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(54) **Title:** COMPOSITION COMPRISING STILBENES SUITABLE FOR ENHANCING AQUACULTURE PRODUCTIVITY

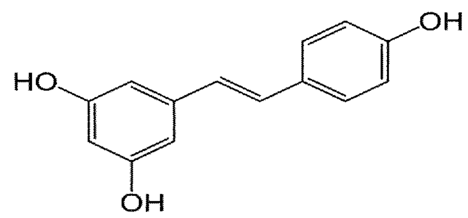

Figure 1. Chemical structure of trans-resveratrol.

(57) **Abstract:** Compositions comprising and methods of administering stilbenes in aquaculture feed, to improve the survival, health, growth and/or efficiency of feed conversion into biomass in fish and crustacean decapoda.

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Title: COMPOSITION COMPRISING STILBENES SUITABLE FOR ENHANCING AQUACULTURE PRODUCTIVITY

Field of the Invention

[0001] The present invention relates to compositions, methods and uses thereof to improve yield and quality of farmed or bred fish and decapod crustaceans.

Background of the invention

[0002] Fish and decapod crustaceans (such as crayfish, shrimp, prawn, crab and lobsters) have been farmed extensively for many centuries, by fostering a natural environment with little or no inputs. This developed into semi-intensive farming with a greater use of inputs. The end of the 20th century saw the development of intensive fish farming, high input systems with high stocking densities. For example, in India, which is the second largest producer of fresh water farmed fishes in the world, there is 2.414 ha of potential area in the country available for aquaculture, almost 60 % of which is at present utilized and there is scope for horizontal expansion to the remaining 40% area. However, due to the burgeoning demand for fish as a protein source, horizontal expansion even up to 100% utilization level of pond resources may not be sufficient to cater for the need. A vertical increase in the average productivity of the culture system from the present level of 2.9 t/ha to a level higher than 5.0- 6.0 t/ha may be required. Such an increase can only be realized with the adoption of new tools and techniques permitting higher stocking density culturing.

[0003] In decapod and fish farming practices, stocking density is a key factor in determining the productivity and profitability of commercial fish farms. However higher stocking density increases stress and in turn affect fish growth, feed utilization and fish yield. Additional problems such as the risk of disease outbreak coupled with regulatory limitations or complete exclusion of the use of antibiotics, together with feed with a high vegetal content, may lead to a reduction in survival and growth as well as a lower efficiency of feed use. Infections caused by bacterial and fungal pathogens are being increasingly recognized as a significant constraint on aquaculture production and constitute the single largest cause of economic losses in this sector. The major contributors of aquatic bacterial diseases include *V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. harveyi, Aeromonas hydrophila, Pseudomonas fluorescens*, *Edwardsiella tarda* and *Streptococcus* sp. Similarly, the other fungal pathogens include *Aspergillus flavus* and *Fusarium solani*.

[0004] There are diverse approaches have been followed as prophylaxis and treatment measures against diseases in aquaculture systems, but administration of antibiotics is currently employed as a preferred method of treatment and/or pretreatment. However, the indiscriminate and inappropriate usage of antibiotics leads to the development of resistance among aquatic pathogens and makes antibiotic treatment ineffective. Administration of antibiotics to aquatic environments presents additional problems regarding lack of containment and lack of specificity of target organism.

[0005] Thus there is a need for cost-effective and environmentally friendly feed additives which enhance the metabolism and energy of fish body cells, raise the efficiency of feed utilization and balance immune modulators appropriate to the stock density, whilst also having inhibitory or curative effects specific to aquatic pathogens without damaging the surrounding aquatic ecosystem or beneficial symbionts.

[0006] Resveratrol and its derivatives are phytoalexin stilbenes found in very low concentrations in grapes, peanuts, cranberries, blueberries, strawberries, *Polygonum cuspidatum* and some other botanical sources. The content of resveratrol and resveratrol glycosides increases in grapes increases upon infection with *Botrytis cinerea* (Roldan 2003; Sahin et al. 2010; Alagawany et al. 2015).

[0007] Resveratrol is an antioxidant and has natural antibacterial properties, which can be used in both food preservation and medicinal use (Paulo et al. 2010), and may also be a potent antiviral molecule against DNA and RNA viruses (Campagna & Rivas 2010). However, although there is much interest in the use of resveratrol in addressing a variety of issues, difficulties associated with producing the compound restrict its use in husbandry, the human environment and food-related industries. In the current market, the most common resveratrol production processes relies on extracting resveratrol from Japanese knotweed, a plant from the polygonaceae family grown commercially in China. Unfortunately, the extraction process is labour intensive and the yield of resveratrol is low, which is especially a problem for applications that would benefit from large volumes of stilbene either over time the course of one year or because of the potentially very broad application of the technology. A further problem with conventional resveratrol extraction processes from Japanese knotweed is the presence of contaminants. For example, commercially available resveratrol derived from Japanese knotweed may be contaminated with ground and/or air pollutants from the environment in which the knotweed plants have grown. For example, very high concentrations of poly-aromatic hydrocarbons have been detected in commercially available resveratrol samples derived from knotweed plants grown in various locations in China. Table 1 shows the results of analysis using gas chromatography coupled to mass spectrometry performed on 40 g samples of commercially available resveratrol derived from knotweed plants grown in China (samples 1, 2 and 3) when compared with fermentation derived resveratrol (samples 4 and 5).

Contaminant	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Anthracene	14 µg/kg	15 µg/kg	250 µg/kg	<0.5 µg/kg	0.66 µg/kg
Benzo(a) anthracene	0.89 µg/kg	0.91 µg/kg	300 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Benzo(a) pyrene	0.50 µg/kg	0.52 µg/kg	350 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Benzo(b) fluoranthene	0.74 µg/kg	0.73 µg/kg	350 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Benzo(ghi) perylene	0.52 µg/kg	<0.5 µg/kg	260 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Benzo(k) fluoranthene	<0.5 µg/kg	<0.5 µg/kg	130 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Chrysene	1.2 µg/kg	1.3 µg/kg	360 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Dibenzo(a,h) anthracene	<0.5 µg/kg	<0.5 µg/kg	31 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Fluoranthene	15 µg/kg	15 µg/kg	940 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Fluorene	94 µg/kg	98 µg/kg	280 µg/kg	<0.5 µg/kg	1.3 µg/kg
Indeno(1,2,3-cd)pyrene	<0.5 µg/kg	<0.5 µg/kg	190 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Phenanthrene	67 µg/kg	69 µg/kg	860 µg/kg	1.5 µg/kg	2.8 µg/kg
Pyrene	13 µg/kg	13 µg/kg	920 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Sum PAHs (4 or less rings)	3.3 µg/kg	3.5 µg/kg	1 400 µg/kg	<0.5 µg/kg	<0.5 µg/kg

Sum of heavy" PAHs (5 or more rings)	1.8 µg/kg	1.3 µg/kg	1 300 µg/kg	<0.5 µg/kg	<0.5 µg/kg
Sum of all positive identified PAHs	210 µg/kg	210 µg/kg	5 200 µg/kg	1.5 µg/kg	4.8 µg/kg

Table 1: Polycyclic aromatic hydrocarbon content in resveratrol samples derived from knotweed grown in China (samples 1, 2 and 3) and in resveratrol derived from recombinant microbial fermentation (samples 4 and 5).

[0008] Polycyclic aromatic hydrocarbons (PAHs), otherwise known as polynuclear aromatic hydrocarbons, are chemicals released from incomplete combustion of organic matter, typically by the burning of fossil fuels in smelting, manufacturing, power stations, vehicles and home fires, as well as burning the by-products of agriculture such as crop residues and dung. PAHs generally have low solubility in water, and are predominantly seen in solid form as particulate air pollution, soil or sediment pollution. Although cancer is the primary health risk of exposure to PAHs, they have also been linked to cardiovascular disease and poor fetal development. Hence, the administration of resveratrol extracts comprising environmental pollutants such as has been observed in knotweed-derived resveratrol may raise concerns from the ultimate human consumers of the resulting aquaculture products (such as fish, shellfish and products derived therefrom such as flesh or eggs).

[0009] The rising global human population is placing increasing stress on the current means of production of food and on the use of land areas. There is an environmental need to maximize the efficient use of land already allocated to farming, whilst simultaneously reducing the impact of such modern farming practices to the surrounding natural environments.

[0010] Therefore, in light of the current challenges to commercial rearing of fish and decapod crustaceans, there is a growing need for effective, sustainable, environmentally friendly compositions and methodologies to benefit the health of farmed animals and birds, and to improve the quality and yield of fish, decapod crustaceans and resulting meat.

Summary of the Invention

[0011] Provided herein are effective natural compositions and methods of their use in the rearing of fish and decapod crustaceans.

[0012] In some embodiments a stilbene or a methylated or glycosylated derivative thereof (such as resveratrol or pinosylvin) is applied to feed or feed additive compositions suitable for administration to fish or decapod crustaceans.

[0013] In some embodiments, the composition suitable for feeding a fish or decapod crustacean comprises 50 to 500ppm of a stilbene.

[0014] In some embodiments, the composition further comprises at least one additional ingredient selected from natural or regenerated mineral material (such as calcium), solvent, surfactant, vegetable oil (such as a rapeseed oil), dispersant, carrier, vegetable mass (such as algae, soybeans, seaweed, barley, rice, peas, canola, lupine, wheat gluten, soy, corn or corn gluten), protein sources (such as insects, fish meal, fish trimmings from the fish industry, or small feeder fish stocks bred or harvested from the environment for incorporation in feed), lipids (such as fish oil), wetting agent, adhesive, thickener, binders, attractant, pigments (such as the carotenoids astaxanthin or canthaxanthin), vitamins, antioxidants, essential oil (thymol) or enzyme (such as phytase).

[0015] In some aspects, provided herein is a method of enhancing weight gain and/or length in fish or decapod crustacean by feeding the fish or decapod crustaceans a feed composition comprising 50 to 500ppm of a stilbene applied once per day, once every two days, once per week, twice per week, once per two weeks, once per month, or once per two months, once per three months, or once per lifecycle of the cultured fish or decapod crustacean.

[0016] In some aspects, provided herein is a method of enhancing weight gain and/or length in fish or decapod crustacean by feeding the fish or decapod crustaceans a feed composition comprising 100 to 300ppm of a stilbene applied once per day, once every two days, once per week, twice per week, once per two weeks, once per month, or once per two months, once per three months, or once per lifecycle of the cultured fish or decapod crustacean.

[0017] In some aspects, provided herein is the use of a stilbene in aquaculture to increase the productivity of fish or decapod crustacean, wherein productivity is measured as at least one of increased survival index, weight gain, gain in length, mass of harvestable flesh or mass of eggs compared to fish or decapod crustaceans from the same initial stock not fed compositions comprising stilbene.

[0018] In any of these aspects, the stilbene may be resveratrol, pinosylvin, or a methylated or glycosylated derivative thereof.

[0019] In preferred aspects of the methods and uses provided herein, the fish is a carp, tilapia or salmonid

[0020] In other preferred aspects of the methods and uses provided herein, decapod crustacean is a crayfish, lobster, crab, shrimp or prawn.

Description of drawings and figures

Figure 1 provides the chemical structure of the stilbene trans-resveratrol.

Figure 2 provides a comparison of fish growth with and without the stilbene resveratrol.

Figure 3 provides photographs of *vibrio* biofilm formation following incubations with and without the stilbene resveratrol.

Figure 4 provides a comparison of fingerling gain in length with and without the stilbene resveratrol over an 82 day period.

Figure 5 provides a comparison of fingerling gain in weight with and without the stilbene resveratrol over an 82 day period.

Figure 6 provides a comparison of fingerling survival rates with and without the stilbene resveratrol over an 82 day period.

Figure 7a Activity of resveratrol against biomarker strain *C. violaceum.*

Figure 7b Anti QS activity of resveratrol against violacein production in biomarker strain C*. violaceum.*

Figure 8 Anti QS activity of resveratrol against prodigiosin production in *S. marcescens.*

Figure 9a Antibacterial activity of resveratrol against *P. aeruginosa*

Figure 9b Anti QS activity of resveratrol against pyocyanin production in *P. aeruginosa*

Figure 10a Inhibitory effect of resveratrol on growth of *V. parahaemolyticus*

Figure 10b Inhibitory effect of resveratrol on growth of *V. alginolyticus*

Figure 10c Inhibitory effect of resveratrol on growth of *V. harveyi*

Figure 10d Inhibitory effect of resveratrol on growth of *V. vulnificus*

Figure 10e Inhibitory effect of resveratrol on growth of *A. hydrophila*

Figure 10f Inhibitory effect of resveratrol on growth of *P. fluorescens*

Figure 11a Effect of resveratrol on biofilm of *V. parahaemolyticus*

Figure 11b Effect of resveratrol on biofilm of *V. alginolyticus*

Figure 11c Effect of resveratrol on biofilm of *V. harveyi*

Figure 11d Effect of resveratrol on biofilm of *A. hydrophila*

Figure 11e Effect of resveratrol on biofilm of *V. vulnificus*

Figure 12a Light microscopic analysis of *V. parahaemolyticus* biofilm grown in the absence and presence of resveratrol.

Figure 12b Light microscopic analysis of *V. alginolyticus* biofilm grown in the absence and presence of resveratrol

Figure 12c Light microscopic analysis of *V. harveyi* biofilm grown in the absence and presence of resveratrol

Figure 13 Effect of resveratrol on EPS production in *V. alginolyticus*

Figure 14a Effect of resveratrol on swarming motility of *V. alginolyticus*

Figure 14b Effect of resveratrol on swimming motility of *V. alginolyticus*

Detailed Description of the Invention

[0021] All publications, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety for all purposes.

Definitions

[0022] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

[0023] The term “comprise” and “include” as used throughout the specification and the accompanying claims as well as variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. These words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows. For the purposes of describing and defining the present invention it is noted that the term “substantially” is utilized herein to represent the inherent degree of uncertainty that can be attributed to any quantitative comparison, value, measurement, or other representation. The term “substantially” is also utilized herein to represent the degree by which a quantitative representation can vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

[0024] As used herein, the term “about” refers to ±10% of any particular value.

[0025] As used herein, the terms “or” and “and/or” are utilized to describe multiple components in combination or exclusive of one another. For example, “x, y, and/or z” can refer to “x” alone, “y” alone, “z” alone, “x, y, and z,” “(x and y) or z,” “x or (y and z),” or “x or y or z.”

[0026] It is noted that terms like “preferably,” “commonly,” and “typically” are not utilized herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that can or cannot be utilized in a particular embodiment of the present invention.

[0027] The term “isolating" or “separating” means any human intervention which change the relative amount of the compound compared to another selected constituent in a given matrix to a higher relative amount of the compound relative to the other constituent. In an embodiment, the compound may be isolated into a pure or substantially pure form. In this context, a substantially pure compound means that the compound preparation contains less than 10%, such as less than 8%, such as less than 6%, such as less than 5%, such as less than 4%, such as less than 3%, such as less than 2%, such as less than 1 %, such as less than 0.5% by weight of other selected constituents. In an embodiment, an isolated compound is at least 50% pure, such as at least 60% pure, such as at least 80% pure, such as at least 90% pure, such as at least 91% pure, such as at least 92% pure, such as at least 93% pure, such as at least 94% pure, such as at least 95% pure, such as at least 96% pure, such as at least 97% pure, such as at least 98% pure, such as at least 99% pure, such as at least 99.5% pure, such as 100 % pure by dry weight.

