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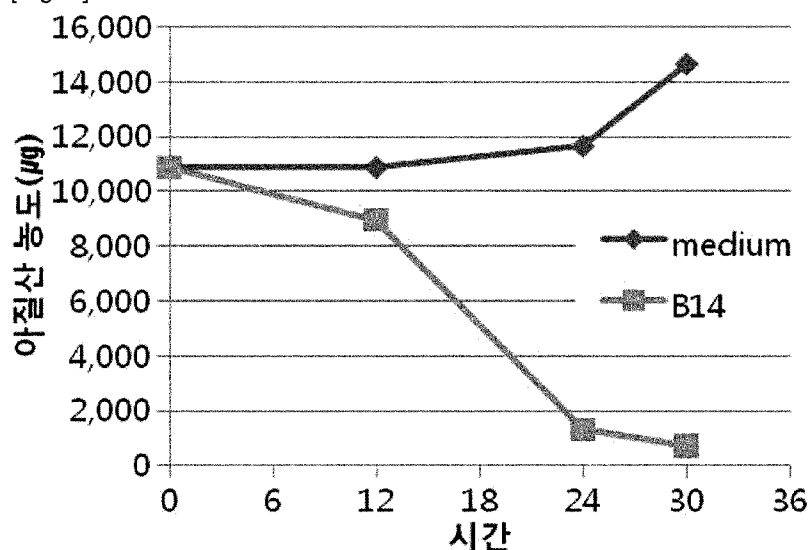
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[Continued on next page]

(54) Title: PROBIOTICS FOR BIOLOGICAL CONTROL AGAINST SAPROLEGNIA SP.

[Fig. 4]



(57) **Abstract:** The present invention relates to probiotics for biological control against Saprolegnia sp., and in particular, to a newly isolated bacillus strain that inhibits the growth of a pathogenic fungus, Saprolegnia sp. and produces siderophores; a culture broth obtained by culturing the strain, a concentrate thereof, or a dry product thereof; a probiotic composition, a feed additive, an antimicrobial agent, an antifungal agent, or a water quality improving agent comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof; and a method for culturing fish or crustaceans, preventing saprolegniosis in animals and improving water quality using the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.

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Description

Title of Invention: PROBIOTICS FOR BIOLOGICAL CONTROL AGAINST SAPROLEGNIA SP.

Technical Field

- [1] The present invention relates to probiotics for biological control against *Saprolegnia* sp., and in particular, to a newly isolated bacillus strain that inhibits the growth of a pathogenic fungus, *Saprolegnia* sp. and produces siderophores; a culture broth obtained by culturing the strain, a concentrate thereof, or a dry product thereof; a probiotic composition, a feed additive, an antimicrobial agent, an antifungal agent, or a water quality improving agent comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof; a method for culturing fish or crustaceans using the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof; a method for preventing saprolegniosis in animals by addition of the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof; and a method for improving water quality by treatment with the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.

[2]

Background Art

- [3] In advanced regions such as the US, Europe and Japan, much attention has been given to the improvement of dietary life in order to prevent adult diseases, as the world's population and people's standard of living are growing. Accordingly, demands for fishery products continue to increase, leading to a rapid growth in aquaculture production. Aquaculture industry has become an important contributor to many countries' economies, and a disease outbreak among cultured fish would result in serious economic losses (Jose Luis Balcazar et al. *Veter.Microbio.* 114(2006):173-186; Laurent venrschuere et al. *Microbio.Mot. Biol.Rev.* Dec. 2000.655-671).
- [4] The occurrence of diseases in fish farms is caused by infected fish or pathogens transmitted from the marine environment near aquaculture farms, and also caused by a supply of contaminated fish feed or unsanitary conditions in the fish farms. Infectious diseases of fish are divided to fungal, bacterial, and viral infections. Among them, a representative fungal infection is caused by *Saprolegnia* sp. (Diagnosis of Fish Diseases and Treatment Measures, The Ministry of Maritime Affairs and Fisheries, 2001). *Saprolegnia* sp. is ubiquitous in freshwater ecosystems and is the main genus of water molds responsible for significant fungal infections of freshwater fish. In fish, *Saprolegnia* sp. invades the epidermal tissues, generally beginning on the head or fins and can spread over the entire surface of the body, leading to the occurrence of serious

diseases in fish farms. It was reported that *Saprolegnia* sp. causes primary infections, but in most cases, occurs as a secondary pathogen, following the primary damage to the fish integument caused by parasites such as viruses and bacteria (Aquatic fungi and fish production in Egypt, II-in vivo studies).

- [5] In order to prevent damages caused by *Saprolegnia* sp., synthetic fungicides (e.g., malachite green, etc.) have been used. However, these synthetic fungicides are not readily biodegradable and tend to persist in the environment to cause environmental degradation, and also cause moderate or severe adverse effects, and some fungi have developed resistance to these chemicals. Hence, the use of these chemicals becomes restricted, and many efforts have been made to develop alternatives thereof (Ebele M. et al. African Journal of Biotechnology Vol.8 (24), 7130-7132, 2009). In one such effort, US Patent No. 6160023 discloses the use of bronopol in the treatment of various diseases of aquatic organisms, including fungal infections, protozoan infections, and bacterial gill disease, and a method of disinfecting fish tanks and/or equipment using bronopol. Likewise, Korean Patent No. 0782500 discloses a therapeutic agent for fungal infection of fish, comprising an extract of sweet flag, (*acorus calamus*) as an active ingredient.

[6]

- [7] Meanwhile, with increasing demand for environmentally friendly aquaculture, the use of probiotics as an immune enhancer is now widely accepted. Probiotic use was popularized by R. Fuller in 1989, and was formulated as a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance. For example, bacteria such as *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Bacillus*, yeasts such as *Saccharomyces*, and fungi such as *Aspergillus* have been used as probiotics. Probiotics are classified as GRAS (Generally Recognized As Safe), and retain no genes toxic to human and animal, and also produce no pathogenic substances. Probiotics are microorganisms approved to have efficacy of improving productivity in livestock. Probiotics are characterized by non-pathogenicity, easy in vitro proliferation, rapid growth in the body, and resistance to acid and bile. In addition, their activity should not be inhibited by feed ingredients, their effects should be maintained at room temperature, and they should be easily blended with feed for convenience.

