



(51) International Patent Classification:

C12N 1/20 (2006.01) *A01N 63/02* (2006.01)
A23K 1/16 (2006.01)

(21) International Application Number:

PCT/KR2012/000763

(22) International Filing Date:

31 January 2012 (31.01.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

10-2011-0009793 31 January 2011 (31.01.2011) KR

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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, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, 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.

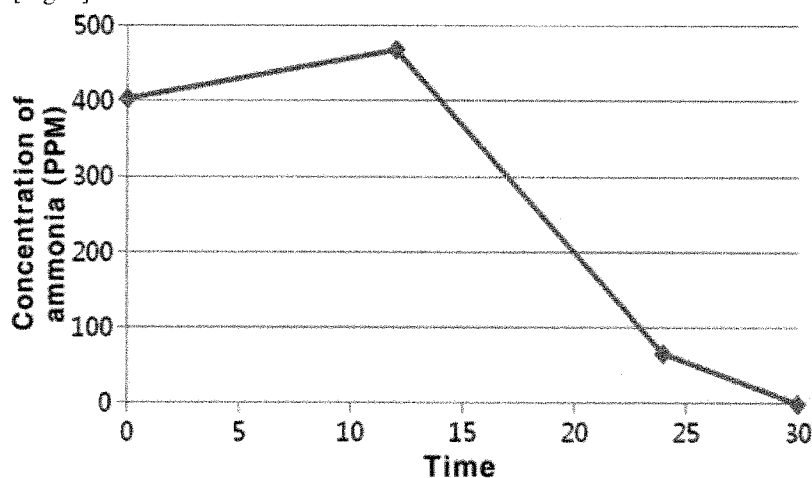
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, 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, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

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

[Fig. 4]



(57) Abstract: The present invention relates to probiotics for biological control against *Vibrio* sp., and in particular, to a newly isolated bacillus strain that degrades quorum-sensing signal molecules of the pathogenic bacteria *Vibrio* sp., and inhibits biofilm formation; 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, 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, degrading quorum-sensing signal molecules of *Vibrio harveyi* and inhibiting biofilm formation, preventing bacterial infection in animals and improving water quality using the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.



Description

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

Technical Field

- [1] The present invention relates to probiotics for biological control against *Vibrio* sp., and in particular, to a newly isolated bacillus strain that degrades quorum-sensing signal molecules of the pathogenic bacteria *Vibrio* sp., and inhibits biofilm formation; 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, 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 degrading quorum-sensing signal molecules of *Vibrio harveyi* and inhibiting biofilm formation by addition of the strain, the culture broth thereof, the concentrate thereof, or the dry product thereof into aquaculture farms; a method for preventing bacterial infection 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 countries in 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 can 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] *Vibrio* sp., one of pathogens that cause bacterial infections in fish and shrimp aquaculture farms, causes toxicity as a primary problem, and also forms biofilms as a secondary problem. That is, *Vibrio* sp. produces and releases low molecular weight signal molecules called autoinducers (or AI) or pheromones. The detection of a minimal threshold stimulatory concentration of the signal molecule recognizes cell density of surrounding *Vibrio* bacteria, leading to an alteration in gene expression and a variety of physiological activities including symbiosis, virulence, competence, con-

jugation, antibiotic production, motility, sporulation, and biofilm formation (Miller, M. B. et al. *Annu. Rev. Microbiol.* 55(2001), 165-199). As such, the process of monitoring population density of microorganisms and regulating correlated gene expression is called quorum sensing. The quorum sensing property is present in Gram-negative and positive bacteria. When bacteria reach a sufficiently high population density, they will alter gene expression required for infection so as to induce the cooperation of a large number of cells for host infection, and they can elude the host's immune responses for successful establishment of the infection (Dangl, J. L. et al., *Nature*. 411(2001) 826-833).