[0028] The term “synthetic” or “non-naturally occurring” means that a compound is not normally found in nature or natural biological systems. In this context, the term “found in nature or in natural biological systems” does not include the finding of a compound in nature resulting from releasing the compound to nature by deliberate or accidental human intervention. Synthetic compounds may include compounds completely or partially synthetized by human intervention and/or compounds prepared by human modification of a natural compound.

[0029] As used herein, the term “aquaculture” relates to the process of rearing a fish or decapod crustacean for at least part of the growth cycle in a semi-controlled environment (aquaculture facility) such that the rate of feeding and any medication may be controlled, and the fish or decapod crustacean is not able to escape into the surrounding environment. The may be reared for fish or decapod crustacean meat for consumption as food or feed, for oil (such as omega 3 or omega 6 oil), eggs (such as caviar), sport, ornamental use, functional use (such as a biological control agent or a cleaner fish) or for breeding programs either for commercial sale or introduction into the wild. Non-limiting examples of an aquaculture facility suitable for such growth of a fish or decapod crustacean may include a fish farm, crustacean farm, hatchery, breeding center, pond, lake, well, tank, enclosure (such as nets or a cage in a river or the sea), freshwater or saltwater aquarium.

[0030] As used herein, the term “fish” refers to any fish farmed or cultured for at least part of the growth cycle. The fish may be a freshwater or saltwater fish. In some aspects the fish may be a carp, catfish, eel, goldfish, tilapia or trout or salmon. Preferably the fish is selected from carp, tilapia or salmonid.

[0031] As used herein, the term “decapod crustacean” refers to crustaceans with 10 legs. The decapod crustacean may be a penaeid. In some aspects the decapod crustacean be a crayfish, lobster, crab (such as the Chinese river crab), shrimp (such as the Pacific White shrimp) or prawn (such as the giant tiger prawn or the giant river prawn).

[0032] As used herein, the term “fish length” and “decapod crustacean length” refer to the total length from front to rear. Figures provided herein are average values of fish length or decapod crustacean length for the whole batch being reared.

[0033] As used herein, the term “fish weight” and “decapod crustacean length” refers to total mass of the fish or decapod. Figures provided herein are average values of fish weight or decapod crustacean weight for the whole batch being reared.

[0034] As used herein, the term “survival index” refers to the proportion, frequently expressed as a percentage, of fish or decapod crustaceans in a batch surviving a specified growth phase; for example, the survival index for fry to fingerling, or the survival index from fingerling to maturity.

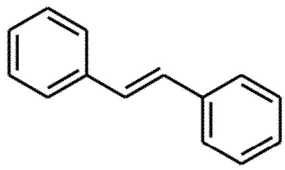
[0035] As used herein, the term “improved health” refers to a general vitality, vigor and absence of disease relative to an equivalent batch of fish or decapod crustaceans from the same stock but not receiving feed supplemented with resveratrol. Typical indications of improved health may include one or more of higher rates of activity, greater appetite for feed, a lack of blemishes or infections in the eyes, scales, shells, legs or fins. Less empirical, but equally valuable perceived indicators of the health of ornamental or farmed fish and crustacean decapods, is a more reflective or shinny appearance of scales or shells. Conversely, “improved health” may also be recognized as a lower incidence of the fish or decapod crustaceans exhibiting moribund characteristics, overt signs of illness, or apathy to external stimuli.

[0036] As used herein, the term “active ingredient” refers to a chemical compound or mixture of chemical compounds capable of enhancing growth, reducing mortality, improving health, or improving the taste, colour or valuable oil content of fish or decapod crustaceans or products derived therefrom.

Compounds

[0037] According to some aspects, the stilbene or a methylated or glycosylated derivative thereof may be resveratrol (trans-resveratrol) or pinosylvin. Resveratrol may be provided at suitable concentrations as purified extracts of Japanese knotweed, yucca, grapes, etc. According to preferred aspects, the resveratrol for inclusion in the feed, feed additives or water may be produced by recombinant microbial fermentation, including but not limited to fermentation extract, purified resveratrol, or dried fermentation cake from recombinant microbes capable of producing resveratrol. Resveratrol derived from fermentation of recombinant microbes is preferred not least because it is inherently free of emodin and hazardous levels of Polycyclic Aromatic Hydrocarbons (PAHs).

[0038] As used herein, the terms "stilbene" and "stilbenoid" are interchangeable and refer to compounds based on the compound of formula (I):


(I),

wherein formula (I) may be substituted at one or more suitable positions. Exemplary substituents include, but are not limited to, halogen, cyano, nitro, C_1-C_6 alkyl, C_1-C_6 haloalkyl, C_1-C_6 hydroxyalkyl, hydroxy, C_1-C_6 alkoxy, thiol, C_1-C_6 alkylthio, amino, C_1-C_6 alkyl amino, di- C_1-C_6 alkyl amino, carboxyl, C_1-C_6 alkoxycarbonyl, amido, methyl, and glycosyl. Generally, stilbenes, including resveratrol, and flavonoids are produced in plants and yeast through the phenylpropanoid pathway as illustrated by the reactions shown in Figure 1.

[0039] As used herein, the term "phenylpropanoid" refers to compounds based on a 3-phenylprop-2-enoate backbone. Examples of such compounds include, but are not limited to, cinnamic acid, coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, sinapinic acid, cinnamoyl-CoA, p-coumaroyl-CoA, and the like.

[0040] As used herein, the term "phenylpropanoid derivative" refers to any compound derived from, synthesized from, or biosynthesized from a phenylpropanoid; *i.e.* a phenylpropanoid derivative includes any compound for which a phenylpropanoid compound is a precursor or intermediate. Examples of phenylpropanoid derivatives include, but are not limited to, stilbenoid compounds and chalcone compounds. Specific examples of phenylpropanoid derivatives include, but are not limited to, resveratrol, pinosylvin, pinocembrin chalcone, and pinocembrin.

[0041] As used herein, the term “dihydrophenylpropanoid” refers to compounds based on a phenylpropanoate backbone. Examples of such compounds include, but are not limited to, dihydrocinnamic acid, phloretic acid, 3,4-dihydroxyhydrocinnamic acid, hydroferulic acid, dihydrocoumaroyl-CoA, dihydrocinnamoyl-CoA, and the like.

[0042] As used herein, the term “dihydrophenylpropanoid derivative” refers to any compound derived from, synthesized from, or biosynthesized from a dihydrophenylpropanoid; *i.e.* a dihydrophenylpropanoid derivative includes any compound for which a dihydrophenylpropanoid compound is a precursor or intermediate. Examples of dihydrophenylpropanoid derivatives include, but are not limited to, dihydrostilbenoid compounds and dihydrochalcone compounds. Specific examples of dihydrophenylpropanoid derivatives include, but are not limited to, phloretin, phlorizin, dihydropinosylvin, dihydropinosylvincarboxylate, 3-O-methyldihydropinosylvincarboxylate, 4-isoprenyl-3-O-methyldihydropinosylvincarboxylate (amorfrutin 1), 3-O-methyldihydropinosylvin, 4-isoprenyl-3-O-methyldihydropinosylvin (amorfrutin 2), 5-hydroxy-lunularic acid, and dihydroresveratrol.

[0043] As used herein, the terms “phenylpropanoid pathway,” “phenylpropanoid derivative pathway,” “phenylpropanoid derivative synthesis pathway,” and “phenylpropanoid derivative biosynthesis pathway” are interchangeable and refer to any biosynthesis pathway in which a phenylpropanoid is a precursor or intermediate.

[0044] The term “stilbene” or a derivative thereof can be hydroxylated derivatives of stilbene and are thus encompassed by the term “stilbene” as used herein. The term “stilbene” includes but is not limited to at least one of resveratrol, dihydroresveratrol, and pinosilvin; a glycosylated stilbene comprising piceid (3-resveratrol monoglucoside or 5-resverarol monoglucoside), resveratroloside (4'-resveratrol monoglucoside), Mulberroside E (3,4'-resveratrol diglucoside), 3,5- resveratrol diglucoside, and 3,5,4'-resveratrol triglucoside and their dihydro- reduced equivalents; a methylated stilbene comprising pterostilbene (3,5-dimethoxy-4'-hydroxy-trans-stilbene), 3,5,4'-trimethoxystilbene, pinostilbene, tetramethoxystilbene, pentamethoxystilbene, and N-Hydroxy-N- (trimethoxphenyl)-trimethoxy-benzamidine and their and their dihydro-reduced equivalents.

[0045] As used herein, the term “resveratrol” refers to a compound seen in Figure 1 that may be synthesized, isolated, and purified from of a mixture of products produced in a host modified to express enzymes of the resveratrol biosynthetic pathway or that can be produced from natually occurring sources, such as grapes. “Resveratrol” further refers to derivatives and analogs analogs thereof, including but not limited to forms of methylated and/or glucosylated resveratrol. For example, the resveratrol compound contemplated for use herein may be produced *in vivo* through expression of one or more enzymes involved in the resveratrol biosynthetic pathway in a recombinant yeast or *in vitro* using isolated, purified enzymes involved in the resveratrol biosynthetic pathway, such as those described in WO2006/089898, WO2008/009728, WO2009/016108, WO2009/124879, WO2009/124967, WO2011/147818, which are incorporated by reference in their entirety. Resveratrol derived from fermentation of recombinant microbes is preferred not least because it is inherently free of emodin and hazardous levels of Polycyclic Aromatic Hydrocarbons (PAHs). Therefore, resveratrol as defined herein can differ chemically from other sources of resveratrol, such as extracts from plants and derivatives thereof, or may include such plant extracts and derivatives thereof.

[0046] As used herein, the terms “modified resveratrol,” “resveratrol derivative,” and “resveratrol analog” can be used interchangeably to refer to a compound that can be derived from resveratrol or a compound with a similar structure to resveratrol. For example, the terms “modified resveratrol,” “resveratrol derivative,” and “resveratrol analog” can refer to resveratrol-like molecules such as to glycosylated resveratrol molecules, methylated resveratrol molecules, or resveratrol molecules that are glycosylated and methylated.

[0047] As disclosed herein, the term “glycosylated resveratrol" refers to resveratrol glycosylated at the 3 hydroxyl group, or the 4’ hydroxyl group, or the 5 hydroxyl group of resveratrol, wherein glycosylation comprises covalently attaching one or a plurality of sugar or saccharide residues at one or more of the 3, 4’, or 5 hydroxyl groups of resveratrol. The saccharide moiety in each position can be independently zero, one, two, three, or multiple sugar residues, wherein all the sugar residues can be the same sugar residues or different sugar residues.

Compositions

[0048] The active ingredients contemplated herein are used in the form of compositions. The active ingredients can be applied to or incorporated into feed, to form a comestible composition suitable for ingestion by fish and/or decapod crustaceans. The active ingredients can be applied, if desired, together with other carriers conventionally used in the formulation art of feed, feed additives, surfactants or other additives which aid application. Suitable carriers and additives can be solid or liquid and are the substances expediently used in the art of formulation, for example natural or regenerated mineral materials, solvents, dispersants, wetting agents, adhesives, thickeners, binders or attractants.

[0049] Fish feed and decapod crustacean feeds typical comprise major nutrients such as protein, fat, carbohydrate, vitamins and minerals, and optionally one or more other feed additive that facilitates feed intake, digestion and absorption of nutrient.

[0050] In one aspect, the resveratrol-containing compositions may comprise the fermentation cake of microorganisms capable of producing resveratrol and/or carotinoids. The microorganism engineered to produce resveratrol and/or carotinoids may be any food or feed-approved microorganism, preferably a yeast, such as baker's or brewer's yeast (*Saccharomyces cerevisiae*), *Kluveromyces marxianus*, *Candida utilis, Rhodotorula glutinis*. Incorporation of fermentation cake comprising resveratrol-producing microorganisms into the feed or feed additives suitable administration to fish or decapod crustaceans has the advantage of providing additional nutrients, vitamins and minerals present in the fermentation broth or produced naturally by the microbes. In a preferred embodiment of this aspect, the resveratrol-producing recombinant microorganism is *saccharomyces cerevisiae* free of antibiotic markers and grown in a fermentation medium not comprising antibiotics. *Saccharomyces cerevisiae* has been included in feed for many years, and is sometimes known as "Feed yeast". The use of feed yeast (an example of a "feed microorganism") has increased since the Bovine Spongiform Encephalopathy crisis, in consequence of the demand for "clean" and sustainable protein sources which could not support the growth or reproduction of viruses or prions able to cross animal species barriers. *Saccharomyces cerevisiae* are now appreciated to have a high Protein Efficiency Ratio, have a high lysine content, are an excellent source of vitamin D and further comprise significant concentrations of the nutrients glutathione, inositol and choline. Yeast beta-glucans and mannans also help stimulate the immune system of many species. Yeast are considered by some to be probiotics for fish aquaculture.

[0051] In some aspects, the resveratrol-producing microorganisms may also be engineered to produce enzymes desirable for incorporation into feeds, additives or supplements suitable for administration to fish and/or decapod crustaceans, such as to improve the efficiency of feed utilization or the health of the fish and/or decapod crustacean.

[0052] In some aspects, the resveratrol-producing microorganisms (such as recombinant *cerevisiae*) are present as whole cells in "direct-fed microbial" compositions such as active dry yeast, yeast culture (such as molasses yeast condensed solubles). In some aspects, the resveratrol-producing microorganisms (such as recombinant *cerevisiae*) are present as whole cells that are heat treated, irradiated, lysed (such as by sonication or by hydrolysis), pasteurized, or in another way rendered "non-fermentive" so that the viability of recombinant microorganisms in the resulting composition is significantly reduced, preferably reduced to the extent that no viable recombinant microbes may be detected. The resveratrol, optionally with saponins, thymol and/or recombinantly produced enzymes suitable for inclusion in fish feed or decapod crustacean feed, additive or supplement thereof, as a "yeast extract" comprising concentrated soluble materials recovered from lysed yeast cells following fermentative growth.

[0053] In other embodiments of the invention, compositions contemplated herein can contain a carrier and at least about 0.0001%, or at least about 0.001%, or at least about 0.01%, or at least about 0.1%, or at least about 1%, or at least about 2%, or at least about 5%, or at least about 7.5%, or at least about 10%, or greater than about 10%, or greater than about 15%, or greater than about 20%, or greater than about 25%, or greater than about 50% by weight stilbene, such as resveratrol. In some applications, stilbene can be present in an amount that is greater than about 60%, about 70%, about 80%, about 90%, about 95% or about 99% by weight of the composition. In one example, the provided compositions contain stilbene in an amount at or about 0.0001% to at or about 2%, or about 0.001% to at or about 5%, or about 0.01% to at or about 75% by weight of the composition. In another example, a composition may contain stilbene in an amount of from at or about 1% to at or about 50% by weight of the composition. In another example, a composition may contain stilbene in an amount of from at or about 5% to at or about 40% by weight of the composition. In another example, a composition may contain stilbene in an amount of from at or about 10% to at or about 30% by weight of the composition. In another example, a composition may contain stilbene in an amount of from at or about 15% to at or about 25% by weight of the composition. In another example, a composition may contain stilbene in an amount of from at or about 1% to at or about 90% by weight of the composition. In another example, a composition may contain stilbene in an amount of about 10%, or about 15%, or about 20%, or about 25%, or about 30%, or about 50% by weight of the composition. In another example, a composition may contain stilbene in an amount of up to about 99% or more by weight of the composition.

