-conditioning networks. The protozoa may be directly added to the water or to the liquids circulating in the pipes or networks to be treated. It is also possible to spray them, for example in the form of an aqueous solution as an aerosol, in the industrial networks, chimneys and plants, and on the industrial surfaces, to be disinfected.

25

Advantageously, the protozoa used in the context of the invention correspond to the strain deposited on August 26, 2006, under number **PTA 7824** at the ATCC, or to the strain deposited on August 26, 2006, under number **PTA 7825** at the ATCC, these two strains having been deposited in the names of the Centre National de la
30 Recherche Scientifique (CNRS) [French National Center for Scientific Research] - 3 rue Michel Ange - 75794 Paris Cedex 16 / France - and the Université Lyon 1 Claude Bernard [Lyon 1 Claude Bernard University] - 43 Boulevard du 11 Novembre 1918 - 69622 Villeurbanne Cedex / France.

35 The protozoa belonging to the *Willaertia* genus corresponding to the strain deposited under number **PTA 7824** at the ATCC or to the strain deposited under number **PTA 7825** at the ATCC are an integral part of the invention. Said

deposited strains PTA 7824 and PTA 7825 are also described in the publication of PCT International application WO 2008/043969.

5 Such protozoa may therefore be used in disinfecting agents, in particular intended for eliminating *Pseudomonas* bacteria and for controlling the proliferation and contamination by *Pseudomonas*.

10 Furthermore, a subject of the invention is a disinfecting agent containing protozoa of the *Willaertia* genus, and in particular of the *Willaertia magna* species. The protozoa corresponding to the strain deposited under number PTA 7824 at the ATCC or to the strain deposited under number PTA 7825 at the ATCC will be preferred. Advantageously, the disinfecting agent according to the invention is in the form of an aqueous solution or suspension, for example in distilled water. The disinfecting agent may be in a sprayable form, for example as an aerosol or any
15 other means of application.

The *Pseudomonas* proliferation-inhibiting activity of the protozoa of the *Willaertia* genus, and in particular of the *Willaertia magna* species, has been demonstrated by the inventors by comparing the replication of *Pseudomonas* in the
20 *Acanthamoeba* and *Hartmannella* genera used as amoebic models with that observed in the *Willaertia* amoebic genus. The activity of the protozoa of the *Willaertia* genus, and in particular of the *Willaertia magna* species, is also demonstrated by demonstrating the predation effect of *Willaertia magna* on biofilms formed by *Pseudomonas aeruginosa*.

25 A subject of the invention is also the use of a disinfecting agent or of a protozoan as described above, as a biocide on *Pseudomonas*.

30 Given the essential role played by amoebae in the proliferation and maintenance of *Pseudomonas* in the external environment, the method and the disinfecting agent according to the invention have numerous advantages, in terms of cost, of effectiveness and of environmental friendliness, in particular.

35 The examples hereinafter make it possible to illustrate the invention but have no limiting nature.

Figure 1 shows the spontaneous evolution of the respective populations of *Hartmannella vermiformis* ("square" symbol ■), *Acanthamoeba castellanii* ("diamond" symbol ◆) and *Willaertia* (*Willaertia magna* - "triangle" symbol ▲) amoebae after placing in coculture with *Pseudomonas* at an initial amoeba/bacterium ratio of 10.

The various free-living amoebae are placed in cocultures (time 0 hour=T0) with *Pseudomonas* at a ratio of 10 (10 bacteria / one amoeba) as described in the materials and methods section. Aliquots of the coculture suspensions are taken every 3 hours: i.e. T0, T0+3h, T0+6h. The percentage of live amoebae is determined by means of a trypan blue exclusion test and a microscopic observation using a Malassez cell. The data are expressed as % of live cells, negative in the trypan blue exclusion test.

Figure 2 shows the growth of *Pseudomonas* in cocultures with *Acanthamoeba* and *Hartmannella* but not with *Willaertia*.