- [5] A representative behavior of quorum-sensing is biofilm formation. When a low density of microorganisms form colonies reaching a certain population, gene expression by quorum-sensing signal molecules produces mucous polysaccharide, leading to formation of structured biofilm that is involved in 65% of bacterial infections (Kjelleberg, S. et al. *Curr Opin Microbiol.* 5(3), 2002, 254-258). The biofilm forms on the host surface to cause infectious diseases. Specifically, in fish, it facilitates nutrient and oxygen supply for the causative microorganism, prevents penetration of antibiotics, blocks the immune defense system, and reduces the effects of disinfectants and probiotics, and also causes antibiotic resistance (Hall-Stoodley, L. et al., *Nat Rev Microbiol.* 2(2), 2004, 95-108).
- [6] Autoinducer-1 (AI-1) and autoinducer-2 (AI-2) are known as chemical signal molecules secreted by microorganisms at an early stage of biofilm formation. Autoinducer-1 is an AHL compound produced by an AHL synthase of LUX-1 family, and contains an invariant homoserine lactone moiety and an acyl group with various chain lengths, saturation levels, and oxidation states, and is exemplified by N-acylhomoserine lactones (AHLs) as signal molecules in Gram-negative bacteria. Autoinducer-1 was also reported to be a strain-specific substance secreted by more than 70 kinds of Gram-negative bacteria, including *Pseudomonas*, *Burkholderia*, *Yersinia*, *Aeromonas*, *Agrobacterium*, *Serratia*, *Erwinia*, *Citrobacter*, *Enterobacter*, *Proteus*, *Chromobacterium*, and *Rhizobium* sp. (Taga, M.E. et al. *Proc. Natl. Acad. Sci. U.S.A.* (2003)). Moreover, it was reported that autoinducer-2 is a compound produced by the protein expressed from the *luxS* gene, has a furanosyl borate diester structure and is a universal signal present in both Gram-positive and negative bacteria (Bassler, B. L. et al. *Mol. Microbiol.* 9(1993)773-786). The *luxS* gene is present in *E.coli* as well as in various bacteria such as *salmonella typhimurium* (*S. typhimurium*), *Hemophilus influenza* (*H. influenzae*), *Helicobacter pylori* (*H. pylori*), *Bacillus subtilis* (*B. subtilis*), *Borrelia burgdoferi* (*B. burgdoferi*), *Neisseria meningitides* (*N. meningitides*), *Campylobacter jejuni* (*C. jejuni*), *Mycobacterium tuberculosis* (*M. tuberculosis*), *Enterococcus faecalis* (*E. faecalis*), *Streptococcus pneumoniae* (*S. pneumoniae*),

Clostridium perfringens (*C. perfringens*), *Clostridium difficile* (*C. difficile*), and *Klebsiella pneumoniae* (*K. pneumoniae*). It was also reported that the *luxS* gene induces luminescence in *Vibrio harveyi*, and affects gene expression required for virulence, motility, biofilm formation, and antibiotic production (Miller, M. B. et al. *Annu. Rev. Microbiol.* 55(2001), 165-199).

- [7] Meanwhile, in the case, the lethality ratio in a population of rainbow trout infected with a fish pathogenic microorganism, *Vibrio anguillarum* (*V. anguillarum*) was 80 to 100%. However, treatment of furanone, which is one of the quorum-sensing inhibitors controlling the quorum-sensing signal molecule AHL, controls the quorum-sensing, so that the lethality ratio was reduced to between 4% to 40% (Rasch, M. et al. *Syst. Appl. Microbiol.* 27(2004), 350-359). Thus, many studies have focused on quorum-sensing signal molecules for reduction of the damages caused by microbial infections.
- [8] For example, US Patent NO. 641685 discloses an antimicrobial agent comprising a photosensitizing agent for phototherapy linked to a polypeptide having a binding affinity to an autoinducer-2 (AI-2), in which the polypeptide killed a bacteria. US Patent NO. 6936453 discloses a method of screening a compound capable of modulating the autoinducer activity by contacting a mixture of the autoinducer and the compound with bacteria cells that react with the autoinducer to be luminescent, and then comparing the luminescence with a luminescence of the bacteria contacted with the autoinducer only. US Patent Publication NO. 2004-0009160 discloses a quorum-sensing inhibitor, furanone, that is regulated by pathogenic bacteria signal molecules, HSL and AHL. Korean Patent Publication NO. 2008-0060777 discloses an antibacterial amide derivative as a quorum-sensing antagonist and a method of preventing biofilm formation using the same. Korean Patent NO. 832565 discloses an antibacterial furanone derivative as a quorum-sensing antagonist and a method of preventing biofilm formation using the same. Korean Patent NOs. 841289, 841294 and 841333 disclose an antibacterial homoserine lactone derivative that has antibacterial activity and functions as a quorum-sensing antagonist at the same time, a method of removing bacteria using the same, and a method of preventing biofilm formation using the same.
- [9] 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 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 humans or animals, and also produce no pathogenic

substances. Probiotics are microorganisms that are approved to have efficacy in 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.

- [10] Probiotics have the efficacies of competing with pathogenic bacteria causing 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, feeding subjects probiotics having 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).
- [11] 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* that does not form endospores. Further, *Bacillus* is able to survive at the low pH of the gastric barrier, and thus most of the 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).
- [12]
- [13] The present inventors have isolated probiotics from a natural source having 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, it was found that the probiotics are *Bacillus* having excellent heat-resistance. In particular, the *Bacillus* controls *Vibrio harveyi* in aquaculture and degrades a quorum-sensing signal molecule, autoinducer-2, so as to inhibit physiological activities that occur upon quorum-sensing (symbiosis, virulence, competence, biofilm formation, etc.)

[14]

Disclosure of Invention

Technical Problem

[15] Accordingly, the present inventors found that the newly isolated *Bacillus* sp. inhibits the growth of *Vibrio harveyi*, and controls quorum-sensing to inhibit biofilm formation, and thus can be used as a probiotic composition for the aquaculture of fish and crustaceans, thereby completing the present invention.