- [8] Probiotics have the efficacies of competing with pathogenic bacteria which cause enteric diseases to prevent their strong adhesion to the intestinal epithelium, and rapidly recovering the intestinal microbial flora altered as a result of antibiotic treatment. Further, probiotics prevent infection of pathogenic microorganisms and inhibit their proliferation, promote the proliferation of intestinal microbial flora by supporting optimal conditions, produce lactic acid or acetic acid constituting intestinal

organic acids, and lower the pH in the intestine to prevent proliferation of harmful, pathogenic bacteria. Therefore, the feeding of probiotics having the above multiple actions maintains intestinal microbial flora and enhances productivity in livestock. In aquaculture, probiotics are also added to fish feed or additionally to water. Recent studies have been made to enhance immunity against pathogens and improve water quality by use of probiotics in the aquaculture of fish or shrimp (Jiqui Li et al. *Aquaculture* 291(2009) 35-40). The probiotics used for biological control in aquaculture are exemplified by beneficial bacteria such as *Lactobacillus*, *Enterococcus*, and *Bacillus*. *Bacillus* is a Gram-Positive, rod-shaped, endospore-forming bacterium, and has a distinctive morphology among the strains used as probiotics. Strains such as *Bacillus subtilis* (*B. subtilis*), *Bacillus cereus* (*B. cereus*), *Bacillus coagulans* (*B. coagulans*), *Bacillus clausii* (*B. clausii*), *Bacillus megaterium* (*B. megaterium*), and *Bacillus licheniformis* (*B. licheniformis*) are used as probiotics. *Bacillus* is more excellent in heat resistance than *Lactobacillus* which does not form endospores. Further, *Bacillus* is able to survive at the low pH of the gastric barrier, and thus most of ingested bacteria reach the small intestine intact (Barbosa, T.M. et al. *Appl. Environ. Microbiol.* 71(2005)968-978; Spinosa, M. R. et al. *Res. Microbiol.* 151(2000):61-368). It has been reported that *Bacillus* is used in humans as dietary supplements and in animals as growth promoters, and also used to promote growth and disease resistance of fish or shrimp in aquaculture, and improves water quality and growth rate of shrimp, and reduces pathogenic vibrios (Dalmin, G. et al. *Indian J. Exp. Biol* 39(2001) 939-942; Wang, Y.B. et al. *Fish. Sci.* 71(2005) 1034-1039).

- [9] Meanwhile, siderophore is a kind of antibiotic produced by microorganisms including pathogenic bacteria that compete with other microorganisms for rapid growth and proliferation under a given environment. Siderophores bind with iron ions (Fe^{3+}), and are then transported into the cells through a high-affinity iron-transport system, and the iron ions (Fe^{3+}) support the growth of microorganisms. Microorganisms produce siderophores because of competition for iron ions (Fe^{3+}), and a high production of siderophores inhibits the growth of other microorganisms. In this regard, siderophore-producing bacteria have been actively studied to be used as a potential biological control agent. In 1970, since it was reported that suppressive soils in agricultural areas was attributable to the antagonistic actions of microorganisms in the soil, competition of iron-specific siderophores produced by *Pseudomonas* sp. has been studied as a biological control method for inhibiting the growth of plant pathogens (Sang-Min Woo and Sang-Dal Kim, *Kor. J. Microbiol. Biotechnol.* Vol.36(4):326-335(2008)). Until now, it has been reported that siderophore is a water-soluble metabolite having a low molecular weight of 1 kDa or less, and high affinity for iron ions. Siderophores are divided into catechol type and hydroxamate type

according to the iron ion-binding functional groups, and the siderophores and probiotics producing the same have been actively studied. In one of the studies, US Patent No. 6746672 discloses a method of inhibiting the growth of *Lactococcus lactis* (L. lactis) using siderophores produced by *Bifidobacteria*. Korean Patent No. 037043 discloses a new antagonistic strain of *Pseudomonas fluorescens* LS20 producing siderophore, a method for inhibiting the growth of a plant pathogenic rhizobacterium, *Fusarium solani* using *Pseudomonas fluorescens* LS20, and a method for treating diseases caused by plant pathogenic fungi using siderophores. Korean Patent No. 0747700 discloses siderophore-producing *Bacillus subtilis* and a composition for the control of *Phytophthora capsici* or *Fusarium oxysporum* including siderophores produced thereby as an active ingredient.

[10] Most of the related patents have mentioned the use of siderophore-producing microorganisms for the biological control of plant pathogenic bacteria and fungi, and there has been no report on a probiotic bacillus used for the inhibition of fish pathogenic bacteria.

[11]

[12] The present inventors have isolated probiotics from a natural source, in which the probiotics have excellent antimicrobial activity against pathogenic bacteria in the aquaculture of fish and shrimp and excellent productivity of digestive enzymes, and they examined the morphological, biochemical and genetic characteristics thereof. As a result, they found that the probiotics are *Bacillus* having excellent heat-resistance, and the bacillus is excellent in siderophore productivity and inhibition of an aquatic fungus, *Saprolegnia* sp.

[13]

Disclosure of Invention

Technical Problem

[14] Accordingly, the present inventors found that the newly isolated *Bacillus* sp. inhibits the growth of an aquatic fungus, *Saprolegnia* sp., and has excellent siderophore productivity to compete with pathogenic bacteria for iron capture, and thus can be used as a probiotic composition for the aquaculture of fish and crustaceans, thereby completing the present invention.

[15]

Solution to Problem

[16] An object of the present invention is to provide a newly isolated *Bacillus* strain that inhibits the growth of *Saprolegnia* sp. and produces siderophores.

[17] Another object of the present invention is to provide a culture broth obtained by culturing the strain, a concentrate thereof, or a dry product thereof.

- [18] Still another object of the present invention is to provide a probiotic composition comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [19] Still another object of the present invention is to provide a feed additive comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [20] Still another object of the present invention is to provide a method for culturing fish or crustaceans using the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [21] Still another object of the present invention is to provide an antimicrobial agent or an antifungal agent comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [22] Still another object of the present invention is to provide a method for preventing saprolegniosis by addition of the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [23] Still another object of the present invention is to provide a water quality improving agent comprising the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.
- [24] Still another object of the present invention is to provide a method for improving water quality by treatment with the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.

Advantageous Effects of Invention

- [25] The newly isolated *Bacillus* sp. CJP-14 of the present invention shows excellent antifungal effect on an aquatic fungus, *Saprolegnia* sp. and also produces siderophores. Therefore, it can be widely applied to various products capable of controlling fungal infections including *Saprolegnia* sp. infection and bacterial infection, such as feed additives for aquaculture, probiotic compositions, and water quality-improving agents.
- [26]

Brief Description of Drawings

- [27] FIG. 1 is a photograph showing that the *Bacillus* sp. CJP-14 of the present invention inhibited the growth of an aquatic fungus *Saprolegnia* sp.;
- [28] FIG. 2 is a photograph showing the antimicrobial activity by siderophore formation of the *Bacillus* sp. CJP-14 of the present invention on the fish pathogens, *Vibrio harveyi* (VH1, VH2, VH3), *Vibrio cholerae* (VC), *Vibrio vulnificus* (VV), and *Aeromonas salmonicida* (AS) in CAS medium;
- [29] FIG. 3 is a photograph showing the presence of hemolysis of the *Bacillus* sp. CJP-14 of the present invention; and
- [30] FIG. 4 is a photograph showing the nitrous acid utilization of the *Bacillus* sp. CJP-14

of the present invention.

[31]

Best Mode for Carrying out the Invention

[32] In one embodiment, the present invention provides a newly isolated *Bacillus* sp. CJP-14 (KCCM11143P). The strain is able to produce siderophores and also inhibit the growth of *Saprolegnia* sp.