[0054] In one particular embodiment, a contemplated stilbene-containing composition, such as a resveratrol-containing composition is provided as a concentrate. For example, a stilbene-containing composition may be provided as a 20X, or a 10X, or a 5X, or a 3X concentrate that can be diluted by an end user with an appropriate solvent to achieve a 1X working concentration. Alternatively, a stilbene-containing composition may be provided to an end user at a 1X working concentration. However, any concentration is contemplated for use herein. For example, compositions provided as concentrates can be used without dilution at all or may be diluted from a highly concentrated concentrate (*e.g.*, about 20X to about 100X) to some multiple of concentration higher than 1X, such as 2X, 2.5X, 3X, etc. or can be used at a more dilute concentration, such as 1/2X, 1/4X, 1/10X, etc. While concentrates are more preferred as commercially available goods, the industry and end consumers typically apply dilute compositions to propagated plants, propagated plant material, materials and surfaces.

[0055] In another embodiment, a contemplated composition may be seen in Table 2, where ingredients can be measured in percent volume per volume, percent weight per volume, or percent by weight.

Table 2. Contemplated composition formulation.

Ingredient	Approximate %
Stilbene (such as resveratrol)	0.001 – 100
Surfactant	0 – 99.999
Additional active ingredients	0 – 99.999
Carrier	0 – 99.999
Additives	0 – 99.999

[0056] In preferred aspects the resveratrol is produced by fermentation of recombinant microbes, which is more cost effective. Preferred production organisms include *Saccharomyces cerevisiae* or other yeasts.

[0057] In some aspects the resveratrol employed in the compositions and methods described herein may be resveratrol derivatives or other forms of resveratrol, particularly more water-soluble derivatives including but not limited to acetyl-resveratrol or pinosylvin.

[0058] Further examples of additional active ingredients include plant essential oil compounds or derivatives thereof. Examples include thymol, aldehyde C16 (pure), α-terpineol, amyl cinnamic aldehyde, amyl salicylate, anisic aldehyde, benzyl alcohol, benzyl acetate, cinnamaldehyde, cinnamic alcohol, carvacrol, carveol, citral, citronellal, citronellol, p-cymene, diethyl phthalate, dimethyl salicylate, dipropylene glycol, eucalyptol (cineole) eugenol, is-eugenol, galaxolide, geraniol, guaiacol, ionone, menthol, methyl salicylate, methyl anthranilate, methyl ionone, methyl salicylate, nootkatone, α-pheliandrene, pennyroyal oil perillaldehyde, 1- or 2-phenyl ethyl alcohol, 1- or 2-phenyl ethyl propionate, piperonal, piperonyl acetate, piperonyl alcohol, D-pulegone, terpinen-4-ol, terpinyl acetate, 4-tert butylcyclohexyl acetate, thyme oil, metabolites of trans-anethole, vanillin, and ethyl vanillin.

[0059] In another embodiment, a contemplated composition may include a stilbene, such as resveratrol, to additional active ingredient ratio of about 1:10, or about 1:8, or about 1:6, or about 1:4, or about 1:2, or about 1:1, or about 2:1, or about 4:1, or about 6:1, or about 8:1, or about 10:1. In some embodiments the additional active ingredient is thymol.

[0060] Carriers may be added to a composition in an amount of about 10%, or about 15%, or about 20%, or about 25%, or about 30%, or about 50% by weight of the composition. In some applications, a carrier can be present in an amount that is at or greater than about 60%, about 70%, about 80%, about 90%, about 95%, or about 99% by weight of the composition.

[0061] According to some aspects, carriers may be included as part of stilbene (such as resveratrol) comprising composition. Non-limiting examples of suitable carriers include an alcohol (such as ethanol), aqueous liquid carrier, water, a saline, a gel, an inert powder, a zeolite, a cellulosic material, a microcapsule, a hydrocarbon, a polymer, a wax, a fat, an oil, and the like. Some carriers include time release materials where a stilbene-containing composition may be released over a period of hours, or days, or weeks.

[0062] Solvents suitable for incorporation into compositions according to some aspects of the current invention include but are not limited to alcohols and glycols and also their ethers and esters, such as ethanol, ethylene glycol, or strongly polar solvents. In some aspects, alcohol solvents are preferred for resveratrol.

[0063] In some embodiments, one or more additional active ingredients efficacious against aquatic bacterial pathogens may also be included in compositions comprising stilbenes (such as resveratrol). In some aspects, one or more of the active compounds listed in Table 3 may be added at concentrations of greater than 10µg/ml).

Table 3 Active compounds against aquatic bacterial pathogens

Active compounds	**Bacterial pathogen**
Palmitic acid	*V. harveyi, V. parahaemolyticus, V. vulnificus, V. alginolyticus*
Curcumin	*V. vulnificus*
Cinnamaldehyde	*V. vulnificus*, *V. harveyi*
Tannic acid	*A. hydrophila*
Rosmarinic acid	*A. hydrophila*
Naringenin, Quercetin and Apigenin	*V. harveyi*
Piper betle (Phytol & hexadecanoic acid)	*V. harveyi*
Pyrogallol	*V. harveyi*
Ellagic acid, catechin	*E. coli* and *Pseudomonas Sp.*
Isoeugenol	*P. fluorescens*

Formulation examples

[0064] The feed-additives are, when manufactured in their concentrated form on the basis of two or more elements, and both as regards insoluble and soluble variants be blended and packed at facilities using equipment approved of for also blending of vitamin and mineral premixes for animals. Non-limiting examples of feed and feed additives include:

a. 50 grams stilbene (such as resveratrol) included in the feed at 50 grams per ton of feed.

b. 100 grams stilbene (such as resveratrol) included in the feed at 100 grams per ton of feed.

c. 200 grams stilbene (such as resveratrol) included in the feed at 200 grams per ton of feed.

d. 300 grams stilbene (such as resveratrol) included in the feed at 300 grams per ton of feed.

e. 400 grams stilbene (such as resveratrol) included in the feed at 400 grams per ton of feed.

f. 500 grams stilbene (such as resveratrol) included in the feed at 500 grams per ton of feed.

g. soluble or solution suitable for incorporating into feed containing 25 grams stilbene (such as resveratrol)per liter water or aqueous solution.

h. soluble or solution suitable for incorporating into feed containing 50 grams stilbene (such as resveratrol)per liter water or aqueous solution.

i. soluble or solution suitable for incorporating into feed containing 100 grams stilbene (such as resveratrol)per liter water or aqueous solution.

j. soluble or solution suitable for incorporating into feed containing 200 grams stilbene (such as resveratrol)per liter water or aqueous solution.

k. soluble or solution suitable for incorporating into feed containing 300 grams stilbene (such as resveratrol)per liter water or aqueous solution.

l. soluble or solution suitable for incorporating into feed containing 400 grams stilbene (such as resveratrol)per liter water or aqueous solution.

m. soluble or solution suitable for incorporating into feed containing 500 grams stilbene (such as resveratrol)per liter water or aqueous solution.

n. soluble or solution suitable for incorporating into feed containing 600 grams stilbene (such as resveratrol)per liter water or aqueous solution.

o. powdered product suitable for incorporating into feed containing 25 grams stilbene (such as resveratrol)per kg powered product.

p. powdered product suitable for incorporating into feed containing 50 grams stilbene (such as resveratrol)per kg powered product.

q. powdered product suitable for incorporating into feed containing 100 grams stilbene (such as resveratrol)per kg powered product.

r. powdered product suitable for incorporating into feed containing 200 grams stilbene (such as resveratrol)per kg powered product.

s. powdered product suitable for incorporating into feed containing 300 grams stilbene (such as resveratrol)per kg powered product.

t. powdered product suitable for incorporating into feed containing 400 grams stilbene (such as resveratrol)per kg powered product.

u. powdered product suitable for incorporating into feed containing 500 grams stilbene (such as resveratrol)per kg powered product.

v. powdered product suitable for incorporating into feed containing 600 grams stilbene (such as resveratrol)per kg powered product.

[0065] Preferred inclusion rates of the stilbene (such as resveratrol or a derivative thereof) in the final feed are at concentrations of less than 700 PPM, 50 to 500 PPM, 100 to 400 PPM, or 100 to 300 PPM.

[0066] In some aspects, the composition further comprises at least one additional ingredient selected from natural or regenerated mineral material (such as calcium), solvent, surfactant, vegetable oil (such as a rapeseed oil), dispersant, carrier, vegetable mass (such as algae, soybeans, seaweed, barley, rice, peas, canola, lupine, wheat gluten, soy, corn or corn gluten), protein sources (such as insects, fish meal, fish trimmings from the fish industry, or small feeder fish stocks bred or harvested from the environment for incorporation in feed), lipids (such as fish oil), wetting agent, adhesive, thickener, binders, attractant, pigments (such as the carotenoids astaxanthin or canthaxanthin), vitamins, antioxidants, enzyme (such as phytase).

Methods

[0067] According to some embodiments of the current invention, compositions comprising active ingredients may be applied once per day, once every two days, once per week, twice per week, once per two weeks, once per month, or once per two months, once per three months, or once per lifecycle of the cultured fish or decapod crustacean. Compositions according to aspects of the current invention may be employed as pure active ingredients or, preferably, together with the auxiliaries conventionally used in the art of formulation and are therefore processed in a known manner to give, for example, emulsion concentrates, spreadable pastes, ready-to-dilute solutions, dilute emulsions, wettable powders, soluble powders, dusts, granules, or encapsulations, for example in polymeric materials. The methods of application, such as spraying, atomizing, dusting, scattering, brushing on, submerging, coating, pouring or rubbing, and the type of composition are selected to suit the intended aims and prevailing circumstances.

[0068] Compositions as described herein may be introduced to fish or decapod crustaceans at any time of the life cycle (i.e. to subjects of all ages), including but not limited to fry, fingerlings, maturing or finishing stages. In some aspects, compositions as described herein are preferably used in the finishing of commercially reared fish or decapod crustaceans. In some aspects, compositions as described herein are administered or incorporated into the feed, feed supplements or additives during the early growth cycle of commercially reared fish or decapod crustaceans, such as the growth phase from hatchlings to fry, and/or from fry to fingerlings. In some aspects, compositions as described herein are administered or incorporated into the feed, feed supplements or additives during the entire growth cycle of commercially reared fish or decapod crustaceans. In one aspect, the compositions described herein may be administered to fish every day from fry until slaughter. In another aspect, the compositions described herein may be administered to fish every day from fry until end of fingerling stage. In another aspect, the fish or decapod crustaceans may be caught or captured at a juvenile stage of growth in the wild, and transported to grow-out aquaculture facilities where they are fed with compositions as described herein until mature enough for harvest or re-release back into the wild. In another aspect, the fish or decapod crustaceans may be fed feed or feed additive compositions as described herein from egg to fingerling stage in a hatchery, released into the wild and optionally recaptured at a later stage of growth.

[0069] Generally and without limitation, compositions contemplated herein can be in the form of an aqueous liquid, an oil-based liquid, a concentrated liquid, a gel, a foam, an emulsion, a slurry, a paint, a clear coat, a wax, a block, a pellet, a puck, a granule, a powder, a capsule, a vesicle, an effervescent tablet, slow release tablet, an impregnated dissolvable sheet or film, an impregnated material, and combinations thereof.

Application and use

[0070] In some methods and aspects, feeding fish or decapod crustaceans (such as, shrimp, prawn, crayfish, lobster or crab) with feed compositions comprising 50 to 500 ppm of stilbene (such as resveratrol) results in the fish or decapod crustaceans reaching a higher live-weight and having a higher incidence of survival.

[0071] In some methods and aspects, feeding fish or decapod crustaceans (such as, shrimp, prawn, crayfish, lobster or crab) with feed compositions comprising 4 to 320 mg/ml of a stilbene (such as resveratrol) inhibits or treats *Vibrio* biofilm formation without adverse effects on environmental microbes that do not form pathogenic biofilms. In some aspects, feeds or feed additive compositions comprising greater than 10µg/ml of a stilbene (such as resveratrol) inhibits or treats *Vibrio* biofilm formation without adverse effects on environmental microbes that do not form pathogenic biofilms. In some aspects, feeds or feed additive compositions comprising greater than 20µg/ml of a stilbene (such as resveratrol) inhibits or treats *Vibrio* biofilm formation without adverse effects on environmental microbes that do not form pathogenic biofilms.

[0072]

[0073] Correctly stocked aquaculture systems maximize productivity and profit. Too often, a culturist will overstock in a mistaken effort to maximize productivity, but this can lead to reduced growth, high incidence of disease, and high predation. However, understocked systems increase production costs and sometimes lead to overfeeding. In an non-limiting example, fish in static water aquaculture (such as a freshwater pond for catfish) may be stocked so as to reach a final density of 1.8kg/m^2 at the end of the growth season. Calculation methods to determine initial stocking density, taking into account typical mortality and growth rates, are known to those skilled in the art (for example the Food and Agriculture Organization of the United Nations documents incorporated herein in their entirety "cage and pen fish farming; Carrying capacity models and environmental impact" *FAO Fisheries Technical Paper T255, 1984* including example calculations in Appendices 1-4"; and "Site selection and carrying capacity in Mediterranean and brackish aquaculture: key issues" 2011). Similarly, some aquariums calculate initial stocking densities based on aquarium volume and adult fish length.

EXAMPLE 1: Resveratrol tested for performance improvement in fish farming.

Objective:

[0074] In the present study, the effects of the stilbene resveratrol as feed additive was examined to improve survival and growth performance in fresh water major carp species *Catla catla* (Catla) fingerlings in high stocking density farming.

Materials:

[0075] 2 duplicates of 5 concrete treatments tanks of 10 m x 5 m x1 m (ten tanks total).

[0076] 2 duplicates each of one negative control (comprising no resveratrol) and 4 otherwise identical batches of standard fish feed made up to further include levels of resveratrol at 15, 30, 100 and 300ppm respectively.

[0077] Carp fry sufficient for an initial stocking density in all tanks of 40 fry /m^2 (4 lakh/ha).

Procedure:

[0078] A typical feed for this species comprising a powdered mixture of groundnut oilcake and rice polish (1:1 w/w) was used as a feed for the rearing of fry to fingerlings. The feed additive resveratrol was incorporated into feed along with appropriate binders. Large concrete tanks of 10 m x 5 m x1 m size in the seed rearing complex were used for the fingerling rearing study. Important water quality parameters were measured periodically (at 15 day intervals) in the tanks. Fish sampling was carried out at 20 day intervals. The experiment was conducted for a period of three months for Catla fingerling rearing.