[16]

Solution to Problem

[17] An object of the present invention is to provide a newly isolated *Bacillus* strain that degrades quorum-sensing signal molecules of a pathogenic bacterium, *Vibrio* sp., and inhibits biofilm formation.

[18] 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.

[19] 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.

[20] 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.

[21] 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.

[22] Still another object of the present invention is to provide an antimicrobial agent comprising 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 method for degrading quorum-sensing signal molecules of *Vibrio harveyi* and inhibiting biofilm formation by addition of 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 preventing diseases caused by pathogens in animals by addition of the strain, the culture broth

thereof, the concentrate thereof, or the dry product thereof.

[25] 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.

[26] 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.

[27]

Advantageous Effects of Invention

[28] The newly isolated *Bacillus* sp. CJS-26 of the present invention controls quorum-sensing of *Vibrio harveyi* in aquaculture, thereby inhibiting physiological activities that occur upon quorum-sensing (symbiosis, virulence, competence, biofilm formation, etc.) and effectively blocking pathogenicity. Therefore, it can be widely applied to various products capable of controlling diseases caused by bacteria including *Vibrio harveyi*, such as feed additives for aquaculture, probiotic compositions, and water quality-improving agents.

[29]

Brief Description of Drawings

[30] FIG. 1 is a graph showing degradation of *Vibrio harveyi* quorum-sensing signal molecule, autoinducer-2 by the *Bacillus* CJS-26 of the present invention;

[31] FIG. 2 is a photograph showing that the *Bacillus* CJS-26 inhibited biofilm formation by *Vibrio harveyi* when it was cultured for 30 hours;

[32] FIG. 3 is a photograph showing the presence of hemolysis of the *Bacillus* CJS-26 of the present invention; and

[33] FIG. 4 is a graph showing ammonia utilization of the *Bacillus* CJS-26 of the present invention.

[34]

Best Mode for Carrying out the Invention

[35] In one embodiment, present invention provides a newly isolated *Bacillus* sp. CJS-26 (KCCM11144P). The strain is able to degrade quorum-sensing signal molecules of *Vibrio harveyi* and also inhibit biofilm formation.

[36] 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. 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*

inae, and *Vibrio haemolyticus*, were selected by primary screening. Among the primary screened strains having excellent antibacterial activity, 6 types of strains having excellent activity of digestive enzymes such as protease, cellulase, amylase, and lipase were selected by secondary screening. Among the secondary screened strains, selected was finally *Bacillus* sp. CJP-26 that degrades quorum-sensing signal molecules of *Vibrio harveyi* and inhibits biofilm formation.

[37] The *Bacillus* sp. CJP-26 has a Gram-positive rod-shaped morphology, and showed 99% homology with *Bacillus* sp. in the result of 16s rDNA base sequence analysis. Accordingly, the present inventors deposited the newly isolated *Bacillus* sp. CJP-26 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-26" (KCCM11144P).

[38]

[39] In another embodiment, the present invention provides a culture broth of the strain, a concentrate thereof, or a dry product thereof. Specifically, the culture broth of the present invention means a media where the *Bacillus* sp. strain CJP-26 was cultured, and preferably a culture medium including the strain.

[40] 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.

[41]

[42] 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.

[43] 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.

[44] Probiotics live in the gastrointestinal tract to inhibit harmful bacteria and proliferation of pathogenic bacteria. In addition, beneficial digestive enzymes produced by probiotics facilitate absorption and utility of nutrients to improve a feed conversion rate.

[45] 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-26.

[46] Examples of the oral dosage forms including the composition of the present invention as an active ingredient 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.

[47]

[48] 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.

[49] Typically, all Bacillus species can form endospores to be very resistant to heat. Therefore, the newly isolated Bacillus sp. CJP-26 may be prepared in the form of feed additive, and then added to feed. Alternatively, the newly isolated Bacillus sp. CJP-26 may be directly added during the feed preparation. The Bacillus sp. CJP-26 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-26 of the present invention may be mixed in a powder form at a ratio of 0.05 to 10% by weight, and preferably at 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-26 of the present invention.

[50] The feeds comprising the Bacillus sp. CJP-26 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, fat, single-cell proteins, zooplankton, and fish

meals, but are not limited thereto.

[51]

[52] In the present invention, the probiotic composition comprising the *Bacillus* sp. CJP-26 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-26 may further include feed premixes, but is not limited thereto.

[53] 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 concentrate thereof, or the dry product thereof.

[54] As described above, the newly isolated *Bacillus* sp. CJP-26 of the present invention has a wide variety of antimicrobial activity and also inhibits symbiosis, virulence, competence, biofilm formation. Thus, the strain is used to prevent diseases caused by common pathogenic bacteria in aquaculture, thereby allowing the culturing of fish or crustaceans with safety.