The amoebae (5×10^4) were suspended in water and inoculated into the wells. After 1 hour, *Pseudomonas* is added to the wells so as to achieve various MOIs as indicated in panel A. The cocultures are incubated at 30°C and examined after 24 and 48 hours. **A.** Note the absence of bacterial proliferation in the well containing *Willaertia* with *Pseudomonas*. **B.** 100 µl of supernatant of the wells containing 100 bacteria / 1 amoeba and 10 bacteria / 1 amoeba (MOI 100 and MOI 10 respectively) are successively diluted (dilutions ranging from 10^{-6} to 10^{-8} with sterile deionized water) and inoculated onto TSA agar. Note the absence of development of bacterial colonies in the supernatant of the cocultures with *Willaertia magna* (W), and, conversely, the strong development of *Pseudomonas* in the presence of *Hartmannella* (H).

Figure 3 shows the compared kinetics of the development of *Pseudomonas* ("diamond" symbol ◆), obtained in coculture with various amoebic genera, including the *Willaertia* genus (*Willaertia magna* - "cross" symbol ×).

The various free-living amoebae are separately placed in cocultures (time 0 hour=T0) with *Pseudomonas* at a ratio of 10 (10 bacteria / one amoeba). Aliquots

of the coculture suspensions are taken every 3 hours: i.e. T0, T0+3h, T0+6h, and the *Pseudomonas* concentrations are determined as described in the materials and methods section. A positive control, comprising only the *Pseudomonas* bacteria in a concentration equivalent to that of the cocultures, will serve as a control for the growth of *Pseudomonas* in the medium. Note the bacterial multiplication in the cocultures with *Acanthamoeba castellanii* ("square" symbol ■) and *Hartmannella vermiformis* ("triangle" symbol ▲).

Figure 4 shows the biocidal effect of *Willaertia magna* on a biofilm formed by *Pseudomonas*.

Pseudomonas and *Willaertia magna* were inoculated onto TSA agar, and incubated at 30°C for 24h. A. The *Pseudomonas* biofilm lysis plaques form on and beyond the deposits of *Willaertia magna*, as indicated by the arrows on figure 4A. B. Front of a biofilm lysis plaque indicated by the arrow. Note, on the right, the site of deposit of *Willaertia magna* in the absence of bacterial film. Note, on the left, the layer of *Pseudomonas*.

1. MATERIALS AND METHODS

1.1. Strains used:

***Pseudomonas*:** the strain used is the CL 5210 strain (Oxoid, France).

- It is maintained on TSA (Tryptone Soya Agar) (ref PO 5012, Oxoid, France) at a rate of one subculturing per week. The strain is inoculated in broad streaks onto a TSA plate and incubated for 2 days at 30°C.

- ***Amoebae*:** the strains used belong to three different amoebic species:

- *Hartmannella vermiformis*,
- *Acanthamoeba castellanii* (ATCC 30010)
- *Willaertia magna* (strains deposited at the ATCC under Nos. PTA7824 and PTA 7825)

These three strains are cultured axenically, in the presence of 10% of fetal calf serum, on SCGYEM medium (Serum Casein Glucose Yeast Extract Medium), distributed into Falcon® tubes (3033) in a proportion of 3 ml per tube. In maintenance, the vegetative forms are subcultured every 8-9 days. For the

cocultures, 3-to-4-day subcultures are used so as to have trophozoites right in the exponential growth phase.

The SCGYEM medium is obtained as follows

5	➤	Casein (Merck 1.02244.010)	10 g
	➤	Na ₂ HPO ₄	1.325 g
	➤	KH ₂ PO ₄	0.8 g
	➤	Glucose	2.5 g
	➤	Yeast extract (Difco 0127-17-9)	5 g
10	➤	Distilled water	900 ml
	➤	Fetal calf serum	100 ml

2.5 ml of NaOH (1N), then Na₂HPO₄ and KH₂PO₄, are added to 900 ml of distilled water. The mixture is heated slightly on a hotplate, and then the casein is gradually added with magnetic stirring. After the casein has dissolved, the glucose and the yeast extract are incorporated.