[33] Specifically, seawater-derived samples were collected in the shrimp farms around Ganghwa Island, and cultured in a BHI solid media supplemented with 3% sodium chloride. Then, the colonies were observed for grouping, and strains were isolated.

[34] Among the isolated strains, strains showing excellent antibacterial activity on the representative pathogenic bacteria attacking the cultured fish, including *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio anguillarum*, *Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio haemolyticus*, were selected by primary screening.

[35] Among the primary screened strains having excellent antibacterial activity, strains having excellent activity of digestive enzymes such as protease, cellulase, amylase, and lipase were selected by secondary screening.

[36] Among the secondary screened strains, finally selected was *Bacillus* sp. CJP-14 having excellent inhibitory effect on *Saprolegnia* sp. which is a pathogenic fungus in freshwater fish having excellent siderophore productivity to capture iron ions (Fe^{3+}), thereby showing competitive antagonistic action on the growth of other pathogenic bacteria.

[37] The *Bacillus* sp. CJP-14 has a Gram-positive rod-shaped morphology, and showed 99% homology with *Bacillus* sp. W30 in the result of 16s rDNA base sequence analysis. When this result is compared to the result that the 16s rDNA base sequence of the known siderophore-producing *Bacillus subtilis* AH18 shows 98% homology with that of *Bacillus subtilis* strain CICC 10088 (see Korean Patent No. 0717700), it can be seen that the strain of the present invention is a novel strain that has never been known. In addition, the strain of the present invention is able to produce siderophore as well as cellulase, protease, amylase, and lipase, whereas *Bacillus subtilis* AH18 is able to produce cellulose and auxin in addition to siderophores, indicating that the strain of the present invention is a novel strain that has never been known.

[38] Accordingly, the present inventors deposited the newly isolated *Bacillus* sp. CJP-14 at the Korean Federation of Culture Collection (in Korean Culture Center of Microorganisms, 361-221, Yurim B/D 3F, Hongje-1-dong, Seodaemun-gu, Seoul) on DEC. 14, 2010 as "*Bacillus* sp. CJP-14" (KCCM11143P).

[39]

[40] In another embodiment, the present invention provides a culture broth obtained by

culturing the newly isolated *Bacillus* sp. strain, a concentrate thereof, or a dry product thereof. Specifically, the culture broth of the present invention means a media where the newly isolated *Bacillus* sp. strain CJP-14 was cultured, and preferably a culture medium including the strain.

[41] As used herein, the culture medium means a medium including nutrients that are required for culturing animal cells, plant cells or bacteria, and the culture broth means a liquid medium where a strain is inoculated and cultured. The culture broth may be a medium including the strain, or a culture filtrate that is prepared by removing the strain from the culture broth where the strain was inoculated and cultured. The concentrate of the culture broth means those prepared by concentrating the culture broth, and the dry product of the culture broth means those prepared by removing water from the culture broth. The drying method may include air drying, natural drying, spray drying, and freeze drying, but is not limited thereto.

[42]

[43] In still another embodiment, the present invention provides a probiotic composition comprising the newly isolated *Bacillus* sp. or the culture broth thereof, the concentrate thereof, or the dry product thereof as an active ingredient, and a pharmaceutically acceptable carrier.

[44] As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. For formulation of the composition into a liquid preparation, a pharmaceutically acceptable carrier which is sterile and biocompatible may be used such as saline, sterile water, buffered saline, albumin infusion solution, dextrose solution, maltodextrin solution, glycerol, and mixtures of one or more thereof. If necessary, other conventional additives may be added such as antioxidants, buffers, bacteriostatic agents, and the like. Further, diluents, dispersants, surfactants, binders and lubricants may be additionally added to the composition to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions, or pills, capsules, granules, or tablets.

[45] The newly isolated *Bacillus* sp. that is included as an active ingredient in the probiotics of the present invention lives in the gastrointestinal tract of cultured fish or crustaceans to inhibit harmful bacteria and proliferation of pathogenic bacteria. In addition, beneficial digestive enzymes produced by the strain facilitate absorption and utility of nutrients to improve a feed conversion rate.

[46] The composition of the present invention includes 5×10^4 to 5×10^{10} CFU/ml and preferably 1×10^6 to 1×10^9 CFU/ml of the *Bacillus* CJP-14.

[47] Preferably, the composition of the present invention may be prepared in oral dosage forms, and examples of the oral dosage forms may include tablets, troches, lozenges,

aqueous or emulsive suspensions, powder or granules, emulsions, hard or soft capsules, syrups, or elixirs. For formulation such as tablets and capsules, useful are a binder such as lactose, saccharose, sorbitol, mannitol, starch, amylopectin, cellulose or gelatin, an excipient such as dicalcium phosphate, a disintegrant such as corn starch or sweet potato starch, a lubricant such as magnesium stearate, calcium stearate, sodium stearyl fumarate, or polyethylene glycol wax. For capsules, a liquid carrier such as a lipid may be further used in addition to the above-mentioned compounds.

[48]

[49] In still another embodiment, the present invention provides a feed additive comprising the newly isolated *Bacillus* sp. strain or the culture broth thereof, the concentrate thereof or the dry product thereof.

[50] Typically, all *Bacillus* species can form endospores to be very resistant to heat. Therefore, the newly isolated *Bacillus* sp. CJP-14 may be prepared in the form of feed additive, and then added to feed. Alternatively, the newly isolated *Bacillus* sp. CJP-14 may be directly added during the feed preparation. The *Bacillus* sp. CJP-14 in the feed of the present invention may be in a liquid or dry form, and preferably in a dry powdery form. The drying method may include air drying, natural drying, spray drying, and freeze drying, but is not limited thereto. The *Bacillus* sp. CJP-14 of the present invention may be mixed in the powder form at a ratio of 0.05 to 10% by weight, and preferably 0.1% to 1% by weight, based on the feed weight. In addition, the feed for aquaculture may further include common additives to improve the preservability, in addition to the *Bacillus* sp. CJP-14 of the present invention.

[51] The feeds comprising the *Bacillus* sp. CJP-14 of the present invention may include plant-based feeds such as grains, nuts, food processing byproducts, algae, fibers, oil, starches, meals, and grain byproducts, and animal-based feeds such as proteins, inorganic substances, fat, minerals, single-cell proteins, zooplankton, and fish meals, but are not limited thereto.

[52]

[53] In the present invention, the probiotic composition comprising the *Bacillus* sp. CJP-14 include additives for preventing quality deterioration, such as binders, emulsifiers and preservatives, and additives for increasing utility, such as amino acids, vitamins, enzymes, flavorings, non-protein nitrogen, silicates, buffering agents, extracts, and oligosaccharides, but is not limited thereto. In addition, the probiotic composition including the *Bacillus* sp. CJP-14 may further include feed premixes, but is not limited thereto.