[55] In still another embodiment, the present invention provides an antimicrobial agent comprising the newly isolated *Bacillus* strain, the culture broth thereof, the concentrate thereof, or the dry product thereof. Further, the present invention provides a method for preventing biofilm formation that is a source of pollution in fish or crustacean farms using the newly isolated *Bacillus* strain, the culture broth thereof, the concentrate thereof, or the dry product thereof, or a method for preventing diseases caused by pathogens in animals excluding human using the newly isolated *Bacillus* strain, the culture broth thereof, the concentrate thereof, or the dry product thereof.

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

[57] The newly isolated *Bacillus* sp. CJP-26 of the present invention has antimicrobial activity against the above described 6 types of pathogenic bacteria in aquaculture, and produces siderophores to show excellent iron-capturing ability. Thus, the newly isolated *Bacillus* sp. CJP-26 degrades a quorum-sensing signal molecule of *Vibrio harveyi*, autoinducer-2 and inhibits biofilm formation, thereby controlling quorum-sensing of *Vibrio harveyi* and inhibiting symbiosis, virulence, competence, and biofilm formation. Therefore, diseases caused by *Vibrio harveyi* can be prevented. In addition, it was confirmed that the newly isolated *Bacillus* sp. CJP-26 has antimicrobial activity against 6 types of representative pathogenic bacteria in aquaculture and shows no hemolysis in a blood agar plate. Thus, the newly isolated *Bacillus* sp. is used to prevent

the diseases caused by the above described pathogenic bacteria in aquaculture.

- [58] The pathogenic bacteria refer to bacteria causing diseases, and the specific pathogenic bacteria that can be prevented comprise *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio anguillarum*, *Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio hemolyticus*, and most preferably, *Vibrio harveyi*.
- [59] 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.
- [60] The newly isolated *Bacillus* sp. CJP-26 of the present invention inhibits biofilms formed by *Vibrio harveyi*, and reduces the content of ammonia present in the aquaculture environment. To improve water quality, the newly isolated *Bacillus* sp. CJP-26 of the present invention may be separately prepared in the form of an agent for improving water quality, or the strain and/or the probiotic composition may be directly sprayed. The *Bacillus* sp. CJP-26 in the agent for improving water quality of the present invention may be in a liquid or dry form, and preferably in a dry powdery form.
- [61] 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, emulsions, pills, capsules, granules, or tablets.
- [62] If the agent for improving water quality is used, the water quality of the 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 may be added to the aquaculture farm before aquaculture and left for a predetermined period. Consequently, the newly isolated *Bacillus* sp. CJP-26 of the present invention is used to degrade quorum-sensing signal molecules, thereby inhibiting biofilm formation. In addition, the agent for improving water quality is added during aquaculture once or more so as to prevent additional biofilm formation.
- [63]
- [64] 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 can 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 the known method.

[65]

Mode for the Invention

[66] 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.

[67]

Example 1: Isolation of Bacillus sp. CJS-26

Example 1-1: Sample Acquisition and Strain Isolation

[70] 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 were 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.

[71]

Example 1-2: Selection of Strains Having High Antimicrobial Activity

[73] 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.

[74] The antimicrobial activity against pathogenic bacteria was evaluated by clear zone analysis. Specifically, 3 ml of 0.7% agar (W/V) and 150 µl each of the shaking culture broth of the 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 clear zone surrounding the dropped strains was observed (Table 1).

[75]

[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] As shown in Table 1, among 53 types of strains, the strains showing antimicrobial activity against any one of 6 types of pathogenic bacteria in fish aquaculture were total 20 strains (Strain Nos. 1, 3, 6, 7, 9, 11, 12, 13, 14, 15, 23, 26, 27, 29, 32, 34, 35, 38, 52 and 53). 6 types of them (Strain Nos. 7, 9, 15, 32, 34 and 38) showed antimicrobial activity against only one type of the pathogenic bacteria, but the rest 14 types of them (Strain Nos. 1, 3, 6, 11, 12, 13, 14, 23, 26, 27, 29, 35, 52 and 53) showed complex antimicrobial activity against two or more types of the pathogenic bacteria. Thus, 14 types of the strains showing complex antimicrobial activity were primarily screened.

[87]

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

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

[90] Among 14 types of the strains showing complex antimicrobial activity, strains having complex digestive enzyme activity were selected.

[91] Specifically, 14 types of the strains having complex antimicrobial activity that were selected in Example 1-2 were cultured in BHI medium for 8 and 24 hours, and their culture broth was centrifuged at 4°C and 13,000 rpm for 5 minutes to obtain each supernatant. Each supernatant was used as a crude enzyme extract for the analysis of enzymatic activity, and activities of protease, cellulase, amylase, and lipase included in the supernatant were determined as follows.