After complete dissolution, the mixture is filtered successively on glass fiber (Sartorius SM 6513400), and then on a 1 µm membrane (Whatman 7190 004). The medium is then aliquoted into glass bottles. The bottles are sterilized in an autoclave for 20 minutes at 120°C. Before the definitive use and distribution of the medium, the fetal calf serum is added sterilely, under a laminar flow hood, in a proportion of 10% of the final volume.

25 **1.2. Monoamoebic coculture of *Pseudomonas***

1.2.1. Preparation of the bacterial inoculum:

A suspension of *Pseudomonas* in sterile distilled water is prepared from a 2-day culture on TSA, so as to obtain 1 Optical Density unit at 550 nm, i.e. a concentration of 10⁸ CFU (colony-forming units)/ml.

1.2.2. Carrying out monoamoebic cocultures

The cocultures are carried out in cell culture tubes (Falcon® 3033) containing 3 ml of autoclave-sterilized water. The inoculation of the tubes is carried out in a proportion of 1×10^5 amoebae/ml, from an axenic amoebic suspension canted beforehand on a Malassez hemocytometer. The infestation of the amoebae with *Pseudomonas* is carried out by fixing a *Pseudomonas* / amoeba ratio of 10, i.e. 1×10^6 bacteria / ml of incubation medium. Immediately after the infestation, the coculture tubes are centrifuged at low speed (760 g for 10 min) in order to promote contact between amoebae and bacteria. After 10 min, the tubes are resuspended manually and are incubated, in the inclined position, in an incubator at 30°C.

The fates of the amoebae and of *Pseudomonas* placed in coculture are determined in the following way:

The cocultures are monitored for 6 hours after the bacterial infestation. At each time interval (every 3 hours), the coculture tubes are sampled and examined from both the amoebic point of view and the bacterial point of view after vigorous stirring on a vortex in order to detach the amoebae from the walls. For each tube examined:

- The amoebae are counted directly on a Malassez cell.
- The *Pseudomonas* concentrations are determined by directly plating the culture medium out on TSA after 10-fold serial dilution in sterile distilled water, in Eppendorf microtubes. Each dilution is plated out in triplicate on TSA in a proportion of 100 µl per plate. The plates are then incubated at 30°C for a minimum of 48 hours. A first reading of the TSAs is carried out 24 hours after the plating out, by counting the colonies; it is followed by a second reading on the 2nd day for confirmation. The *Pseudomonas* concentrations are expressed in CFU/ml of incubation medium, taking into account the dilution factor and assuming that each colony corresponds to one bacterium initially present in the diluted suspension.

For each amoebic genus, the *Pseudomonas* growth curves are represented as a function of time.

In addition, the possible cytotoxic effect of *Pseudomonas* on the various amoebic species is determined in the following way:

- by counting the proportion of amoebae which are positive in the trypan blue exclusion test. This test is carried out under a microscope by counting, in a Malassez cell, the number of trypan blue-positive cells / number of total cells.

1.3. Effect of *Willaertia magna* on *Pseudomonas* biofilms

The *Willaertia* were deposited on the layer of *Pseudomonas* which had just been plated out on the TSA. The agars are placed at 30°C for 24 hours so as to allow the development of a bacterial film on the surface of the agar. The agars are then observed under an optical microscope (magnification × 400) in order to detect therein the formation of possible bacterial layer lysis plaques.

2. RESULTS

2. 1. *Willaertia magna* exhibits resistance to *Pseudomonas*

The effect of *Pseudomonas* on the survival of the various amoebic species tested was determined by means of a trypan blue exclusion test. Very rapidly, after placing *Acanthamoeba castellanii* in coculture with the bacterium, a major cytotoxic effect occurs in this amoebic species, with a drop of ~ 30% in the viability after 3 hours of coculture (see **figure 1**). Conversely, this phenomenon is never observed when *Willaertia magna* is placed in coculture with *Pseudomonas*, including up to 9 hours of incubation with a viability which is maintained close to 100% (**figure 1**). Like *Willaertia magna*, the free-living *Hartmanella vermiformis* amoeba does not exhibit any drop in terms of viability determined by trypan blue exclusion (**figure 1**). All of these observations (no encystment and no cytotoxicity induced by *Pseudomonas*) clearly demonstrate that *Willaertia magna* and *Hartmanella vermiformis*, contrary to the other amoebic species of the *Acanthamoeba castellanii* type, exhibit the initial ability to resist *Pseudomonas*.