Using this system, two duplicates of each of the following were established.

- Control
- 15 ppm (15 mg/kg of feed)
- 30 ppm (30 mg/kg of feed)
- 100 ppm (100 mg/kg of feed)
- 300 ppm (300 mg/kg of feed)

Observations:

[0079] On average, fingerlings exposed to the negative control treatment (normal feed with no resveratrol) gained 10 grams of additional body weight per fingerling over the course of the study.

[0080] On average, fingerlings exposed to the normal feed supplemented with 100 PPM resveratrol gained 12 grams of additional body weight per fingerling over the course of the study.

[0081] On average, fingerlings exposed to the normal feed supplemented with 300 PPM resveratrol gained 14 grams of additional body weight per fingerling over the course of the study.

Conclusions:

[0082] After 2 months of feeding, resveratrol resulted in a significant increase in body weight and length of the fingerlings compared to the negative control. This was especially the case for administration of feed comprising 300 PPM resveratrol. Without being bound by theory, this relative increase in growth and weight gain of fingerlings may reflect enhancement or efficiency of the metabolism and energy of fish body cells, efficient feed utilization and/or balanced immune response at high socking densities.

EXAMPLE 2: Resveratrol tested for activity against shrimp Acute Hepatopancreatic Necrosis Disease.

Objective:

[0083] To study the effect of a representative stilbene against *Vibrio alginolyticus* formation. Early Mortality Syndrome (EMS) also named Acute Hepatopancreatic Necrosis Disease (AHPND) is one of the most destructive diseases in shrimp populations. EMS/AHPND is caused by vibriosis, a consequence of biofilm formation by gram-negative bacteria in the family Vibrionaceae. Vibriosis may be caused by a number of vibrio species of bacteria, including: *Vibrio alginolyticus, Vibrio harveyi, Vibrio vulnificus, Vibrio parahaemolyticus* and *Vibrio penaeicida.* Essentially, Vibrio bacteria are transferred through the oral cavity and then localize in the shrimp gastrointestinal tract and create a poison that causes tissue devastation and invalidism of the shrimp digestive system (hepatopancreas). The Food and Agricultural organization (FAO) of the UN has estimated that EMS leads to annual losses of more than USD 1 billion in shrimp aquaculture globally. Attempts in the art at prevention and cure using antibiotics and /or disinfectants have thus far not proved successful and there is no known cure. Many bacteria, including vibrio, require the formation of a biofilm in order to colonise a surface, for example during the early stages of infection.

Materials:

[0084] 50 ml glass test tubes.

[0085] Difco - Marine Broth 2216.

[0086] 100 % Ethanol.

[0087] 0.1 % crystal violet.

[0088] Commercially available *Vibrio alginolyticus* stock cutulturee.

[0089] Incubator - Scigenics.

[0090] Resveratrol standard preparation: 50 mg of resveratrol solubilised in 1 ml of 100 % Ethanol (50 mg/ml Resveratrol).

Procedure:

[0091] 5mL marine broth liquid cultures were prepared from a streaked plate of stocks of *Vibrio alginolyticus.* The cultures were grown overnight at 37 degrees Celsius in a shaking incubator. 100 µl of these overnight cultures were inoculated into 10 ml of marine broth (1:l00 dilutions) in test tubes. After inoculation, 20, 30, 40 and 50 PPM of Resveratrol was introduced into the duplicate culture broths in numbered test tubes. One broth comprising only media was inoculated with overnight culture and the same volume of ethanol (without resveratrol) was then incubated alongside the resveratrol-treated as a positive control for biofilm formation. Media containing 20PPM resveratrol and not inoculated with overnight culture was kept as a negative control. The test tubes were incubated in static condition (not in a shaker) at 37°C for 6 days. Once visual inspection confirmed biofilm formation in the positive control tube, the test tubes were carefully removed from the incubator without disturbing any biofilm that had formed. All tubes were inspected for biofilm formation or inhibition, and the cultures then decanted into a beaker and autoclaved for proper disposal. After decanting the cultures, the test tubes were immersed three times into a larger beaker containing water. The test tubes were inverted and placed on tissue paper to drain any remaining liquid from the tubes. All of the test tubes were stained with 15 mL of 0.1% crystal violet for 10 minutes. After the staining incubation period, the crystal violet solution was decanted into a large beaker of water. Any excess crystal violet in the test tubes was removed by gently rinsing, such that the only crystal violet remaining was that bound to a biofilm ring at the sides of the glass tubes. It was then possible to assess biofilm formation and inhibition by examining the depth and intensity of the crystal violet stained ring at the location of the meniscus of the culture broth.

Observations:

[0092] Photographs (Figure 3) were taken at the end of the incubation and crystal violet staining to demonstrate reduced *Vibio* biofilm. *Vibrio* biofilm formation was observed to be somewhat inhibited at resveratrol levels of 30 ppm and more so at 40 ppm, though the most effective inhibition of *Vibrio* biofilm formation was observed in 50 ppm resveratrol.

Conclusions:

[0093] This study demonstrated that resveratrol could significantly inhibit biofilm formation by *Vibrio alginolyticus* in a concentration-dependent manner.

EXAMPLE 3: Resveratrol tested for effects on key performance indicators of shrimp aquacultures

Objective:

[0094] Intensive aquaculture is associated with a high level of stress in farmed fish, shrimp or crustaceans, due to poor water quality and/or high amount of competing biomass. Stress can result in an impaired or reduced immunity, which results in increased rates of infections and severity of infections by pathogens. The positive ability of resveratrol on *Vibrio* pathogenic bacteria was confirmed in the above study (Example 2). The present study was performed to investigate the more general effects of a representative stilbene (resveratrol) on aquaculture productivity, using a shrimp farm as an example.

Materials:

[0095] Pacific White Leg Shrimp (*Penaeus vannamei*) was selected as a test subject. *Vannamei* is a variety of prawn native to the eastern Pacific Ocean and is considered to be one of the most important aquaculture crops worldwide. They are characterised by fast growth rate, good tolerance to high stocking density and efficient utilization of plant proteins in formulated diets. They are omnivorous scavengers and are less aggressive and less carnivorous than black tiger shrimp. The trial was conducted in a recirculating aquaculture system in fiberglass tanks (with a capacity of 100 litres) containing 70 litres of water and 15 ppm saline.

Procedure:

[0096] Fifteen *Vannamei* white leg shrimp were placed in each 100L tank. There were 6 repetitions per treatment. Commercial aquaculture feed was ground and the different test treatments were applied and mixed in. The shrimp were fed 3 times a day. Any uneaten feed was collected, dried and weighed. There were five groups: a control group and 4 treatment groups. The treatment groups were the following:

TG0: Negative control group: No inclusion beyond the commercial aquafeed.

TG1: Trial group I: Inclusion of resveratrol at 30 ppm final concentration.

TG2: Trial group II: Inclusion of resveratrol and thymol at final concentrations of 10 ppm resveratrol and 10 ppm thymol.

TG3: Trial group III: Inclusion of resveratrol and thymol at final concentrations of 15 ppm resveratrol and 15 ppm thymol.

[0097] Key aquaculture production parameters were measured for each treatment of shrimps: average daily weight gain (ADG) (g/shrimp/day), total gain, total feed consumption, and feed conversion rate (FCR) (kg feed/kg gain).

Observations:

[0098] Table 2 shows the aggregate results (i.e. totals across duplicate treatments) for survival and total weight gain at the end of the entire course of the 8 week trial for each treatment group.

	TG0	**TG1**	**TG2**	**TG3**
Number of shrimp at start	90	90	90	90
Number of shrimp at end	70	73	72	73
Survival rate %	77.8%	81.1%	80.0%	81.1%
Total shrimp mass at start (g)	335.1	336.3	333.6	335.0
Total shrimp mass at end (g)	582.3	629.8	630.9	629.1
Total aggregate mass gain for all shrimps in each treatment (g)	247.2	293.5	297.3	294.1
Average start mass per shrimp (g)	3.72	3.74	3.71	3.72
Average end mass per shrimp (g)	8.33	8.63	8.76	8.62
Average mass gain per shrimp (g)	4.60	4.89	5.05	4.89
Average mass gain per day per shrimp (g/day)	0.082	0.087	0.090	0.087
Average feed consumption per shrimp (g)	6.97	7.14	7.16	7.02
Efficiency of biomass gain per mass feed (Feed conversion ratio)	1.51	1.46	1.42	1.44

[0099] The shrimp in the negative control group had the lowest total mass gain, whilst the shrimp fed aquaculture comprising resveratrol exhibited significantly improved mass gain representing an approximately 19% higher final biomass than untreated shrimp.

[00100] The shrimp in the negative control group had the lowest survival rate, whilst the shrimp fed aquafeed comprising resveratrol exhibited improved survival rates over untreated shrimp.

[00101] The significantly higher total end mass of resveratrol-treated shrimp compared to negative control shrimp was not explained by survival rates alone however; the average shrimp end mass, and thus the average mass gain per day per shrimp were also significantly greater for resveratrol-fed shrimp than for negative control shrimp.

[00102] Of very significant importance to the aquaculture industry, the feed conversion ratio of all groups of resveratrol-treated shrimp were lower (indicating greater efficiency) than the negative control shrimp fed only standard aquafeed.

Conclusions:

[00103] This study demonstrated that the stilbene resveratrol included in aquafeed significantly improved key aquaculture production efficiency parameters: survival rate, total mass gain, growth rate, feed conversion ratio.

EXAMPLE 4: Resveratrol tested as an aquafeed additive during an extended fish trial rearing fingerlings onwards.

Objective:

[00104] In fish farming practices, stocking density is a key factor in determining the productivity and profitability of commercial fish farms. However, higher stocking density increases stress and in turn affect fish growth, feed utilization, fish yield, and promotes concerns for animal welfare. High stocking densities also increase the risk of disease outbreak and overall economic impact when an outbreak does occur, and this is especially a concern in aquacultures forbidden by local legislation from using antibiotics. At the same time, feed usage typically constitutes more than 70% of the recurring expenditure of commercial-scale aquaculture practices, so the provision of suitable aquaculture feed (aquafeed) and the efficiency of its conversion into biomass, are commonly considered to be the key factors in determining aquaculture profitability. The aim of the present study is to examine the effects of a representative stilbene, resveratrol, in key performance indicators of high density aquaculture of a representative fish species under typical conditions.

Materials:

[00105] The three families of fish species with the highest aquaculture production tonnages per annum are carps, tilapias and salmonids, which account for respectively 33%, 13% and 7% of total global farmed fish production tonnage. The species selected for the current studies were Indian major carp (*Catla Catla*). In an effort to assess the potential of resveratrol to enhance key performance indicators in an already well optimised system, aquaculture feed optimised for most efficient biomass production in these species for rearing in the Asian environment was chosen as the base to which various levels of resveratrol were added in distinct treatment group.

[00106] Table. 3 Composition of basal optimised aquafeed showing ingredients and approximate nutritional content

Feed Ingredients	mass (g)	Approximate Composition	(%)
Fish meal	35	Protein	31.8
Rice bran	16	Fat	9.54
Soybean meal	14	Carbohydrate	10.13
Ground nut cake	35	Fibre	4.01
Total	100	Moisture	8.14

[00107] To supplement the above basal feed composition, feed mixtures for application to different treatment groups were made containing 15mg (group T1), 30mg (groupT2), 100mg (group T3) and 300 mg (group T4) resveratrol per kilogram mixture. The resveratrol in this study was present on a sodium caseinate carrier. Considering the possible interference of sodium caseinate in fish growth due to its protein content, a negative control (NC), i.e, feed with addition of only sodium caseinate, was also included in the study to detect any effects attributable to the carrier. Additionally, the standard aquafeed without any supplements of sodium caseinate nor resveratrol was used as a control (C). Both control and negative control feeds were virtually iso-proteinous with the treatment feeds. All resulting feed mixtures were formulated as physically identical 3 mm diameter extruded floating pellets using the same feed mill.

[00108] Analyses of feed content were determined following the method provided in Association of Officials Analytical Chemistry (AOAC), (1990) Official methods of analysis. 15th Edn. Edited by S. Williams, AOAC, Virginia, 1298 pp. Moisture content was determined gravimetrically in a hot air oven at 100+ 10°C for 24 h. Crude protein contents were determined by the micro-kjeldahl method. Crude lipid contents were determined by the soxhlet extraction method using petroleum ether (Boiling point: 40-60°C) in the electro-thermal soxhlet apparatus. After extraction of lipid the samples were estimated for crude fibre content following Patra (Patra BC, Maity J, Debnath J, Patra S (2002) Making Aquatic weed useful II: Nymphoides cristatum (Roxb.) O .Kuntze as fish feed for an Indian major carp *Labeo rohita* (Ham.). Aquaculture Nutrition 8:33-42).

Procedure:

[00109] The study on the influence of resveratrol as a feed additive to improve growth performance of Catla juveniles was carried out for a period of 82 days in a set of 12 large concrete tanks (50 m^2 each) of the fish farm. Water from a nearby pond, filtered through a bag net (60 mesh/inch) was filled in the tanks to a depth of 1.0 m seven days prior to stocking. Each tank was fertilized with a total of 1.25 kg ground nut oil cake (GOC), 350 g cow dung (CD) and 80 g single super phosphate (SSP) four days prior to fish stocking as standard procedure increase the plankton population. Two replicate tanks were maintained for each of the control, negative control and the treatments. The fingerlings (also known as fry) were acclimatized to the new tank environment prior to stocking. Each tank was stocked with 250 fingerlings (equating to an initial stocking density of 5 fingerlings/m^3) of Catla raised under standard conditions in the farm (all between 9.02- 9.73 cm, 11.2-11.8 g).

[00110] Sampling of water in the tanks was carried out every two weeks. Water samples were collected between 07:00 and 08:00 hours from the tanks. Water temperature and pH were measured *in situ*. Dissolved oxygen (Wrinkler's method), total alkalinity, total hardness and inorganic nutrients *viz*., total ammonia nitrogen (TAN), nitrite, nitrate and phosphate were analyzed in the laboratory following standard methods (American Public Health Association (APHA) (2005) Standard method for examination of water and wastewater, 21st edn. APHA, AWWA, WPCF, Washington). The water quality in the tanks were maintained according to standard protocols in this farm, through periodic liming ($CaCO_3$ at 1.0 kg/tank equivalent to 200 kg/ha) and fertilization (urea at 10 kg/ha and SSP at15 kg/ha). The water loss due to evaporation in the tanks was replaced at week intervals.

[00111] For first five days of the experiment, the fry were provided with extruded floating pellet at a feed rate equivalent to 2% of the initial biomass in each treatment group. The feed amount was increased to 3% on sixth day onwards and subsequently provided as per the consumption. The daily feed amount was recorded for each tank. Fish sampling was carried out at periodic intervals. At every sampling, length and weight of 25 randomly selected fish in every replicate tank were recorded before returning to their tanks. The data from these samplings was used to estimate the biomass present in each tank using an assumption of 80% fingerling survival based on an earlier preliminary trial, though it should be noted that untreated Catla survival rates are usually under 50%. The juveniles were harvested after 82 days by netting followed by complete draining of the tanks to confirm complete harvesting. Consequently the final harvest data points are definitive, whereas the biomass estimates were calculated using a single best case survival probability that was not achieved in the lower dosage and control groups.