[92]

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

[94] A YM (Difco, USA) medium supplemented with 2% skim milk (Sigma, USA) as a protease substrate was prepared. Each crude enzyme extract obtained in Example 1-3-1 was added to the substrate medium, and then reacted at 30°C for 2 hours. After completion of the reaction, protease activity in each crude enzyme extract was determined by measuring the area of clear zone, which is formed by degradation of the substrate in the medium by the protease included in each crude enzyme extract.

[95]

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

[97] A YM medium supplemented with 1% CMC (carboxyl methyl cellulose) as a cellulase substrate was prepared, and each crude enzyme extract obtained in Example 1-3-1 was added thereto, and then reacted at 30°C for 2 hours. After completion of the reaction, cellulase activity in each crude enzyme extract was determined by measuring the area of the clear zone, which is formed by degradation of the substrate in the medium by the cellulase included in each crude enzyme extract.

[98]

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

[100] A YM medium supplemented with 1% soluble starch as an amylase substrate was

prepared, and each crude enzyme extract obtained in Example 1-3-1 was added to the substrate medium, and then reacted at 30°C for 2 hours. After completion of the reaction, amylase activity in each crude enzyme extract was determined by measuring the area of the clear zone, which is formed by degradation of the substrate in the medium by the amylase included in each crude enzyme extract.

[101]

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

[103] A YM medium supplemented with 1% tricaptylin as a lipase substrate was prepared, and each crude enzyme extract obtained in Example 1-3-1 was added thereto, and then reacted at 30°C for 2 hours. After completion of the reaction, lipase activity in each crude enzyme extract was determined by measuring the area of the clear zone, which is formed by degradation of the substrate in the medium by the lipase included in each crude enzyme extract.

[104]

[105] **Example 1-3-6: Comparison of Enzymatic Activity in Selected Strains**

[106] Each enzymatic activity determined in Examples 1-3-2 to 1-3-5 was compared, and 6 types of the strains having excellent activities of the four enzymes (Strain Nos. 11, 12, 14, 23, 26 and 52) were secondarily selected (Table 2). 6 types of the strains were designated as CJS11, CJS12, CJS114, CJS26, CJS59 and CJS83, respectively.

[107]

[108] Table 2

[Table 2]

Enzyme productivity of secondary screened strains

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

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

[110]

[111] **Example 2: Selection of Strain Having Quorum-Sensing Controlling Effect and**

Biofilm Inhibitory Effect

[112] From 6 types of the strains having excellent antimicrobial activity and enzymatic activity selected in Example 1, strains capable of degrading quorum-sensing-including autoinducers and inhibiting biofilm formation were selected.

[113]

Example 2-1: Selection of Strain Having Quorum-Sensing Controlling Effect

[115] *Vibrio harveyi* BB120 (ATCC BAA-1116, USA) that is a standard quorum-sensing strain responding to autoinducer-1 and autoinducer-2, and *Vibrio harveyi* BB170 that is a mutant strain responding to autoinducer-2 only were used to select strains capable of inhibiting activity of autoinducer-2 inducing quorum-sensing in *Vibrio harveyi*, among the 6 types of strains having excellent antimicrobial activity and enzymatic activity selected in Example 1. In this regard, the method of selecting the strain capable of inhibiting autoinducer-2 activity was performed on the basis of the fact that *Vibrio harveyi* BB120 and *Vibrio harveyi* BB170 are luminescent by quorum-sensing reaction when they are close to each other.

[116]

[117] First, a quorum-sensing evaluation medium (AB medium) in which the two type of *Vibrio harveyi* can be cultured was prepared according to the composition of the following Table 3.

[118]

[119] Table 3

[Table 3]

Medium composition (AB medium) for quorum-sensing evaluation

Sodium chloride (NaCl)	17.5 g
Magnesium sulfate (MgSO ₄)	12.3 g
Casamino acid	2.0 g
1M Potassium phosphate	10
0.1M L-arginine	10
Glycerol	10

[120]

[121] Subsequently, 0.5% (v/v) of *Vibrio harveyi* BB120 was inoculated in the AB medium, and cultured at 30°C and 200 rpm for 12 hours, and 0.02% (v/v) of *Vibrio harveyi* BB170 was inoculated in the AB medium, and cultured at 30°C and 200 rpm for 3 hours. Each of the 6 types of the strains selected in Example 1 was inoculated with 0.1% (v/v) in BHI medium and cultured for 8 hours.

[122] After completion of the cultivation, each culture broth was centrifuged at 4°C and

13,000 rpm, and each supernatant was filtered using a 0.45 µm filter to obtain the active fraction of each strain.

[123]

[124] In order to evaluate that the 6 types of probiotic strains selected are able to degrade the *Vibrio harveyi* quorum-sensing signal molecule, autoinducer-2 (AHL), the *Vibrio harveyi* BB120 culture supernatant and the culture broth of each selected probiotic strain were mixed at a ratio of 1 : 1 and reacted for 3 hours. Then, 1% thereof was added to *Vibrio harveyi* BB120 cultured for 3 hours, and further cultured at 30°C and 200 rpm for 4 hours to measure luminescence using a Luminometer.