2.2 Predation of *Pseudomonas* by *Willaertia magna*

The results of the *Pseudomonas* cocultures carried out in the presence of amoebae belonging to the *Hartmannella* and *Acanthamoeba* genera demonstrate a considerable multiplication of the bacterium in the presence of these two amoebic genera since an increase in the bacterial concentrations is noted in 6 hours (see **figure 2**). Conversely (although the cocultures are carried out under strictly identical conditions), a reduction of about one log in the detectable *Pseudomonas* concentrations is noted in the presence of the *Willaertia magna* amoeba, compared with the control containing only *Pseudomonas* (see **figure 2 and figure 3**). The measured drop in the *Pseudomonas* concentrations demonstrates a massive predation effect of *Willaertia magna* toward *Pseudomonas*.

Willaertia magna and *Hartmannella vermiformis* survive, but only *Willaertia magna* prevents bacterial proliferation. This effect of *Willaertia magna* on *Pseudomonas* is further illustrated in **figures 3 and 4**. After incubation for 48 hours in water, the cocultures of *Acanthamoeba* and *Hartmannella* with the bacterium demonstrate a proliferation of *Pseudomonas* (note the cloudy appearance of the wells containing *Pseudomonas* and *Hartmannella* or *Acanthamoeba*, due to bacterial proliferation) (**figure 3, panel A**). The *Pseudomonas* concentrations determined in the supernatant of the coculture wells demonstrate the absence of bacteria with *Willaertia magna* (**figure 3, panel B**) when said bacteria were incubated at an MOI of 10 (10 bacteria / one amoeba).

The predation effect of *Willaertia magna* on *Pseudomonas* is also demonstrated in **figure 4**. Indeed, after 24 hours in the presence of *Willaertia magna*, surfaces of the agar where the bacterial layer has disappeared appear very clearly (these zones are referred to here as bacterial layer/biofilm lysis plaques - **figure 4, panel A**). The microscopic examination of the agars also shows that the *Willaertia magna* are concentrated at the limit of this lysis plaque; this effect is also illustrated in **figure 4, panel B**, where the disappearance of the bacterial layer under the action of the *Willaertia magna* is clearly apparent. All of these data and observations clearly show the predation effect of *Willaertia magna* toward the pathogenic bacterium *Pseudomonas* having developed a biofilm.

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Eljárás *Pseudomonas* biológiai gátlására

SZABADALMI IGÉNYPONTOK

1. Eljárás a *Pseudomonas* terjedésének megakadályozására, emberi, vagy állati szervezetre alkalmazott kezelési eljárásoknak a kivételével, azzal jellemezve, hogy az eljárás *Willaertia magna* fajba tartozó protozoákat alkalmaz.
2. Az 1. igénypont szerinti eljárás, azzal jellemezve, hogy egy gáz- vagy folyadékáramot, vagy szilárd felszínt a *Willaertia magna* fajba tartozó protozoákkal kezelünk.
3. Az 1. vagy 2. igénypont szerinti eljárás, azzal jellemezve, hogy ezek a protozoák az ATCC-nél PTA 7824 számon letétbe helyezett törzsnek, vagy az ATCC-nél PTA 7825 számon letétbe helyezett törzsnek felelnek meg.
4. Az 1-3. igénypontok bármelyike szerinti eljárás, azzal jellemezve, hogy háztartási vizet vagy ipari vizet elosztó rendszerek, ipari üzemek hűtőkörei, vagy légkondicionáló rendszerek fertőtlenítésére fogatosítjuk.
5. Az 1-3. igénypontok bármelyike szerinti eljárás, azzal jellemezve, hogy vízvezetékben, vagy emberi, vagy állati táplálékkal kapcsolatba kerülő (vagy nem kerülő) felületeken biofilmek kialakulásának megakadályozására használjuk.
6. A *Willaertia magna* fajba tartozó protozoát tartalmazó fertőtlenítőszer alkalmazása biocidként *Pseudomonas*-ra, emberi, vagy állati szervezetre alkalmazott kezelési eljárások kivételével.
7. A 6. igénypont szerinti alkalmazás, amelyben a protozoa az ATCC-nél PTA 7824 számon letétbe helyezett törzsnek, vagy az ATCC-nél PTA 7825 számon letétbe helyezett törzsnek felel meg.
8. A 6. vagy 7. igénypont szerinti alkalmazás, amelyben a fertőtlenítőszer oldat, vagy vizes szuszpenzió formájában van.