[00112] The technological indicators were calculated using the formulas: Weight gain (WG) = (mean final weight – mean initial weight)/culture duration in days

[00113] Specific growth rate (SGR) = [(final weight – initial weight)/culture duration in days] x 100

[00114] Feed conversion ratio (FCR) = Total feed intake/total biomass gain

[00115] Survival percentage = (number of fish harvested/number of fish stocked) x 100

[00116] The data on length and weight increment, survival and biomass production were subjected to one way analysis of variance (ANOVA). Further, Duncan's multiple range test was performed to compare the mean growth and yield of the different treatments.

Observations:

[00117] The water quality parameters in the experimental tanks are presented in Table 4. The ranges of all the parameters were within the tolerance range of juvenile Catla. Periodic liming and fertilization, standard measures during the rearing phase, helped to maintain the water quality within tolerable ranges.

[00118] Table 4. Water quality parameters during rearing of the Catla juveniles in concrete tanks with feed containing varied levels of resveratrol. Values are expressed as mean ±SD; figures in parentheses represent the minimum to maximum range of the water quality parameters during the study

Parameter	**Control (C)**	**Negative control (NC)**	**T-1**	**T-2**	**T-3**	**T-4**
Temperature (°C)	27.8 ±1.9 (24.2-30.0)	27.8 ±1.8 (24.5-30.2)	27.9 ±1.9 (24.2-29.8)	27.7 ±1.6 (24.3-29.0)	27.3 ±1.5 (24.5-29.0)	27.3 ±1.5 (24.3-29.5)
pH	8.9 ±0.6 (7.4-9.7)	8.9 ±0.4 (8.3-9.5)	9.1 ±0.6 (8.0-9.9)	9.3 ±0.5 (8.5-10.3)	9.0 ±0.4 (8.2-9.6)	9.2 ±0.4 (8.2-9.9)
Dissolved oxygen (mg/l)	3.4 ±0.4 (2.8-4.0)	3.4 ±0.5 (2.8-4.0)	3.6 ±0.6 (2.4-4.4)	3.4 ±0.5 (2.8-4.0)	3.2 ±0.5 (2.8-4.0)	3.5 ±0.5 (2.8-4.0)
Total alkalinity	68.3	68.0	77.3	69.7	69.0	68.0

(mg/l)	±9.1 (52.0-80.0)	±9.6 (44.0-76.0)	±3.6 (72.0-84.0)	±7.1 (60.0-80.0)	±7.5 (56.0-80.0)	±8.0 (56.0-80.0)
Total hardness (mg/l)	62.3 ±6.0 (52.0-76.0)	56.9 ±5.6 (48.0-64.0)	68.7 ±6.1 (60.0-80.0)	59.3 ±6.3 (48.0-72.0)	61.1 ±5.7 (56.0-72.0)	62.0 ±5.3 (56.0-68.0)
Transparency (cm)	47.0 ±9.7 (40.0-66.00)	52.8 ±14.4 (38.0-80.0)	45.9 ±12.2 (33.0-69.0)	41.7 ±9.9 (29.0-65.0)	41.9 ±8.9 (32.0-57.0)	38.8 ±7.7 (29.0-52.0)
Total ammonia – N (mg/l)	0.12 ±0.08 (0.02-0.26)	0.09 ±0.0 7 (0.02-0.24)	0.09 ±0.07 (0.001-0.21)	0.06 ±0.05 (0.01-0.17)	0.07 ±0.04 (0.02-0.15)	1.00 ±0.05 (0.04-0.19)
Nitrite-N (mg/l)	0.009 ±0.010 (0.001-0.037)	0.004 ±0.005 (0.001-0.015)	0.009 ±0.007 (0.001-0.016)	0.005 ±0.004 (0.001-0.012)	0.013 ±0.016 (0.002-0.064)	0.003 ±0.003 (0.001-0.009)
Phosphate-P (mg/l)	0.030 ±0.023 (0.013-0.0930	0.018 ±0.003 (0.016-0.025)	0.029 ±0.016 (0.015-0.067)	0.036 ±0.012 (0.020-0.059)	0.036 ±0.036 (0.018-0.157)	0.032 ±0.025 (0.015-0.112)

[00119] Figures 4 and 5 depict the growth curves (length and weight gain respectively) of the juvenile Catla in the controls and treatments. There was noted to be a relative increase in average length in the treatments (T1 to T4) as compared to control baseline by end of study; this effect became increasingly noticeable from 40 days of culture onwards as the slight variations in initial length grew out and became progressively less of a factor in determining fish length at each sampling time. Similarly, the relative increase in weight compared to control was most significant in T3 and T4 ($P<0.05$) as compared to controls (C and NC) after 40 days. A slight relative increase in weight in T-1 and T-2 compared to controls suggested that the enhanced weight gain effect of resveratrol may be dose dependent within the tested range.

[00120] Table 5. Yield attributes of the Catla juveniles reared with varied levels of resveratrol in feed.

Treatment	Initial body weight (g)	Harvested body weight (HBW) (g)	Survival (%)	Gross biomass/ tank (kg)	Net Biomass/ tank (kg)	Specific growth rate (SGR) (%)	FCR
C	11.26 ±0.1	19.49 ±0.2[c]	54.2 ±5.4[b]	2.64 ±0.26	1.11 ±0.10[d]	0.67 ±0.02[c]	5.85 ±0.34[c]
NC	11.77 ±0.2	20.07 ±0.2[cd]	55.6 ±4.0[b]	2.78 ±0.25	1.15 ±0.11[d]	0.65 ±0.01[c]	5.65 ±0.33[c]
T-1	11.59 ±0.0	20.89 ±0.1[cd]	56.0 ±4.8[b]	2.92 ±0.29	1.30 ±0.16[d]	0.71 ±0.01[d]	5.00 ±0.26[c]
T-2	11.29 ±0.1	21.07 ±0.8[b]	64.0 ±3.1[c]	3.37 ±0.02	1.56 ±0.05[bc]	0.76 ±0.03[d]	4.16 ±0.07[d]
T-3	11.46 ±0.5	22.35 ±0.6[b]	82.6 ±4.2[a]	4.61 ±0.12	2.24 ±0.09[b]	0.81 ±0.02[b]	2.81 ±0.11[ab]
T-4	11.41 ±0.2	24.63 ±0.1[a]	87.2 ±6.8[d]	5.36 ±0.37	2.88 ±0.13[a]	0.93 ±0.03[a]	2.25 ±0.12[a]

[00121] The key production attributes of harvested body weight, survival, net biomass yield and specific growth rate in the fishes in the negative control (NC) were very similar to those in control (C) group (P>0.05), confirming the procedural correctness of the study, and indicating minimal effects of the sodium caseinate resveratrol carrier. The controls, therefore, could be considered as the baseline to study the contribution of resveratrol in the various treatment feeds towards the production performance, and comparison of the results among the treatments revealed the relative advantages of adding more resveratrol in aquaculture feed.

[00122] Fishes fed aquafeed comprising resveratrol (T1 to T4) showed dose response improvements in survival rates as compared to the NC, with T3 and T4 showing significant (P<0.05) increases in survival rate (>80%). The control group (C) without resveratrol also showed similar survival as that of NC. Such results clearly indicated the positive effects of resveratrol for improving fish survival. The 87% survival rate measured in T4 perhaps indicated resveratrol

dosage may have reached the saturation limit under these test conditions, beyond which increasing the dosage may not have resulted in significant improvements on survival rate. Treatments T-3 and T-4 also resulted in significantly higher ($P<0.05$) body weight at harvest (HBW), which was surprisingly achieved despite the significantly higher survival rates and hence more crowding in the tanks of these treatment groups compared to controls. This suggests the especially beneficial use of resveratrol for weight gain in high density aquacultures. In fact, the biomass production in the treatments was significantly higher in T-3 and T-4 ($P<0.05$), compared to the NC control, which is a metric highly correlated with aquaculture profitability. This beneficial effect is replicated in the feed conversion ratio, which demonstrated a dose-dependent effect of resveratrol in treatments T-1 to T-4. Surprisingly, T-3 and T-4 show that resveratrol dosages of 100 ppm and higher were able to halve the Feed Conversion Ratio in densely stocked aquaculture when compared to control feed compositions already considered by local aquaculture practitioners to be optimized for highest growth and most efficient FCR. In general, the measured attributes improved from T-1 to T-3 and became highest in T4 ($P<0.05$) revealing an especially strong influence on fish growth at the higher incorporation levels tested herein.

Conclusions:

[00123] The present study revealed the positive role of resveratrol to facilitate weight gain in fish despite testing in crowded conditions representative of densely stocked commercial aquacultures thought to induce stress in farmed fish. Incorporation of resveratrol into the aquafeed resulted in improved survival rates and specific growth rates, thereby leading to significantly higher overall biomass production. Perhaps the most notable conclusion for commercial aquaculture practices was that the resveratrol tested herein significantly lowered the feed conversion ratio.

EXAMPLE 5: Testing the *in vitro* anti-QS and antibiofilm potentials of resveratrol against aquatic microbial pathogens.

[00124] In recent years, the discovery of Quorum Sensing (QS) mechanism has provided a new perspective to understand the pathogenicity of microbes. QS is a cell density dependent gene expression regulatory mechanism, which correlates with signal molecules called autoinducers (AIs). Many aquatic pathogens utilize the QS mechanism for the expression of various phenotypic characters, including biofilm formation and production virulence factors – both processes are critical to pathogenicity in aquaculture. Therefore, the below investigation evaluated the anti-infective and anti-biofilm potentials of resveratrol in reducing QS dependent pathogenicity of aquatic bacterial and fungal pathogens.

Materials and methods

[00125] The bacterial strains used as test pathogens inculded *V. parahaemolyticus* (ATCC 17802), *V. alginolyticus* (ATCC 17749), *V. harveyi* (MTCC 3438), *V. vulnificus* (MTCC 1145), *A. hydrophila* (ATCC 7966) and *P. fluorescens* were. All the test bacterial pathogens were cultivated in Luria-Bertani (LB) broth (pH 7.5±0.2) at 28°C. For experimental purposes, all the test bacterial pathogens were subcultured in LB until achieving an OD of 0.4 at 600 nm (~1×10^8 CFU/ml). *Chromobacterium violaceum*, *Serratia marcescens* PS1 (GenBank accession number: FJ584421) and *P. aeruginosa* PAO1 were used as QS biomarker strains.

***In vitro* Determination of Minimum Inhibitory Concentration (MIC) of resveratrol**

[00126] For assay purposes, the resveratrol stock solution was prepared by dissolving 50 mg of resveratrol in 1 mL of DMSO. The MIC of resveratrol against test bacterial pathogens was determined as per the CLSI guidelines (2012). Briefly, 1% of bacterial culture was added to 1 mL of LB broth supplemented with varying concentrations of resveratrol and incubated at 28°C for 24 hours in a shaker at 120 rpm. The MIC was recognized as the minimum concentration that completely prevents visible growth.

Screening for anti-quorum sensing (anti-QS) activity of resveratrol

i) Violacein quantification assay

[00127] The anti-QS activity of resveratrol was assessed by violacein inhibition assay using *C. violaceum* (Zhu et al. 2011). Briefly, 1% of *C. violaceum* (~OD 0.4 at 600 nm) cells were

added to 1 ml of LB broth in a polystyrene micro-titre plate (MTP) and treated with various concentration of resveratrol. The plates were incubated at 30°C for 24 hours. After incubation, the cell pellets were collected from the control and resveratrol treated samples by centrifugation at 16,000 × g for 10 mins. Violacein pigment was then extracted from the collected cell pellets using DMSO and the OD was measured at 585 nm.

ii) Prodigiosin quantification assay

[00128] The effect of resveratrol on the production of prodigiosin was assessed as described by Morohoshi et al (2007). Briefly, 1% of *S. marcescens* was grown in 1 mL of LB broth in the absence and presence of resveratrol for 18 h at 28 °C. During the late stationary phase, the cell pellets were collected from each sample by centrifugation at 16,000 × g for 10 mins. Then the prodigiosin pigment was extracted from the cell pellets using acidified ethanol (4% of 1M HCl in absolute ethanol). Finally, the supernatants were collected from each sample and the level of extracted prodigiosin was measured at $OD_{534\ nm}$.

iii) Pyocyanin quantification assay

[00129] Supernatants from overnight cultures of *P. aeruginosa* PAO1 grown in the presence or absence of resveratrol were collected and the pyocyanin pigment extracted using chloroform followed by 0.2 M HCl. Finally, the level of pyocyanin present in each sample was measured at $OD_{520\ nm}$.

Assessment of antibiofilm activity of Resveratrol

i) Biofilm inhibition assay

[00130] The effect of resveratrol on biofilm formation of test bacterial pathogens was determined by biofilm biomass quantification assay (Luo et al. 2017). Briefly, 1% of bacterial culture was added to 1 mL of LB broth in the presence and absence of resveratrol in 24 well MTP and incubated statically for 24 h at 28°C. After incubation, the planktonic cells were discarded and the wells were washed thrice with distilled water, air dried and stained with 0.4% crystal violet. Biofilm bound crystal violet was eluted with 20% glacial acetic acid and the absorbance was read at $OD_{570\ nm}$. The percentage of biofilm inhibition was calculated using the following formula:

$$\%\ \text{of inhibition} = [(\text{Control } OD_{570\ nm} - \text{Treated } OD_{570\ nm}) / \text{Control } OD_{570\ nm}] \times 100$$

ii) Microscopic visualization of biofilm formation

[00131] Microscopic analyses were also performed in order to validate the antibiofilm activity of resveratrol. In the presence and absence of resveratrol, the test bacterial strains were allowed to form biofilm on glass slides (1 × 1 cm) in wells of MTP for 18 hours at 28°C. The slides were then washed with sterile PBS and stained with 0.4% crystal violet for 5 minutes. The slides were washed again with sterile distilled water to remove any excess stain, then the stained slides were viewed using a light microscope (Nikon Eclipse Ti 100, Japan) at 400× magnification (Gowrishankar et al 2012) and images taken.

iii) Exopolysaccharides (EPS) quantification

[00132] The effect of resveratrol on EPS production was quantified through total carbohydrate assay (Viszwapriya *et al.* 2016). Briefly, 1% of bacterial culture was grown in 1 mL of LB broth in the absence and presence of resveratrol for 18 h in a shaker at 120 rpm. After incubation the planktonic cells were discarded from each control. The treated sample and the adhered biofilm cells were harvested using 0.9% NaCl. An equal volume of 5% phenol was added to the cell suspension, followed by the addition of 5 volumes of H_2SO_4 and incubation for 1 hour in the dark. After incubation, the absorbance of supernatants was measured at $OD_{490\ nm}$.

Motility inhibitory assay

Swimming and swarming assay

[00133] The effect of resveratrol on swarming motility was determined by placing 2 µL (OD adjusted to 0.4 at 600 nm) of bacterial culture in the centre of swarming agar medium consisting of 1% peptone, 0.5% NaCl, and 0.5% agar supplemented either with or without resveratrol. The plates were incubated at 28°C for 18 hours. Activity was determined by the decrease in the radius of swarmed area (Sethupathy et al. 2016).