[54] In still another embodiment, the present invention provides a method for culturing fish or crustaceans, comprising the step of treating aquaculture farm of fish or crustaceans using the newly isolated *Bacillus* strain, the culture broth thereof, the con-

centrate thereof, or the dry product thereof.

[55] As described above, the newly isolated *Bacillus* sp. CJP-14 of the present invention has a wide variety of antimicrobial activity and also competitive antagonistic action to inhibit the growth of other pathogenic bacteria. Thus, the strain is used to prevent the diseases caused by the common pathogenic bacteria in aquaculture, thereby culturing fish or crustaceans with safety.

[56] In still another embodiment, the present invention provides an antimicrobial agent or an antifungal agent comprising the newly isolated *Bacillus* strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.

[57] As used herein, the term "prevention" is intended to encompass all actions for restraining or delaying disease progress through the administration of the composition.

[58] The newly isolated *Bacillus* sp. CJP-14 of the present invention has antimicrobial activity against the above described 6 types of pathogenic bacteria in aquaculture, and produces siderophores to show an excellent iron-capturing ability. Thus, the newly isolated *Bacillus* sp. CJP-14 of the present invention has competitive antagonistic action to inhibit the growth of other pathogenic bacteria. In addition, it was confirmed that the newly isolated *Bacillus* sp. CJP-14 is able to inhibit the growth of a pathogenic aquatic fungus, *Saprolegnia* sp. Thus, the newly isolated *Bacillus* sp. is used to prevent the diseases caused by the above described pathogenic bacteria in aquaculture.

[59]

[60] In still another embodiment, the present invention provides an agent for improving water quality, comprising the newly isolated *Bacillus* sp., the culture broth thereof, the concentrate thereof, or the dry product thereof.

[61] The newly isolated *Bacillus* sp. CJP-14 of the present invention can be used to reduce the content of nitrous acid present in the aquaculture environment.

[62] To improve water quality, the newly isolated *Bacillus* sp. CJP-14 of the present invention may be separately prepared in the form of an agent for improving water quality, or the newly isolated *Bacillus* sp. CJP-14, the culture broth thereof, the concentrate thereof, or the dry product thereof may be directly sprayed. The *Bacillus* sp. CJP-14 in the agent for improving water quality of the present invention may be in a liquid or dry form, and is preferably in a dry powdery form.

[63] For the agent for improving water quality, a carrier which is sterile and bio-compatible may be used such as saline, sterile water, buffered saline, albumin infusion solution, dextrose solution, maltodextrin solution, glycerol, and mixtures of one or more thereof. If necessary, other conventional additives may be added such as antioxidants, buffers, bacteriostatic agents, and the like. Further, diluents, dispersants, surfactants, binders and lubricants may be additionally added to the composition to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions,

or pills, capsules, granules, or tablets.

[64] If the agent for improving water quality is used, the water quality of an aquaculture farm can be improved. Specifically, the agent for improving water quality may be added to the aquaculture farm before aquaculture or during aquaculture. Preferably, it is added to the aquaculture farm before aquaculture and left for a predetermined period. Consequently, the newly isolated *Bacillus* sp. CJP-14 of the present invention sufficiently produces siderophores to show an excellent iron-capturing ability, and thus inhibits the growth of other pathogenic bacteria and *Saprolegnia* sp. In addition, the agent for improving water quality is added during aquaculture one or more times so as to prevent additional growth of *Saprolegnia* sp.

[65]

[66] In still another embodiment, the present invention provides a method for improving water quality, comprising the step of treating water with the newly isolated *Bacillus* sp., the culture broth thereof, the concentrate thereof, or the dry product thereof. The improved water can be used in an aquaculture farm, and may also be used as drinking water for livestock or humans. In this regard, the water treated with the newly isolated *Bacillus* sp., the culture broth thereof, the concentrate thereof, or the dry product thereof is preferably purified before use, and the step of purifying water is performed by a known method.

Mode for the Invention

[67] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[68]

Example 1: Isolation of *Bacillus* sp. CJP-14

Example 1-1: Sample Acquisition and Strain Isolation

[71] Considering the characteristics of probiotics that have high environment and host specificity, the present inventors collected seawater-derived samples in the shrimp farms that show low occurrence of domestic diseases and high productivity. The collected samples were serially diluted and spread on a BHI agar (Difco, USA) supplemented with 3% sodium chloride, followed by cultivation at 37°C for 24 hours. Grouping of the strains isolated from each sample were performed by colony observation, and the strains was selected. The selected colonies were cultured in fresh medium three times for isolation. The isolated strains were stored in a medium containing 20% glycerol at -70°C or lower.

[72]

Example 1-2: Selection of Strains Having High Antimicrobial and Antifungal

Activities

- [74] For primary selection of strains having antimicrobial activity against representative pathogenic bacteria in aquaculture, test of antimicrobial activity against 6 types of bacteria including *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio anguillarum*, *Edwardsiella tarda*, and *Vibrio haemolyticus* was performed.
- [75] The antimicrobial activity against pathogenic bacteria was evaluated by clear zone analysis. 3 ml of 0.7% agar (W/V) and each 150 µl of the shaking culture broth of 6 types of pathogenic bacteria (OD₆₀₀=2.0) were mixed and covered onto BHI medium to prepare a top-agar. 10 µl of the culture broth of the selected strain was dropped onto the prepared top-agar, and cultured for 18 hours at 30°C. Then, the presence of the clear zone surrounding the dropped strains was observed. Among 53 types of the selected strains, 20 strains showing antimicrobial activity against any one of 6 types of pathogenic bacteria in aquaculture were primarily screened (Table 1).
- [76] Table 1

[Table 1]

Antimicrobial activity of primary screened strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AS	+	-	+	-	-	+	-	-	+	-	+	+	+	+	-	-	-	-
SI	+	-	+	-	-	-	+	-	-	-	+	+	+	+	+	-	-	-
ET	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	-	-
VH	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-
VA	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
AS	-	-	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	-
SI	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	+	-
ET	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-
VH	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
VA	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	
AS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
SI	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	
ET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
VH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	
VA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

[77] -: no Activity, +; Activity

[78]

[79] AS: *Aeromonas salmonicida*;[80] SI: *Streptococcus iniae*;[81] ET: *Edwardsiella tarda*;[82] VH: *Vibrio harveyi*;[83] VP: *Vibrio hemolyticus*;[84] VA: *Vibrio anguillarum*.

[85]

[86] **Example 1-3: Selection of Strains Having High Enzymatic Activity**

[87] **Example 1-3-1: Collection of Crude Enzyme Extract**

[88] In order to select strains having complex digestive enzyme activity, the 20 types of the strains having antimicrobial activity were cultured in BHI medium for 8, 24, and 48 hours, respectively and their culture broth was collected, followed by centrifugation at 4°C and 13,000 rpm for 5 minutes. The final supernatant was used as a crude enzyme extract for the analysis of enzymatic activity, and media including each substrate for the following enzymes were used to determine the substrate degradation.