[125]

[126] As a result, 6 types of the selected strains significantly degraded *Vibrio harveyi* autoinducer-2 (AHL) ($p < 0.001$). Among them, 3 types (CJS11, CJS26 and CJS83) showing the most excellent degradation of autoinducer-2 (AHL) were selected (FIG. 1). FIG. 1 is a graph showing degradation of *Vibrio harveyi* quorum-sensing signal molecule, autoinducer-2 (AHL) by the *Bacillus* CJS-26 of the present invention.

[127]

[128] **Example 2-2: Selection of Strain Having Inhibitory Effect on Biofilm formation by *Vibrio harveyi***

[129] In order to select the probiotic strains capable of inhibiting biofilm formation by *Vibrio harveyi*, the above strains capable of effectively controlling quorum-sensing were evaluated. After *Vibrio harveyi* was cultured, a biofilm-forming group was used as a control group, and a treatment group was treated with each of the selected probiotic strains and the evaluation was repeated three times as follows.

[130] 200 µl of *Vibrio harveyi* was inoculated in 3 ml of LB medium (Difco, USA) supplemented with 3% sodium chloride, and cultured at 30°C for 6 hours. Thereafter, each 200 µl of the selected probiotic strains was added, and further cultured for 24 hours. Each culture broth cultured for total 30 hours was removed, and stained with 0.4% crystal violet for 10 minutes to examine the presence of biofilm.

[131]

[132] Table 4

[Table 4]

Evaluation of inhibitory effect on biofilm formation by 30-hr cultivation

<i>Vibrio harveyi</i>	+++
CJS114	+
CJS26	-
CJS53	+

[133]

[134] As shown in Table 4, *Vibrio harveyi* formed a violet biofilm band stained with crystal violet, whereas all the probiotic strains inhibited biofilm formation, during 30-hr cultivation (FIG. 2). FIG. 2 is a photograph showing that the *Bacillus* CJS-26 inhibited biofilm formation by *Vibrio harveyi* when it was cultured for 30 hours.

[135]

[136] **Example 3: Identification of Selected Strain and Physiological and Biochemical Characterization thereof**

[137] **Example 3-1: Strain Identification**

[138] Among the 3 types the selected, the CJS-26 strain that effectively controls quorum-sensing and inhibits biofilm formation was finally selected, identified, and analyzed. Identification of the strain was performed by physiological and biochemical methods and a molecular systematic method. 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.

[139] The base sequence analysis was performed by amplification of 16s rDNA using a PCR premix (Bioneer, Korea) and universal primers 27F and 1492R having the following base sequence.

[140] 27F: 5'-AGAGTTTGATCMTGGCTCAG-3'(SEQ ID NO. 2)

[141] 1492R: 5'-GGYTACCTTGTTACGACTT-3'(SEQ ID NO. 3)

[142]

[143] The gene amplification was performed in a total reaction volume of 20 μ l 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. 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. CJS-26" (KCCM11144P).

[144]

[145] **Example 3-2: Physiological and Biochemical characterization**

[146] In order to analyze the biochemical characteristic of the *Bacillus* sp. CJP-26 of the present invention, sugar fermentation patterns of the strain were analyzed using API 50 CHB system (Korean Culture Center of Microorganisms, Korea) (Table 5).

[147]

[148] Table 5

[Table 5]

Result of sugar fermentation patterns

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

[149] +, Positive; -, Negative

[150]

[151] **Example 4: Safety and Utilization**[152] **Example 4-1: β-Hemolysis**

- [153] β -Hemolysis is a complete lysis of red blood cells, which is caused by hydrolysis of phospholipid by phospholipase-producing bacteria.
- [154] In order to examine hemolysis of the *Bacillus* sp. CJP-26, blood agar plate containing TSA(tryptic soy agar)(Difco, USA) and 5% sheep blood (Kisan Biotech, Korea) was prepared. After the *Bacillus* strain CJS26 of the present invention was streaked on the prepared blood agar plate, incubation was performed at 37°C for 24 hours to examine hemolysis. In this regard, when the agar turned white or yellow around the cultured strain, it was determined as hemolysis. When no color change was observed around the cultured strain, it was determined as non-hemolysis (see FIG. 3). FIG. 3 is a photograph showing the presence of hemolysis of the *Bacillus* CJS-26 of the present invention. As shown in FIG. 3, the *Bacillus* CJS-26 of the present invention showed no hemolysis.

[155]

[156] **Example 4-2: Ammonia Utilization**

- [157] In order to examine the ammonia utilization of the *Bacillus* CJS-26 of the present invention, a medium containing ammonia was prepared as shown in the following Table 6. 0.1% of the *Bacillus* sp. CJS-26 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 ammonia in the obtained supernatant was measured to quantify the consumption (see FIG. 4).