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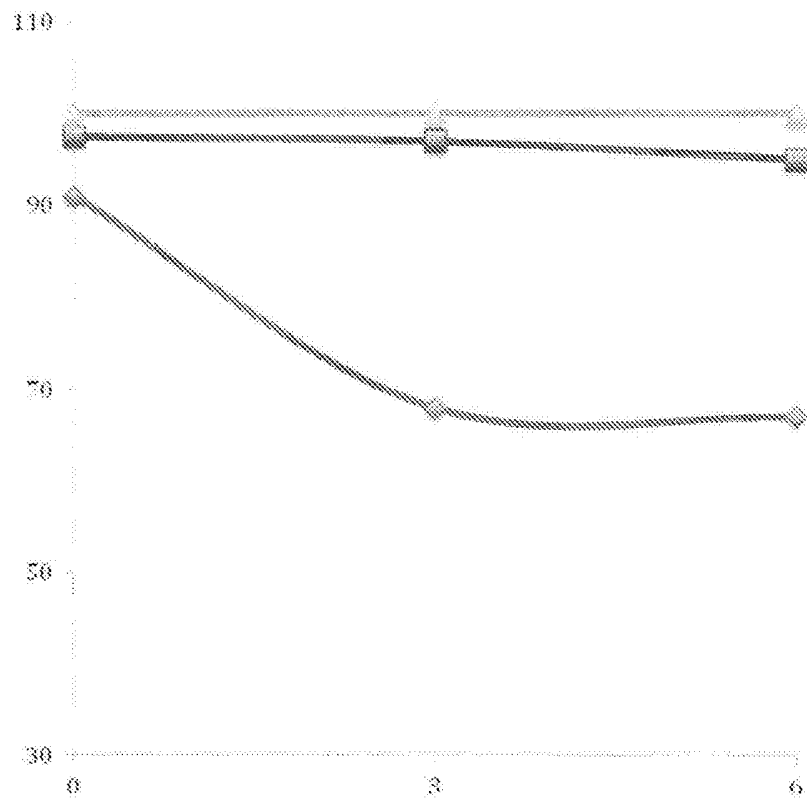


Figure 1.