[89]

[90] **Example 1-3-2: Protease Activity**

[91] A YM (Difco, USA) medium supplemented with 2% skim milk (Sigma, USA) as a substrate was prepared. 3 µl of the collected crude enzyme extract was added to each substrate medium, and then reacted at 30°C for 2 hours. Enzyme activity was determined by the presence of the clear zone surrounding the crude enzyme extract, which is formed by substrate degradation.

[92]

[93] **Example 1-3-3: Cellulase Activity**

[94] A YM medium supplemented with 1% CMC (carboxyl methyl cellulose) as a substrate was prepared. 3 µl of the collected crude enzyme extract was added to each substrate medium, and then reacted at 30°C for 2 hours, followed by straining with a 0.2% congo red aqueous solution for 30 minutes and destaining with 1 M NaCl aqueous solution. Enzyme activity was determined by the presence of the clear zone surrounding the crude enzyme extract, which is formed by substrate degradation.

[95]

[96] **Example 1-3-4: Amylase Activity**

[97] A YM medium supplemented with 1% soluble starch as a substrate was prepared. 3 µl of the collected crude enzyme extract was added to each substrate medium, and then reacted at 30°C for 2 hours, followed by straining with an aqueous solution containing 0.1% I₂ and 2% KI. Enzyme activity was determined by the presence of the clear zone surrounding the crude enzyme extract, which is formed by substrate degradation.

[98]

[99] **Example 1-3-5: Lipase Activity**

[100] A YM medium supplemented with 1% tricaptylin as a substrate was prepared. 3 µl of the collected crude enzyme extract was added to each substrate medium, and then reacted at 30°C for 2 hours. Then, enzyme activity was determined by the presence of the clear zone surrounding the crude enzyme extract, which is formed by substrate degradation.

[101] Among the 20 types of strains having antimicrobial activity, 6 types of the strains

producing 4 kinds of digestive enzymes including protease, cellulase, amylase, and lipase were selected as shown in the following Table 2.

[102]

[103] Table 2

[Table 2]

Digestive enzyme productivity of secondary screened strains

Enzyme	Protease		Amylase		Cellulase		Lipase	
	8hr	24hr	8hr	24hr	8hr	24hr	8hr	24hr
11	+++	+++	+	++	+++	++	-	-
12	+++	++	+++	++	+++	++	-	+
14	++	+	+	++	+++	+++	-	+++
23	+++	++	+	++	+++	++	-	-
26	++	+++	+	+++	+++	+++	-	+++
52	+++	++	+	+	+++	+++	-	++

[104] -: no activity, +; activity, ++: excellent activity, +++: very excellent activity

[105]

[106] **Example 2: In Vitro Test for Inhibitory Effect on Pathogenic Fungus in Freshwater Fish**

[107] The inhibitory effect on a pathogenic fungus *Saprolegnia* sp. in freshwater fish was examined in vitro using 4 candidate strains having excellent antimicrobial and antifungal activities, and complex enzyme productivity.

[108] *Saprolegnia* sp. was cultured by stationary culture on a PDA (Difco, USA) solid medium at 30°C for 72 hours. *Saprolegnia* sp. cultured on the solid medium was cut into a size of 5 mm x 5 mm (width x length), and inoculated on the center of a fresh PDA solid medium, and cultured for 24 hours. Each 20 µl of the 4 candidate strains were placed on sterile paper discs, and then inoculated on the solid medium where *Saprolegnia* sp. was cultured, followed by cultivation at 30°C for 48 hours. As shown in Table 3, the presence of clear zone surrounding the 4 candidate strains was observed (FIG. 1).

[109]

[110] Table 3

[Table 3]

Inhibitory effects on *Saprolegnia* sp. growth

Candidate strain	Presence of clear zone
CJP11	++
CJP12	+
CJP14	+++
CJP26	+++

[111] -: no activity, +; activity, ++: excellent activity, +++: very excellent activity

[112]

[113] In order to evaluate the inhibitory effects on *Saprolegnia* sp. in the liquid medium, the 4 candidate strains were cultured in BHI media at 37°C, 200 rpm for 8 hours. Each culture broth was serially diluted at a density of 10⁸ CFU, 10⁷ CFU, 10⁶ CFU, 10⁵ CFU, and 10⁴ CFU, and added to each well of 24 well plates (Falcon, USA), and *Saprolegnia* sp. was added thereto at a density of 10² to a final volume of 1 ml. 0.5 ml of PDB liquid medium (Difco, USA) supplemented with 0.5 g/L peptone and 20 g/L yeast extract was added thereto, followed by cultivation at 25°C for 48 hours. Inhibition on fungal activity was evaluated by solidification, and two candidate strains inhibited the growth of *Saprolegnia* sp., as shown in the following Table 4.

[114]

[115] Table 4

[Table 4]

Inhibitory effects of two candidate strains on *Saprolegnia* sp. growth

Test strain	10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴
CJP14	+++	+++	++	-	-
CJP26	+++	+	+	-	-

[116] +, Positive; -, Negative

[117]

[118] **Example 3: Selection of Strains having Excellent Siderophore Productivity**

[119] In order to examine the siderophore productivity of the 4 candidate strains having excellent antimicrobial and antifungal activities and complex enzyme productivity, CAS assay was performed. CAS medium was prepared as follows.

[120]

[121] Table 5

[Table 5]

CAS medium composition for evaluation of siderophore productivity

Fe solution (1 mM FeCl ₃ · 6H ₂ O in 10 mM HCl)	1.5
CAS solution (0.121 g CAS in 100 ml distilled water)	7.5
HDTMA (0.0219 g HDTMA in 50 ml distilled water)	50
pH 6.5 piperazine buffer(4.307g piperazine in 30 ml distilled water)	30
Distilled water	100(the rest)

[122]

[123] Each of the 4 candidate strains was inoculated with 0.1% in the BHI liquid medium, and cultured at 37°C and 200 rpm for 18 hours. In addition, each of pathogenic bacteria, *Vibrio harveyi*, *Vibrio cholerae*, *Vibrio vulnificus*, and *Aeromonas salmonicida* was inoculated with 0.1% in LB medium supplemented with 2% sodium chloride, and cultured at 30°C, 200 rpm for 18 hours. Each culture supernatant was filtered using a 0.45 µm filter, and the filtered culture supernatant was used to perform the experiment. A hole with a diameter of 0.5 cm was made in the CAS agar, and each 50 µl of the filtered culture supernatant was inoculated in the hole, and reacted at 37°C for 8 hours. As shown in FIG. 2, an orange-colored clear zone surrounding the hole, which is formed upon utilization of iron, was observed. The *Bacillus* sp. CJP-14 having most excellent siderophore productivity among the 4 candidate strains and above 4 types of pathogenic bacteria was finally selected (Table 6).