[00134] For the swimming assay, 3 µL (OD adjusted to 0.4 at 600 nm) of bacterial culture was stab inoculated at the center of swimming agar medium consisting of 1% peptone, 0.5% NaCl, and 0.3% agar supplemented either with or without resveratrol.

Results

A) The antibacterial and anti-quorum sensing (anti-QS) activity of resveratrol in biomarker strains

i) *Chromobacterium violaceum*

[00135] In the MIC determination assay, resveratrol exhibited visible growth reduction at ≥10µg/ml against the QS biomarker strain *C. violaceum* (Fig. 7a). At a concentration of 4µg/ml, resveratrol exhibited the inhibition of QS mediated violacein pigment production in *C. violaceum* without inhibiting cell growth. However, growth inhibition was observed with concentrations of 6 µg/ml and greater.

ii) *Serratia marcescens*

[00136] The QSI potential of resveratrol was assessed using the prodigiosin inhibition assay. Resveratrol exhibited a concentration dependent prodigiosin inhibitory activity. At a concentration of 50µg/ml, resveratrol reduced the production of prodigiosin pigment to 83% of normal production in *S. marcescens* (Fig. 8). No growth inhibition was observed, even at the highest concentration of 300 µg/ml, which suggests that resveratrol may act as an anti-QS agent.

iii) *Pseudomonas aeruginosa*

[00137] The anti-QS potential of resveratrol was further tested for an ability to reduce QS-dependent pyocyanin production in *P. aeruginosa*. The *P. aeruginosa* cells were cultivated either in the presence or absence of resveratrol in the concentration range between 2.5 to 300 µg/ml. A significant decrease in pyocyanin production of *P. aeruginosa* to 77% of normal production was observed after treatment with resveratrol at 100µg/ml concentration (Fig. 9b). Complete visible growth inhibition was not observed, even at the higher concentrations of resveratrol; an 80% growth inhibition of *P. aeruginosa* growth was seen at the highest tested concentration of 300 µg/ml (Fig. 9a).

EXAMPLE 6: Testing the *in vitro* anti-QS and antibiofilm potentials of resveratrol against aquatic microbial pathogens.

[00138] Resveratrol demonstrated a complete visible growth inhibition at 50µg/ml and 100µg/ml concentration against *Vibrio parahaemolyticus* and *V. alginolyticus* respectively (Fig. 10a and 10b). At a concentration of 320µg/ml, resveratrol exhibited complete antibacterial activity against *V. harveyi* (Fig. 10c). However, resveratrol only displayed 20% growth inhibition of *V. vulnificus,* even at 640 µg/ml (Fig. 10d).

EXAMPLE 7: Antibacterial activity of resveratrol against fresh water pathogens

[00139] Resveratrol showed significant antibacterial activity at 50µg/ml against *Aeromonas hydrophila*. However no further significant increase in growth inhibition was observed even up to 200 µg/ml.

[00140] When used against *Pseudomonas fluorescens,* 100 µg/ml resveratrol showed 70% growth inhibition and no further significant increase growth inhibition was observed at higher concentration (Fig. 10e).

EXAMPLE 8: Antibiofilm activity of resveratrol against aquatic bacterial pathogens

[00141] To further investigate resveratrol inhibition of biofilm formation of further bacterial pathogens of relevance to aquaculture, the biofilm forming potential of all the test strains was assessed in the absence or presence of resveratrol at various concentrations. The obtained results showed biofilm formation was reduced by resveratrol in a concentration dependent manner without inhibiting the growth of the test pathogens at the tested concentrations. At 10 µg/ml, resveratrol effectively reduced 57% of biofilm in *V. parahaemolyticus* without affected their planktonic counterparts (Fig. 11a). Likewise, 63, 69% and 40% of biofilm inhibition were observed in *V. alginolyticus, V. harveyi* and *A. hydrophila,* respectively at 20 µg/ml (Fig. 11b-d).

EXAMPLE 9: Light microscopy analysis

[00142] Microscopic analysis was performed to study any resveratrol-induced alterations on the architecture of bacterial biofilm. Resveratrol was found to be effective in preventing the initial stages of bacterial adhesion and consequent biofilm formation at concentrations as low as 10 µg/ml in *V. parahaemolyticus* (Fig. 12a) and 20 µg/ml in *V. alginolyticus* and *V. harveyi* respectively. In light microscopic studies, the results of resveratrol treatment was clearly visible on the glass slides as a reduction in biofilm formation (Fig. 12b&c).

EXAMPLE 10: Estimation of EPS inhibition in *V. alginolyticus*

[00143] Expression of exopolysaccharides (EPS) is an essential factor for the development of biofilm architecture in aquatic pathogenic bacteria. It is a crucial part of biofilm and it covers almost 50% - 90% of the entire organic matter of biofilm architecture, forming a matrix that retains the biofilm cells in close vicinity. This study determined the effect of resveratrol on EPS production of *V. alginolyticus*. Resveratrol at a concentration of 20 µg/ml was found to effectively inhibit the EPS production in *V. alginolyticus* (Fig. 13).

EXAMPLE 11: Swimming and swarming motility inhibition in *V. alginolyticus*

[00144] Flagellar-mediated motility plays an important role in QS mediated biofilm formation in several aquatic bacterial pathogens. Hence, the anti-QS potential of resveratrol against the swimming and swarming motility of *V. alginolyticus* was studied. The results indicated profound anti-swimming and anti-swarming activities of resveratrol at 20 µg/ml concentration (Fig. 14a and 14b). This inhibition of swimming and swarming motility provides further evidence of the utility of resveratrol for QS-mediated anti-biofilm activity against bacterial aquatic pathogens.

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Claims

1. A composition for feeding a fish or decapod crustacean comprising 50 to 500ppm of a stilbene and at least one additional ingredient selected from thymol, saponins, carriers, solvents, surfactants, antioxidants or enzymes.

2. A composition according to claim 1, wherein the composition comprises 100 to 300 ppm resveratrol.

3. A composition for the treatment of *Vibrio* biofilm formation in and/or on fish or decapod crustaceans comprising greater than 4µg/ml of a stilbene.

4. A composition comprising 4µg/ml to 320 mg/ml of a stilbene for the reduction of biofilm formation of aquatic bacterial pathogens of fish or decapod crustaceans, wherein the pathogenic species is one or more selected from *V. parahaemolyticus, V. alginolyticus, V. harveyi* and *A. hydrophila*.

5. A composition according to any preceeding claim, further comprising at least 4µg/ml of palmitic acid, curcumin, cinnamaldehyde, tannic acid, rosmarinic acid, naringenin, pyrogallol, ellagic acid, or isoeugenol.

6. A method of increasing the productivity of aquacultures of fish or decapod crustaceans by administering a composition of any of claims 1 to 5, wherein productivity is measured as an improvement in survival index, gain in mass, gain in length, gain in biomass, feed conversion ratio, mass of harvestable flesh or mass of eggs, compared to fish or decapod crustaceans from the same initial stock not fed a composition comprising stilbene.

7. A method of reducing or preventing *Vibrio* biofilm formation and improving one or more of survival index, gain in length, gain in biomass, or food conversion ratio of fish or decapod crustaceans, by feeding the fish or decapod crustaceans a feed composition according to any of claim 1 to 5.

8. Any of the methods according to claims 6 or 7, wherein the fish or decapod crustacean are fed with the composition comprising stilbene at least once per week, at least once per three days, or once per day.

9. Use of a stilbene in aquaculture to increase the productivity of fish or decapod crustacean stock, wherein productivity is measured as an improvement in two or more assays selected from survival index, gain in mass, gain in length, feed conversion ratio, mass of harvestable flesh or mass of eggs compared to fish or decapod crustaceans from the same initial stock not fed compositions comprising stilbene.

10. A method of treating or pretreating *Vibrio* biofilm in a fish or decapod crustacean comprising contacting the fish or decapod crustacean with a composition comprising at least 4μg/ml of a stilbene.

11. A method according to claim 10, wherein the composition comprising stilbene further comprising at least 4μg/ml of palmitic acid, curcumin, cinnamaldehyde, tannic acid, rosmarinic acid, naringenin, pyrogallol, ellagic acid, or isoeugenol.

12. A composition, method or use according to any of the preceding claims, wherein the stilbene is resveratrol, pinosylvin, or a methylated or glycosylated derivative thereof.

13. A composition, method or use according to any of the preceding claims, wherein the fish is a carp, tilapia or salmonid.

14. A composition, method or use according to any of claims 1 to 12, wherein the decapod crustacean is a crayfish, lobster, crab, shrimp or prawn.

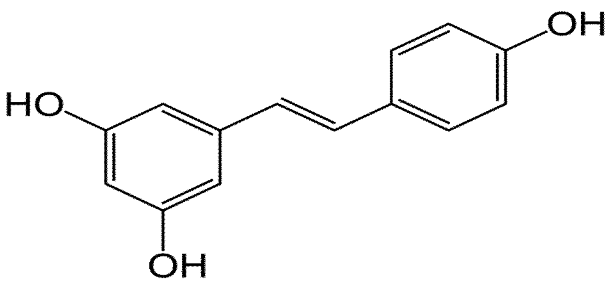


Figure 1. Chemical structure of trans-resveratrol.

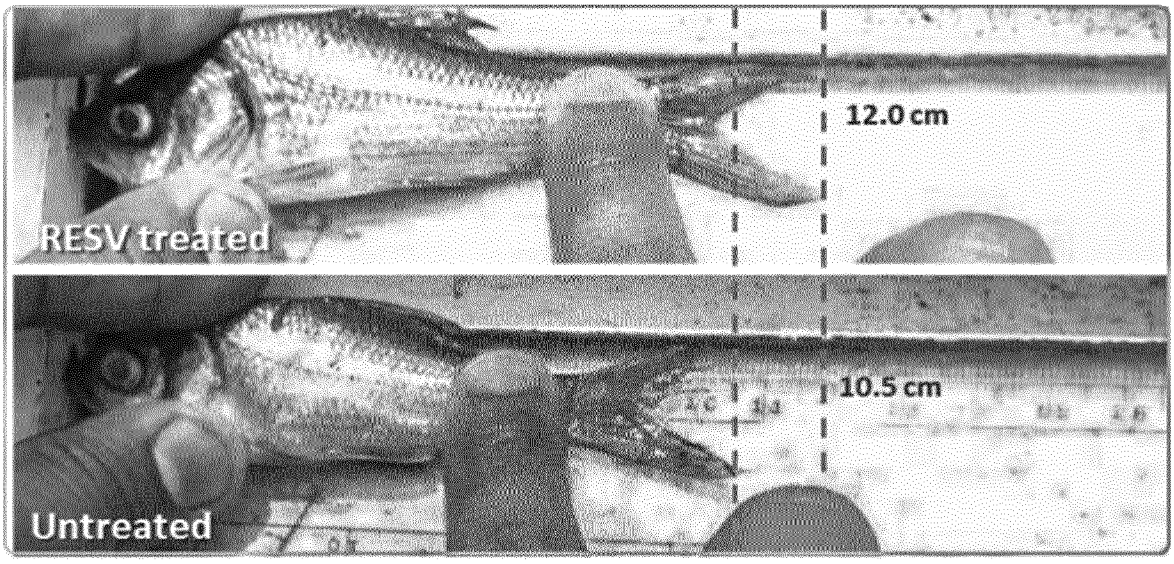


Figure 2: a representative comparison of fingerlings grown from the same batch of fry fed standard feed supplemented with and without 100 PPM of the stilbene resveratrol.

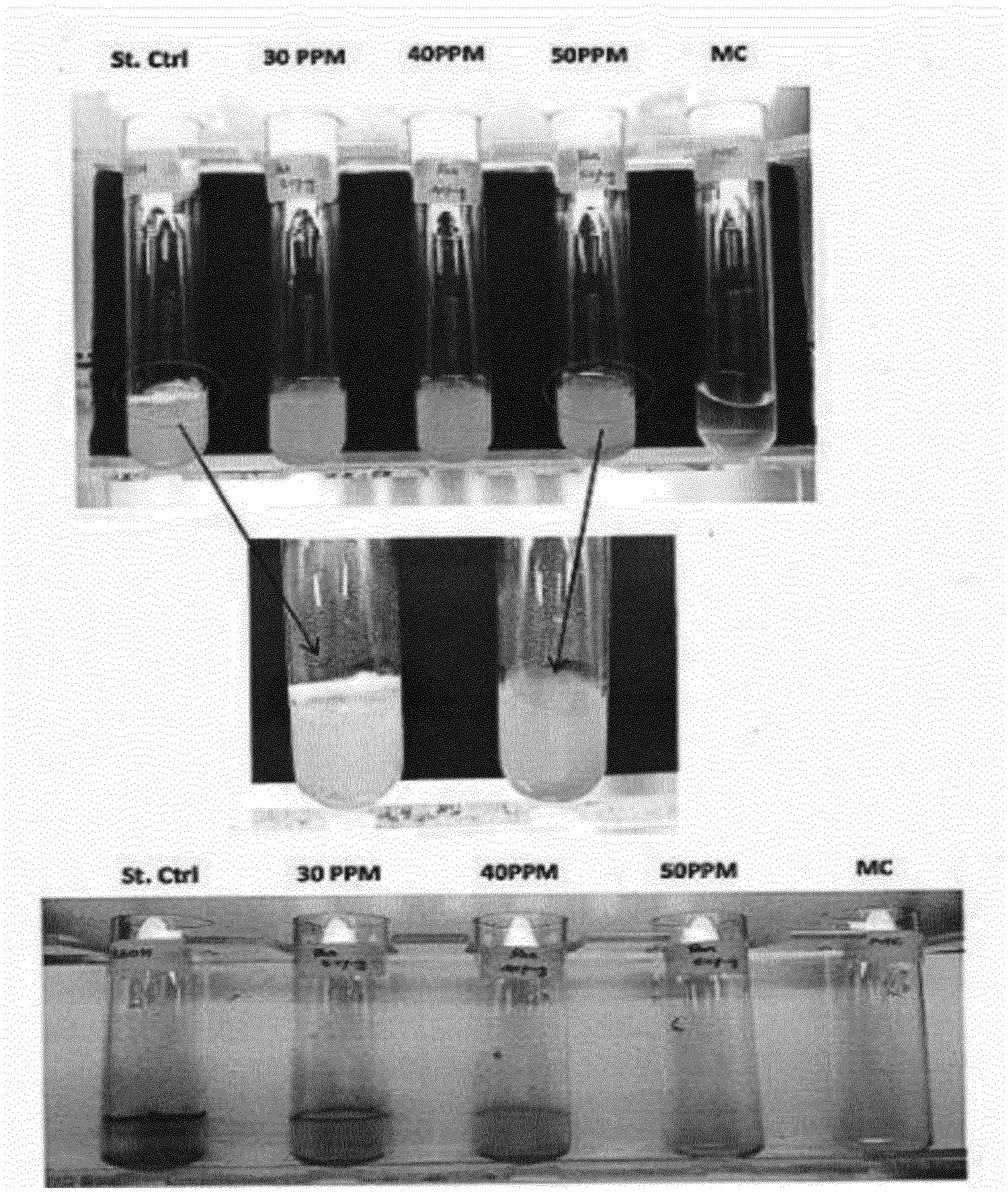


Figure 3: photographs of *vibrio* biofilm formation following incubations with and without the stilbene resveratrol.