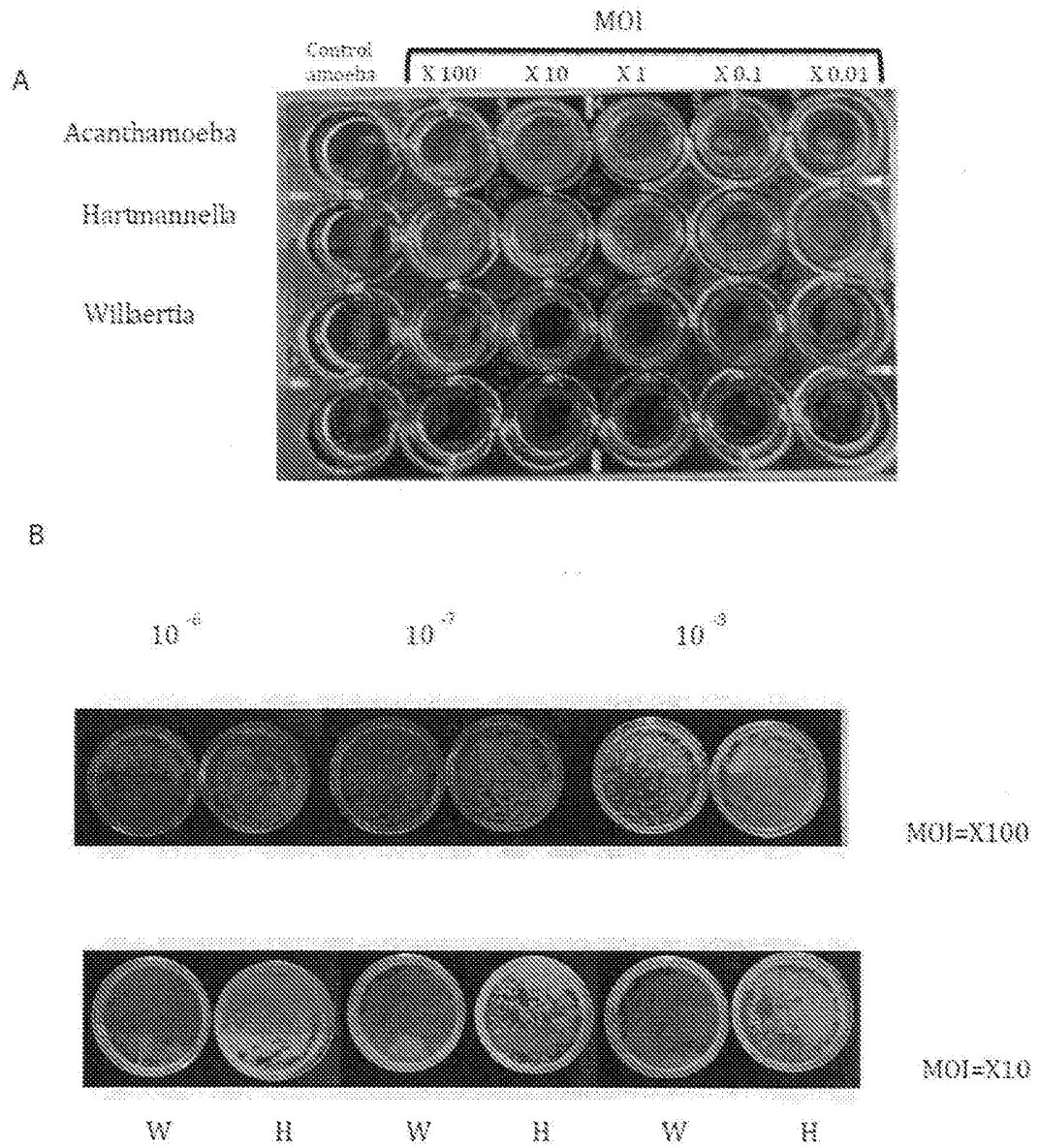


Figure 2.