[124]

[125] Table 6

[Table 6]

Evaluation of siderophore productivity

Candidate strain	siderophore productivity	Pathogenic bacteria in cultured fish	siderophore productivity
CJP11	++	<i>Vibrio harveyi</i>	++
CJP12	+	<i>Vibrio cholerae</i>	++
CJP14	+++	<i>Vibrio vulnificus</i>	++
CJP26	++	<i>Aeromonas salmonicida</i>	+

[126] -: no activity, +; activity, ++: excellent activity, +++: very excellent activity

[127]

[128] **Example 4: Identification of Selected Strain and Physiological and Biochemical**

Characterization thereof**Example 4-1: Strain Identification**

[129] **Example 4-1: Strain Identification**
[130] Among the 4 candidate strains, the CJP-14 strain showing excellent inhibitory effect on *Saprolegnia* sp. and excellent siderophore productivity was finally selected, identified, and analyzed. Identification of the strain was performed by physiological and biochemical methods and molecular systematic methods. The strain was found to have the morphological characteristic of Gram-positive rod-shaped bacterium. Analysis of the 16s rDNA sequence showed that the strain has 99% homology with *Bacillus* sp., indicating a novel microorganism. The base sequence of 16s rDNA of the isolated strain is represented by SEQ ID NO. 1. The base sequence analysis was performed by amplification of 16s rDNA using a PCR premix (Bioneer, Korea) and universal primers 27F and 492R having the base sequence below. The gene amplification was performed in a total reaction volume of 20 µl for total 30 cycles consisting of at 94°C for 1 minute, at 56°C for 1 minute, and at 72°C for 1 minute to analyze the base sequence of the amplified DNA.

[131]

[132] 27F: 5'-AGAGTTTGATCMTGGCTCAG-3'(SEQ ID NO.2)

[133] 92R: 5'-GGYTACCTTGTTACGACTT-3'(SEQ ID NO.3)

[134]

[135] The novel microorganism of the present invention that was identified by the above method was deposited at the Korean Federation of Culture Collection (in Korean Culture Center of Microorganisms, 361-221, Yurim B/D 3F, Hongje-1-dong, Seodaemun-gu, Seoul) on DEC. 14, 2010 as "*Bacillus* sp. CJP-14" (KCCM11143P).

[136]

Example 4-2: Physiological and Biochemical characterization

[137] **Example 4-2: Physiological and Biochemical characterization**
[138] In order to analyze the biochemical characteristic of the strain of the *Bacillus* sp. CJP-14 of the present invention, sugar fermentation patterns of the strain were analyzed using API 50 CHB system (Korean Culture Center of Microorganisms, Korea) (Table 7).

[139]

[140] Table 7

[Table 7]

Result of sugar fermentation patterns

Control	-	Esculine	+
Glycerol	+	Salicine	+
Erythritol	-	Cellobiose	+
D-Arabinose	-	Maltose	+
L-Arabinose	+	Lactose	+
Ribose	+	Melibiose	-
D-Xylose	+	Saccharose	+
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β Methyl-xyloside	-	Melezitose	-
Galactose	-	D-Raffinose	+
D-Glucose	+	Amidon	+
D-Fructose	+	Glycogene	+
D-Mannose	+	Xylitol	-
L-Sorbose	-	β Gentiobiose	-
Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	+	D-Tagatose	-
Mannitol	+	D-Fucose	-
Sorbitol	+	L-Fucose	-
α Methyl-Dmannoside	-	D-Arabitol	-
α Methyl-glucoside	+	L-Arabitol	-
N Acetyl glucosamine	-	Gluconate	-
Amygdaline	+	2 ceto-gluconate	-
Arbutine	+	5 ceto-gluconate	-

[141] +, Positive; -, Negative

[142]

[143] **Example 5: Safety and Utilization**[144] **Example 5-1: β -Hemolysis**

[145] β -Hemolysis is a complete lysis of red blood cells, which is caused by hydrolysis of phospholipid by phospholipase-producing bacteria.

[146] In order to examine hemolysis of the *Bacillus* sp. CJP-14, blood agar plate containing TSA(tryptic soy agar)(Difco, USA) and 5% sheep blood (Kisan Biotech, Korea) was prepared. After streaking with *Bacillus* sp. CJP-14 on the prepared blood agar plate, incubation was performed at 37°C for 24 hours to examine hemolysis. As shown in FIG. 3, no hemolysis was observed.

[147]

[148] **Example 5-2: Nitrous Acid Utilization**

[149] In order to examine the nitrous acid utilization of the strain of the present invention, a medium containing nitrous acid was prepared as shown in the following Table 8. 0.1% of the *Bacillus* sp. CJP-14 was inoculated in the prepared medium, and then cultured at 30°C, 200 rpm for 6 hours, 12 hours, and 24 hours. The culture broth sample was collected at each cultivation time. The collected culture broth samples were centrifuged at 4°C, 13,000 rpm for 5 minutes to obtain supernatant, and the content of nitrous acid in the obtained supernatant was measured to quantify the consumption.

[150]

[151] Table 8

[Table 8]

Medium composition for evaluation of nitrous acid utilization

Nitrite (NaNO ₂)	0.5 g/L
Sodium phosphate dibasic (Na ₂ HPO ₄)	13.5 g/L
Potassium Phosphate dibasic (K ₂ HPO ₄)	0.7 g/L
Magnesium sulfate(MgSO ₄ 7H ₂ O)	0.1 g/L
Calcium chloride(CaCl ₂)	0.18 g/L
Sodium bicarbonate(NaHCO ₃)	0.5 g/L
Ferric chloride (FeCl ₃ 6H ₂ O)	0.014 g/L
Glucose(glucose)	0.5 g/L

[152]

[153] The initial concentration of nitrous acid in the medium was approximately 10 mg. As the *bacillus* sp. CJP-14 grew to utilize nitrous acid, consumption of nitrous acid was approximately 94% after cultivation for 30 hours (see FIG. 4).

[154]

[155] The above results demonstrate that the *bacillus* sp. CJP-14 shows inhibitory effect on the aquatic fungus *Saprolegnia* sp., siderophore productivity and antimicrobial activity.

[156]

[157] **Example 6: Efficacy on Freshwater Fish-Pathogenic Fungus, Saprolegnia sp.**

[158] The efficacy of Bacillus sp. CJP-14 having inhibitory effect on freshwater fish-pathogenic fungus, Saprolegnia sp. in vitro and having excellent siderophore productivity was evaluated. The present inventors infected fertilized eggs of rainbow trout with Saprolegnia sp. in the presence of the Bacillus sp. CJP-14 of the present invention, and then the eyed rate (%) was examined.

[159] Specifically, 500 fertilized eggs of rainbow trout were placed into a beaker containing sterilized freshwater, and then the eggs are immersed with comprising Saprolegnia sp. and the each of contents described in Table 9 for 30 minutes once a day for 3 days, and then the eyed rate (%) was determined as follows (Table 9). Sodium chloride and formalin were used as a control group.

[160] $\text{Egg-eyed rate (\%)} = (\text{the number of eyed eggs} / \text{the number of used fertilized eggs}) \times 100$

[161]

[162] Table 9

[Table 9]

Efficacy on Saprolegnia sp.