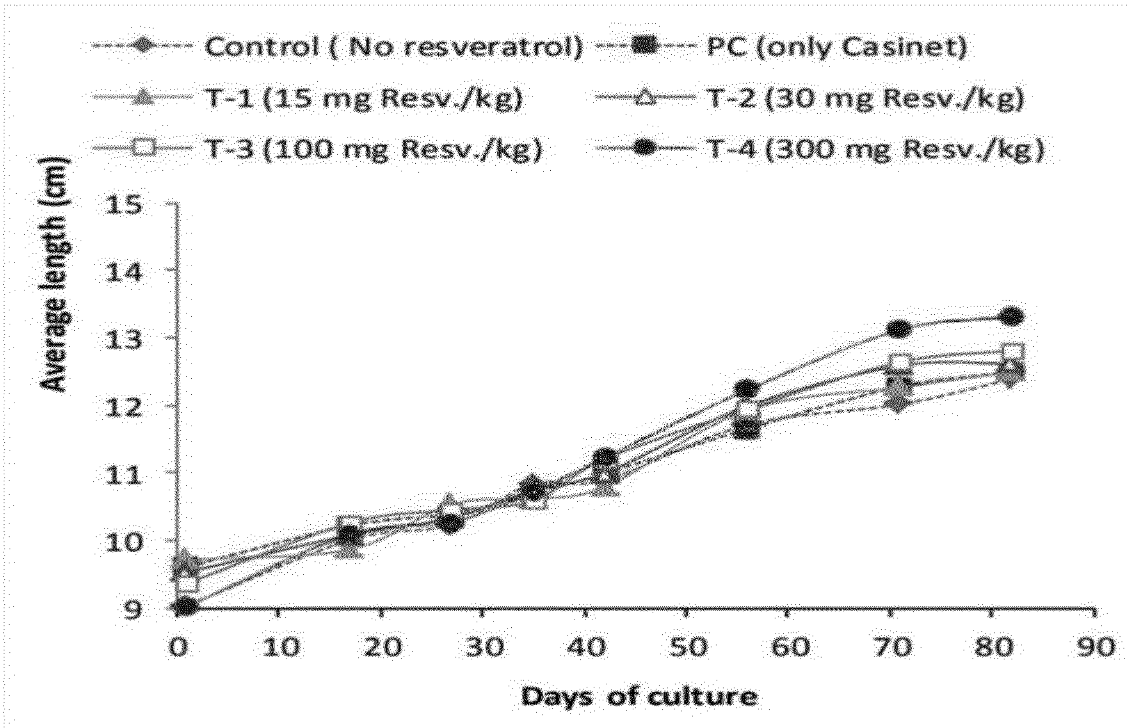


Figure 4: a comparison of fingerling gain in length with and without the stilbene resveratrol over an 82 day period.

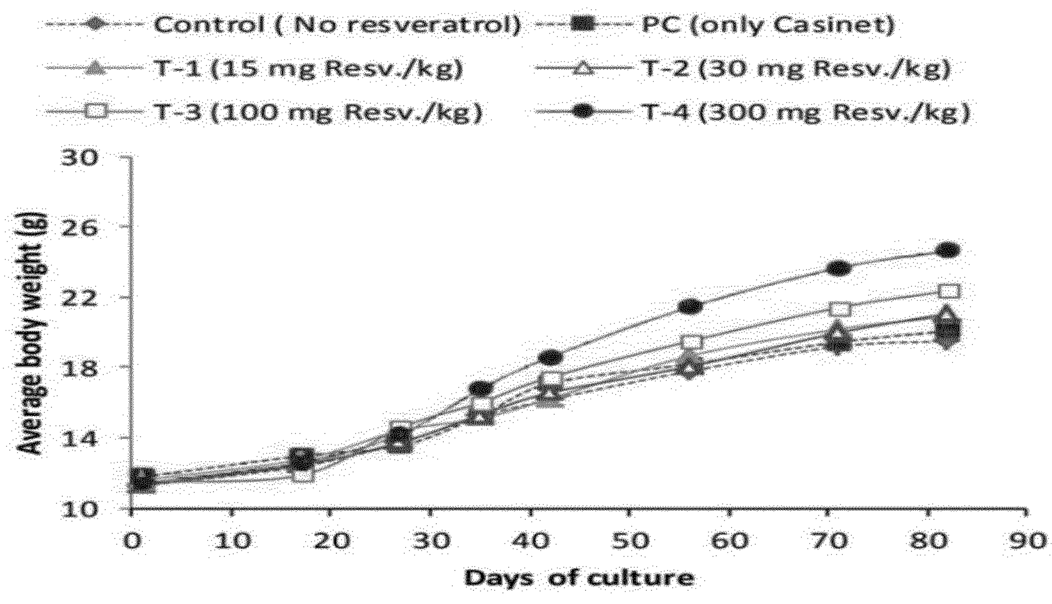


Figure 5: a comparison of fingerling gain in weight with and without the stilbene resveratrol over an 82 day period.

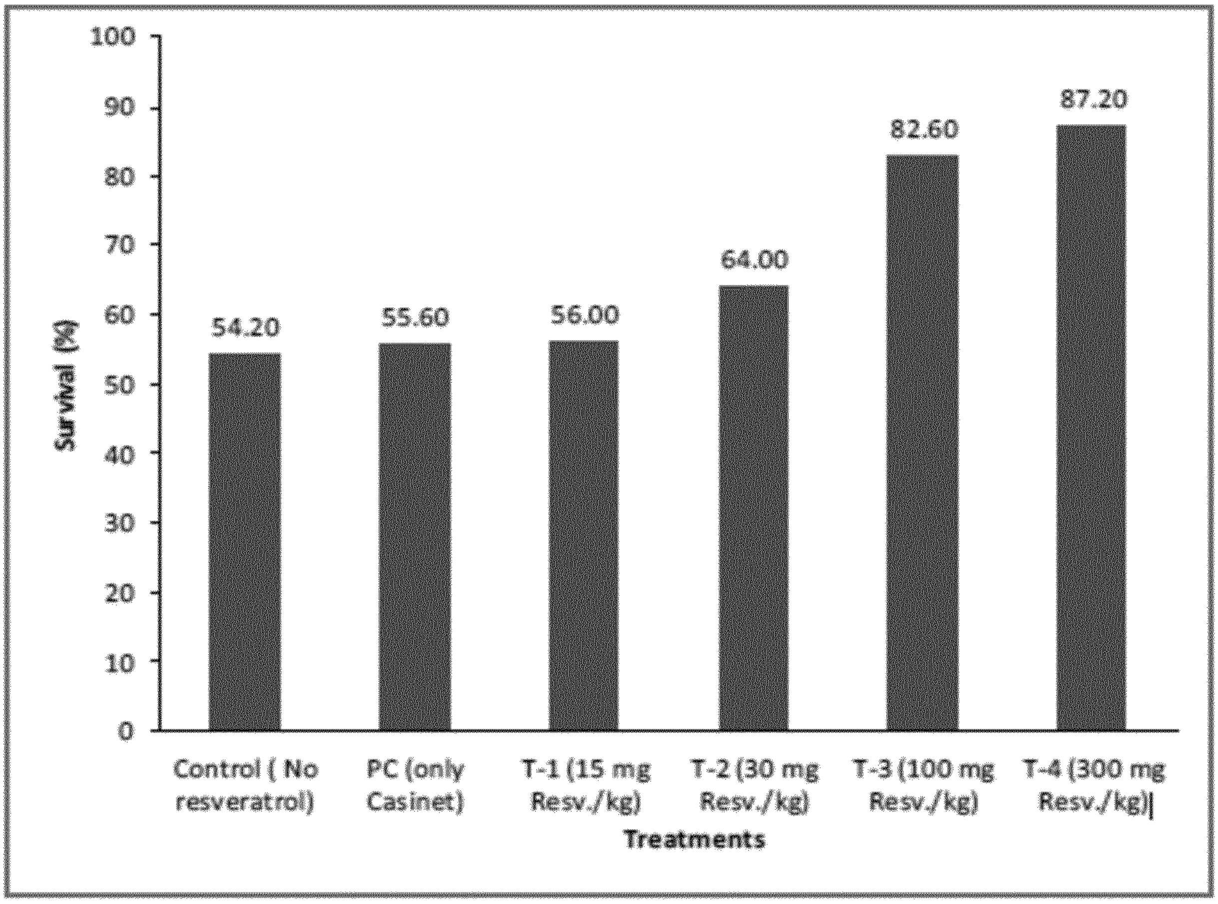


Figure 6: a comparison of fingerling survival rates with and without the stilbene resveratrol over an 82 day period.

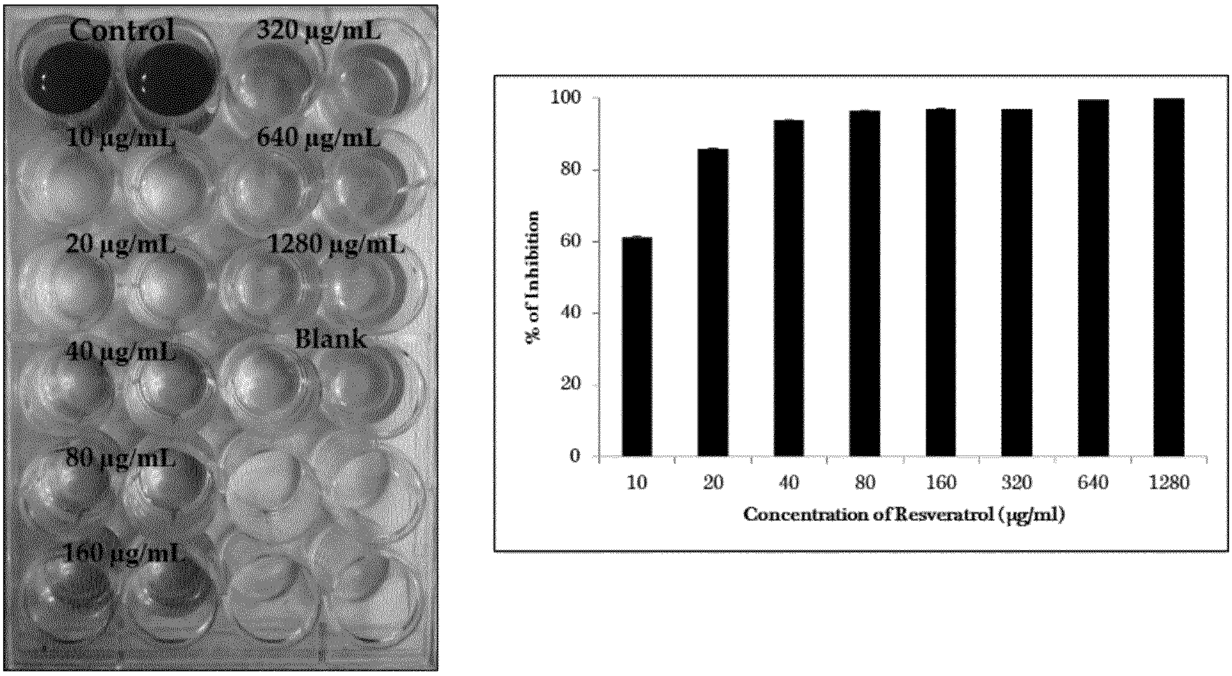


Figure 7a Activity of resveratrol against biomarker strain *C. violaceum*

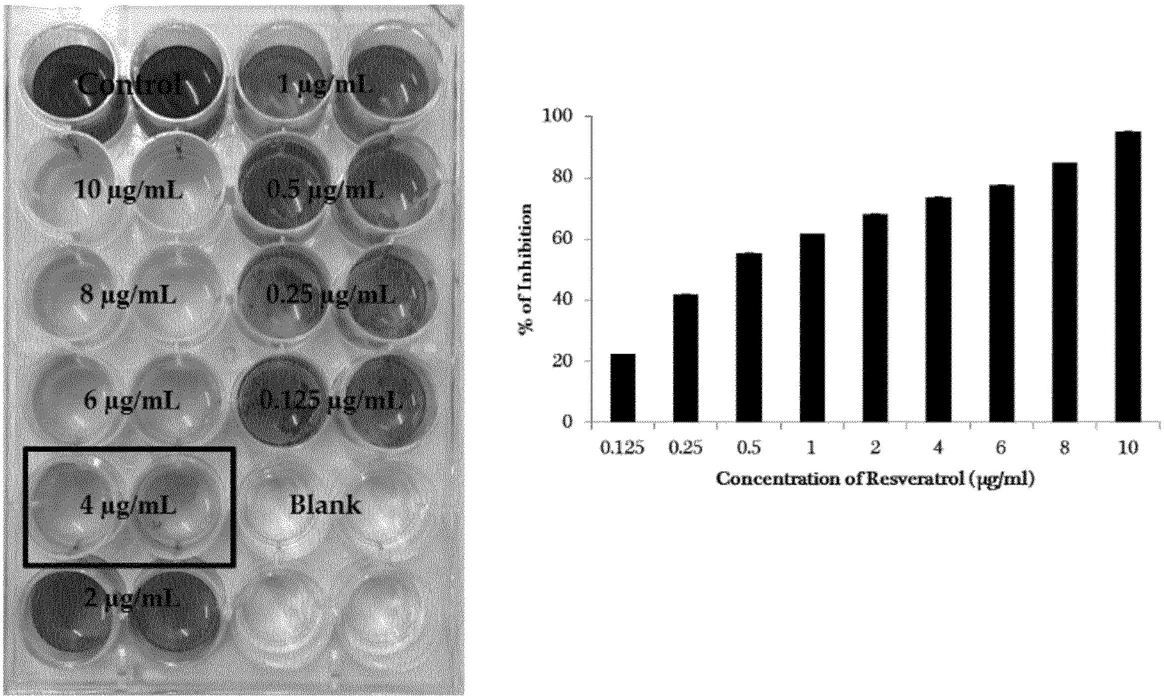


Figure 7b Anti QS activity of resveratrol against violacein production in biomarker strain *C. violaceum.*