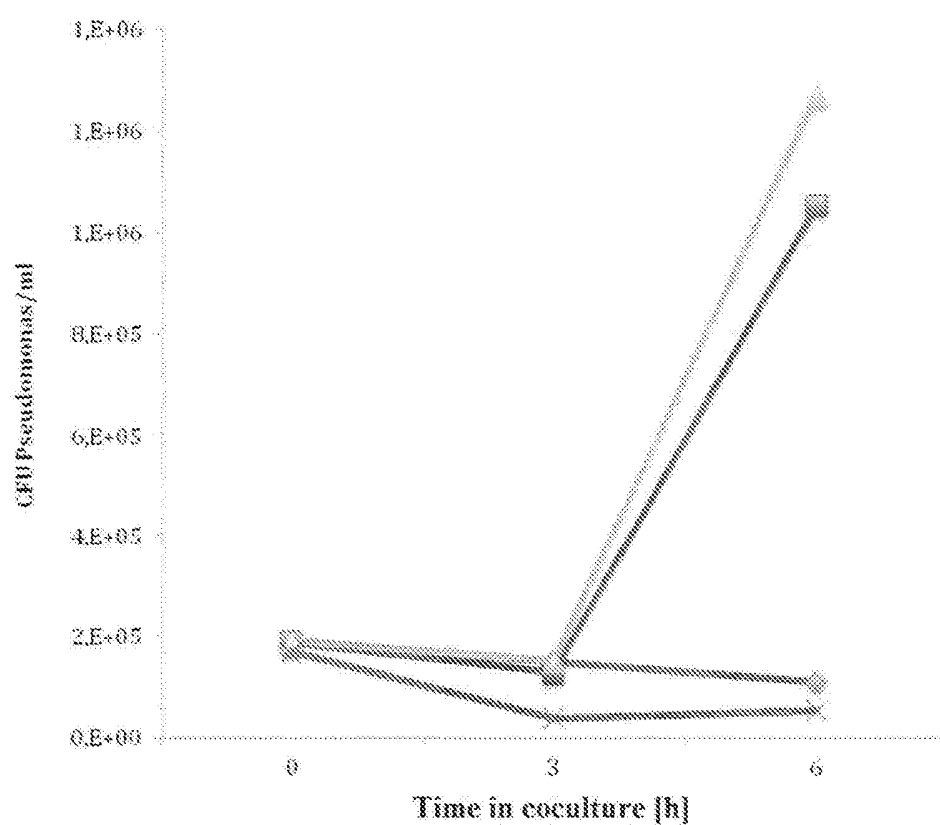


Figure 3.

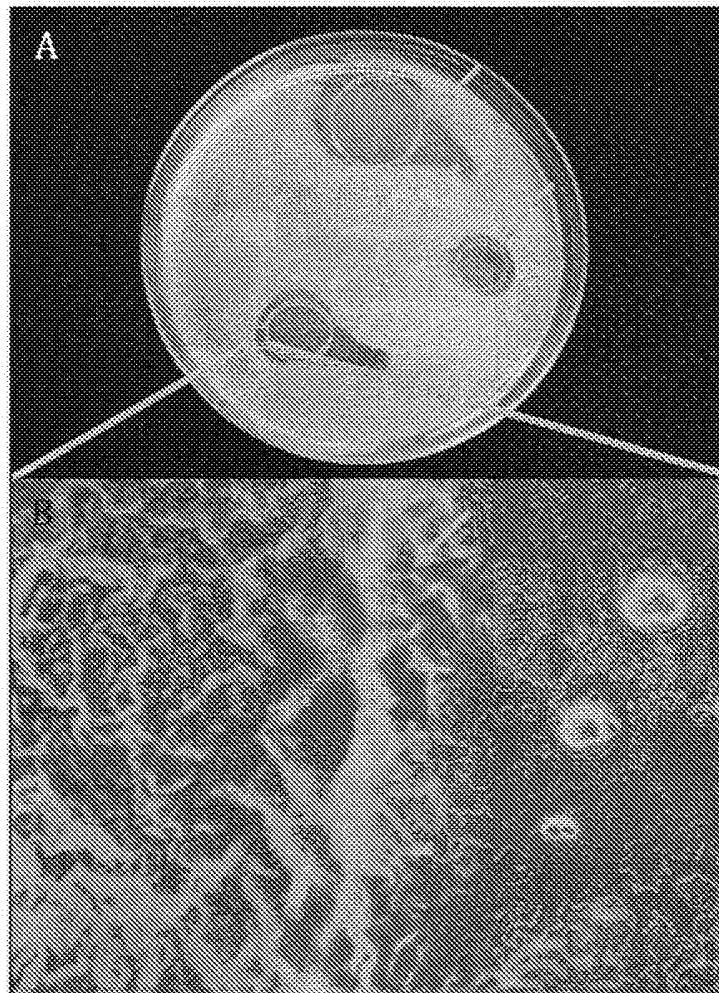


Figure 4.