Treatment group	concentration	Number of fertilized eggs	Eyed rate (%)
Bacillus sp. CJP14	1 x 10 ⁹ CFU	300	54.7
	1 x 10 ⁸ CFU	300	53.2
	1 x 10 ⁷ CFU	300	40.5
Sodium chloride	10 ppm	300	52.3
Formalin (37% formaldehyde solution)	120 ppm	300	19.5
Control group	-	300	0.5

[163]

[164] As shown in the Table 9, the eyed rate of the control group was 0.5%, and those of sodium chloride and formalin currently used as an antifungal agent were 52.3% and 19.5%, respectively. Treatment with the Bacillus sp. CJP-14 of 1 x 10⁸ CFU or higher showed more excellent efficacy than sodium chloride and formalin solution, indicating the inhibitory effect of the bacillus sp. CJP-14 of the present invention on the freshwater fish-pathogenic fungus, Saprolegnia sp.

[165]

[166] **Example 7: Efficacy of Probiotics**

[167] In order to examine whether the *Bacillus* sp. CJP-14 of the present invention practically shows the probiotic action when it is added to feed and fish are fed with the feed, the *Bacillus* sp. CJP-14 of the present invention was mixed with feed, and juvenile flounder was fed with the mixed feed for 8 weeks, so as to determine the rate of weight gain, daily growth rate, feed conversion ratio, protein efficiency ratio, and survival rate.

[168] Specifically, a basal feed(control group) was prepared to have energy content equal to 30% crude protein, as shown in Table 10. The *Bacillus* sp. CJP-14 of the strain of the present invention was added to the basal feed to make mixed feed containing 5% *Bacillus* sp. CJP-14, and the amount of cellulose was reduced to the addition amount of probiotics for equal energy content.

[169]

[170] Table 10

[Table 10]

Basal feed composition

Raw materials	%
White fish meal	36.0
Soybean meal	12.0
Corn gluten meal	8.0
Wheat flour	30.4
Squid liver oil	5.0
Soy bean meal	5.0
CMC	1.0
Cellulose	0.5
Mineral premix	1.0
Vitamin premix	1.0

[171]

[172] The juvenile flounder used in the experiment had the initial average weight of 25 g, and 25 fish were randomly assigned to a 150 L round PP tank. Sand-filtered seawater was used as culture water, and a flow rate was controlled to 2-3 L/min. The tank was equipped with an air stone for oxygen supply and culture water circulation. A photoperiod was maintained under the condition of 12L:12D using a fluorescent lamp. The culture water temperature was maintained within the natural temperature conditions of

21-29°C during the entire experimental period. The fish were fed to satiation twice a day. The growth rate was measured every three weeks, and all fish were starved for 24 hours before measurement (see Table 11).

[173]

[174] Table 11

[Table 11]

Efficacy of probiotics in flounder

	Control group	Experimental group
Final average weight (g)	50.6 ± 1.7 ^a	57.4 ± 3.8 ^b
Rate of weight gain (%)	103.03 ± 5.6 ^a	130.05 ± 9.3 ^b
Daily growth rate (%)	1.42 ± 0.06 ^a	1.67 ± 0.08 ^b
Survival rate (%)	77.3 ± 15.1 ^a	97.3 ± 2.3 ^b
Feed conversion ratio	15.9 ± 0.15 ^a	1.16 ± 0.05 ^b
Protein efficiency ratio	1.10 ± 0.1 ^a	1.40 ± 0.0 ^b

[175]

[176] As shown in Table 11, the addition of the *Bacillus* sp. CJP 14 increased the growth rate to approximately 13%, compared to the control group, and also increased the feed conversion ratio and protein efficiency ratio, compared to the control group. The survival rate was also increased to approximately 25%, compared to the control group.

[177]

[178] These results demonstrate that the *Bacillus* sp. CJP 14 has excellent productivity of complex enzymes to facilitate fish ingestion rate and rate of weight gain, and to reduce excretion, leading to improvement of water quality.

[179]



BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To, CJ Cheiljedang Corporation
500 5-GA NAMDAEMUN-RO,
CHUNG-KU, SEOUL,
REPUBLIC OF KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

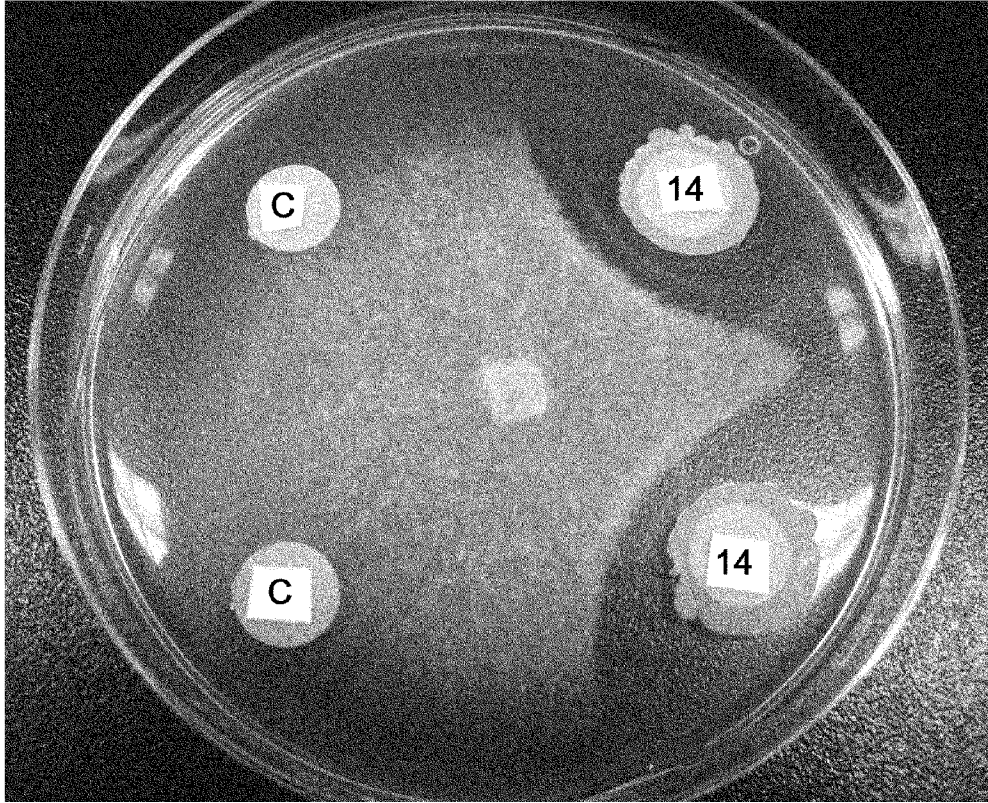
I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>Bacillus</i> sp. CJP-14	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11143P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on December, 14, 2010. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodae-mun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: December, 14, 2010.