[158]

[159] Table 6

[Table 6]

Medium composition for evaluation of ammonia utilization

Ammonium sulfate ((NH ₄) ₂ SO ₄)	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	0.5 g/L

[160]

[161] FIG. 4 is a graph showing ammonia utilization of the Bacillus CJS-26 of the present invention. As shown in FIG. 4, the initial concentration of ammonia in the medium was approximately 400 ppm. As the bacillus sp. CJS-26 grew to utilize ammonia, ammonia was not detected after cultivation for 30 hours (see FIG. 4). No detection of ammonia in the medium suggests that the Bacillus CJS-26 of the present invention is able to prevent water pollution by ammonia, and reduce ammonia causing stress for fish or crustaceans, thereby improving the quality of the cultured fish or crustaceans.

[162]

[163] The above results demonstrate that the Bacillus CJS-26 controlling quorum-sensing of pathogenic bacteria including *Vibrio harveyi*, has antimicrobial activity and produces complex digestive enzymes to facilitate fish ingestion rate and rate of weight gain and to reduce excretion, leading to improvement of water quality.

[164]

[165] **Example 5: Efficacy of Probiotics**

[166] In order to examine whether the Bacillus sp. CJP-26 of the present invention practically shows probiotic action when it is added to feed and fish is fed with the feed, the Bacillus sp. CJP-26 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.

[167] Specifically, a basal feed (control group) was prepared to have energy content equal to 30% crude protein, as shown in Table 7. The Bacillus sp. CJP-26 of the present invention was added to the basal feed to make mixed feed containing 5% Bacillus sp. CJP-26, and the amount of cellulose was reduced to the addition amount of probiotics for equal energy content.

[168]

[169] Table 7

[Table 7]

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

[170]

[171] 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. 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 8).

[172]

[173] Table 8

[Table 8]

Efficacy of probiotics in flounder

	Control group	Experimental group
Final average weight (g)	50.6 ± 1.7^a	55.1 ± 2.5^b
Rate of weight gain (%)	103.03 ± 5.6^a	121.16 ± 18.3^b
Daily growth rate (%)	1.42 ± 0.06^a	1.58 ± 0.12^b
Survival rate (%)	77.3 ± 15.1^a	98.7 ± 2.3^b
Feed conversion ratio	1.59 ± 0.15^b	1.24 ± 0.05^b
Protein efficiency ratio	1.10 ± 0.1^a	1.30 ± 0.1^b

[174]

[175] As shown in Table 8 the addition of the *Bacillus* sp. CJP 26 increased the growth rate to approximately 16%, 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 by approximately 21%, compared to the control group. Therefore, the strain of the present invention effectively facilitates fish ingestion rate and rate of weight gain.

[176]



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. CJS-26	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11144P
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, Seodaemun-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

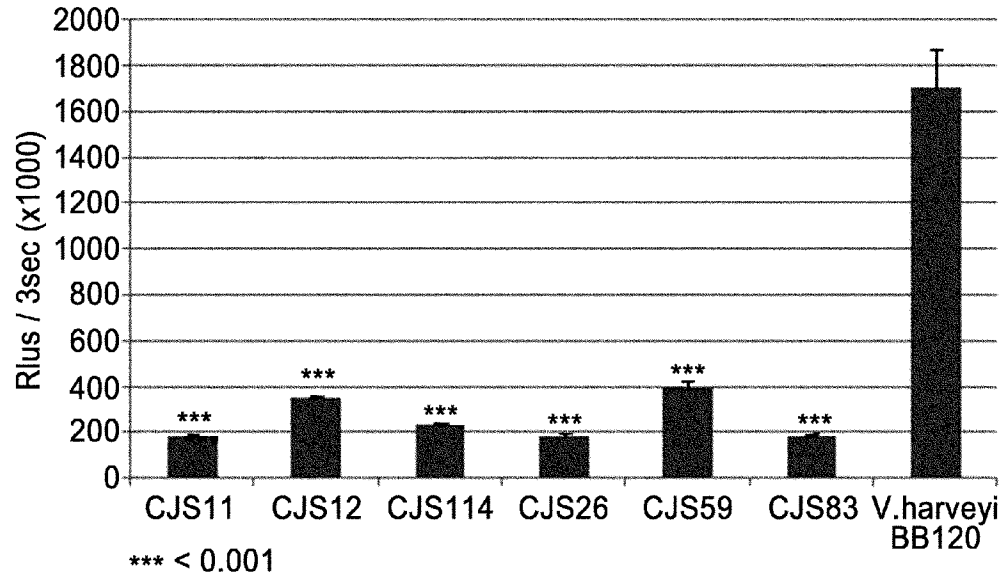
- [Claim 1] A *Bacillus* sp. CJS-26 (KCCM11144P).
- [Claim 2] A culture broth of the *Bacillus* sp. CJS-26 (KCCM11144P) of claim 1, a concentrate thereof, or a dry product thereof.
- [Claim 3] A probiotic composition comprising the strain of claim 1, or the culture broth, the concentrate thereof, or 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 for the 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 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 degrading quorum-sensing signal molecules of *Vibrio harveyi* and inhibiting biofilm formation, comprising the steps of:
(a) culturing fish or crustaceans in an aquaculture farm; and
(b) adding the strain of claim 1, or the culture broth, the concentrate thereof, or the dry product thereof according to claim 2, to the aquaculture farm.
- [Claim 9] The method according to claim 8, wherein step (a) and step (b) are performed sequentially, simultaneously, or in reverse order.
- [Claim 10] A method for preventing diseases caused by pathogenic bacteria 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 11] The method according to claim 10, wherein the pathogenic bacteria is selected from the group consisting of *Aeromonas salmonicida*, *Vibrio harveyi*, *Vibrio anguillarum*, *Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio hemolyticus*.
- [Claim 12] 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, as an active ingredient.