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

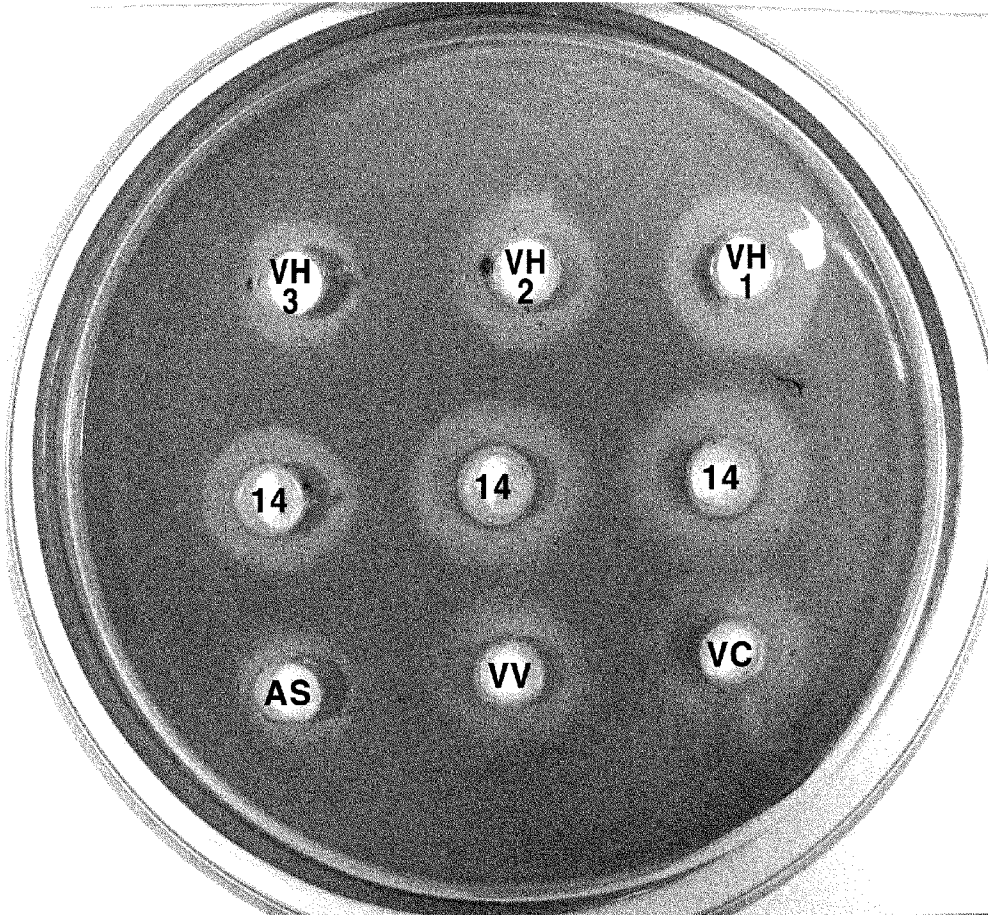
Claims

- [Claim 1] A *Bacillus* sp. CJP-14 (KCCM11143P).
- [Claim 2] A culture broth of the *Bacillus* sp. CJP-14 (KCCM11143P) of claim 1, a concentrate thereof, or a dry product thereof.
- [Claim 3] A probiotic composition comprising the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2, as an active ingredient.
- [Claim 4] A feed additive comprising the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2, as an active ingredient.
- [Claim 5] The feed additive according to claim 4, wherein the feed additive is used for aquaculture of fish or crustaceans.
- [Claim 6] A method for culturing fish or crustaceans, comprising the step of treating fish or crustacean farm with the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2.
- [Claim 7] An antimicrobial agent or antifungal agent comprising the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2.
- [Claim 8] A method for preventing diseases caused by an aquatic fungus in animals, comprising the step of adding the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2.
- [Claim 9] The method according to claim 8, wherein the aquatic fungus is *Saprolegnia* sp.
- [Claim 10] An agent for improving water quality comprising the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2.
- [Claim 11] A method for improving water quality, comprising the step of treating water with the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2.

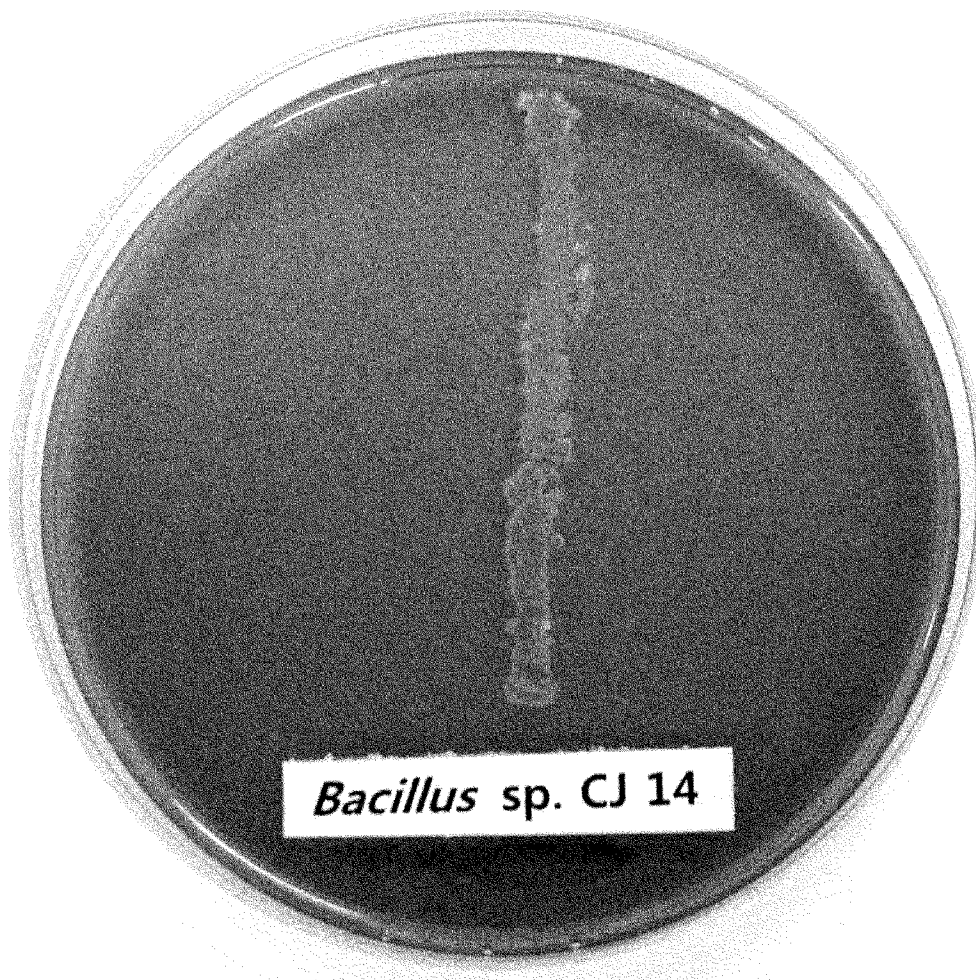
[Fig. 1]



[Fig. 2]



[Fig. 3]



[Fig. 4]