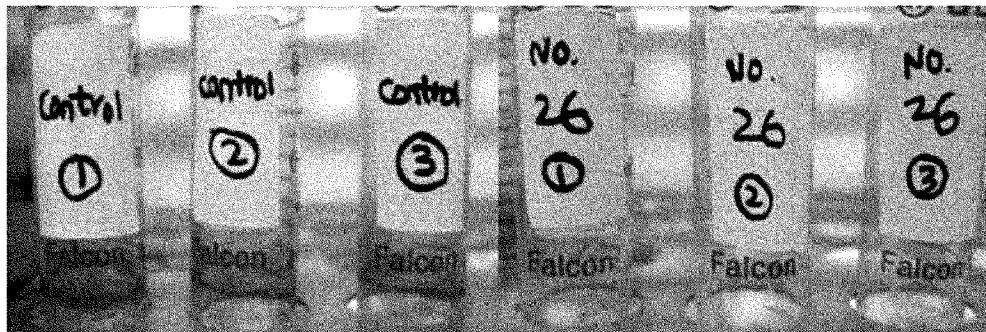
[Claim 13]

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.

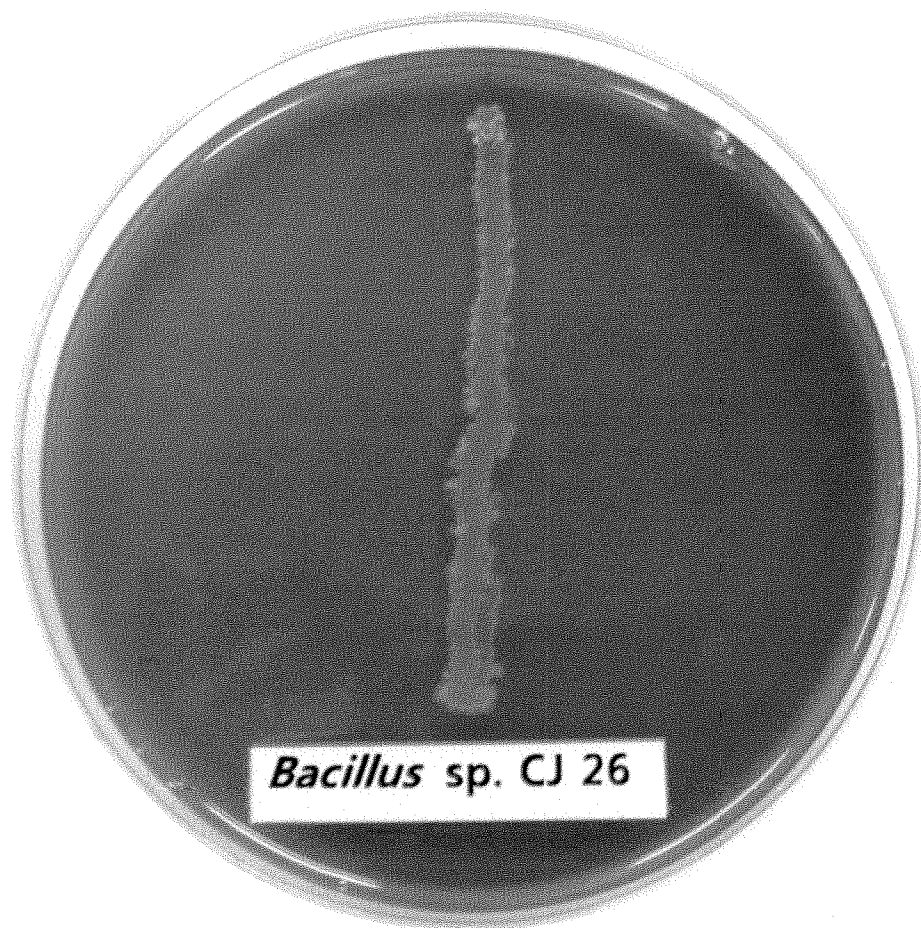
[Fig. 1]



[Fig. 2]



[Fig. 3]



[Fig. 4]