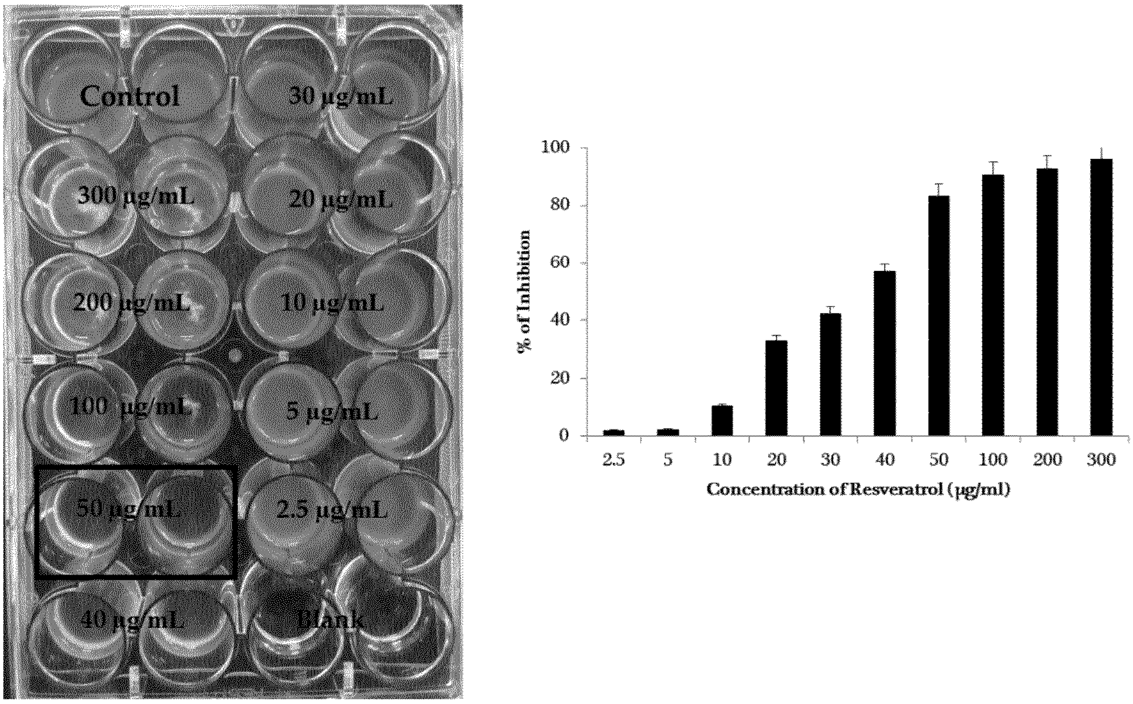


Figure 8 Anti QS activity of resveratrol against prodigiosin production in *S. marcescens.*

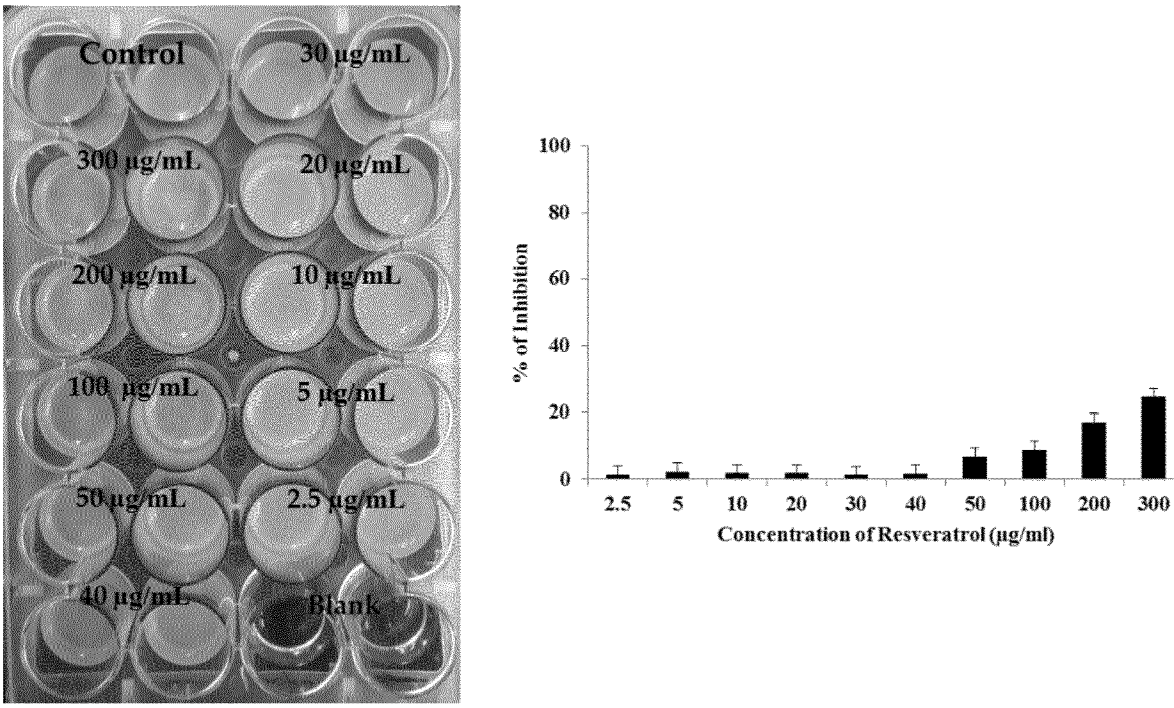


Figure 9a Antibacterial activity of resveratrol against *P. aeruginosa*

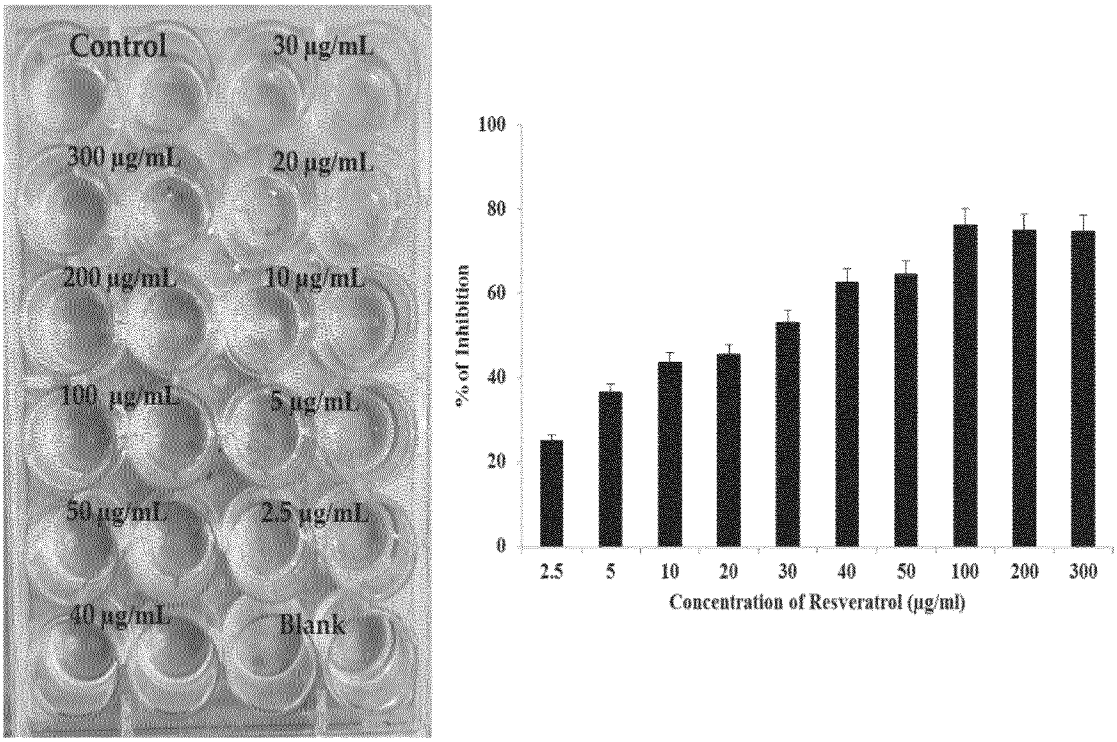


Figure 9b Anti QS activity of resveratrol against pyocyanin production in *P. aeruginosa*

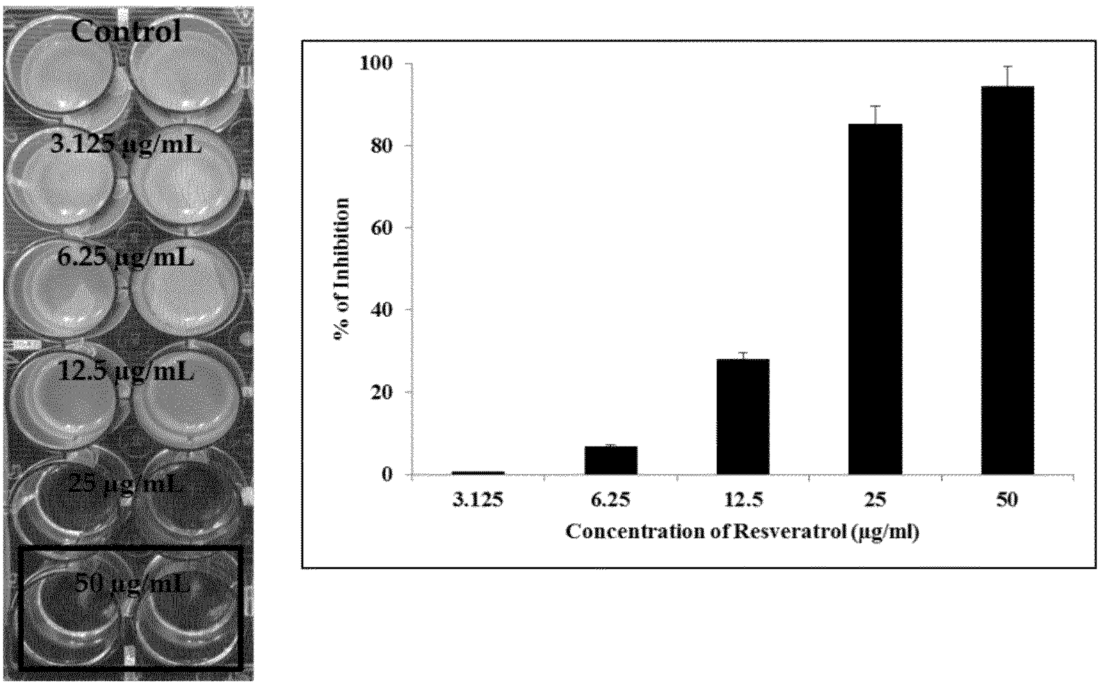


Figure 10a Inhibitory effect of resveratrol on growth of *V. parahaemolyticus*

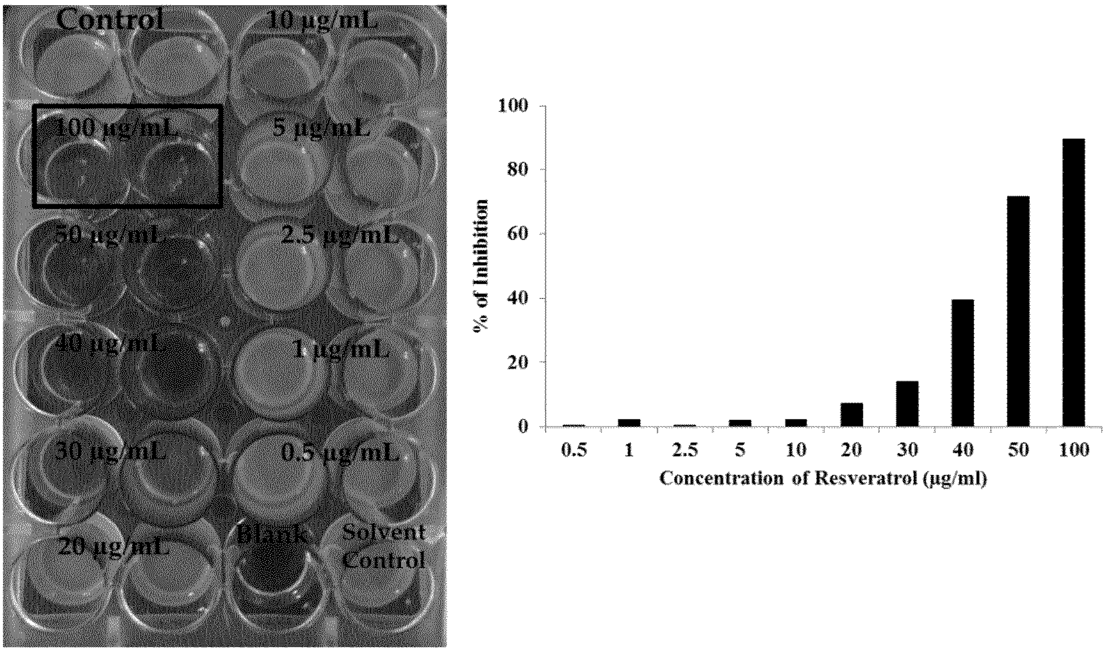


Figure 10b Inhibitory effect of resveratrol on growth of *V. alginolyticus*

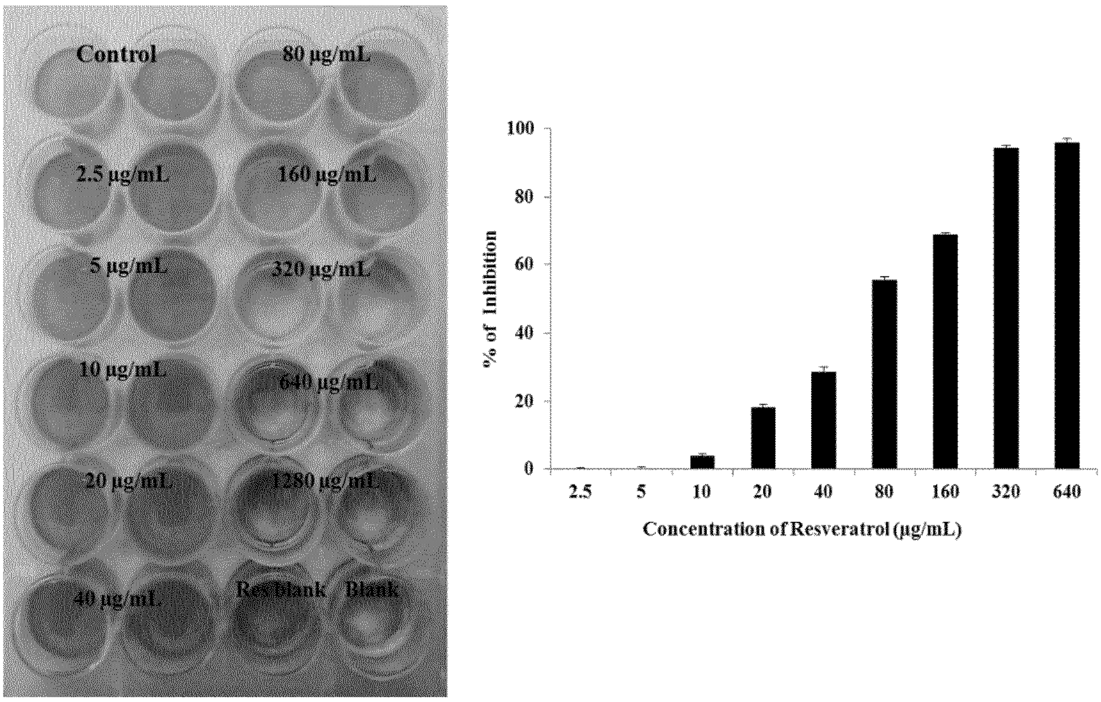


Figure 10c Inhibitory effect of resveratrol on growth of *V. harveyi*

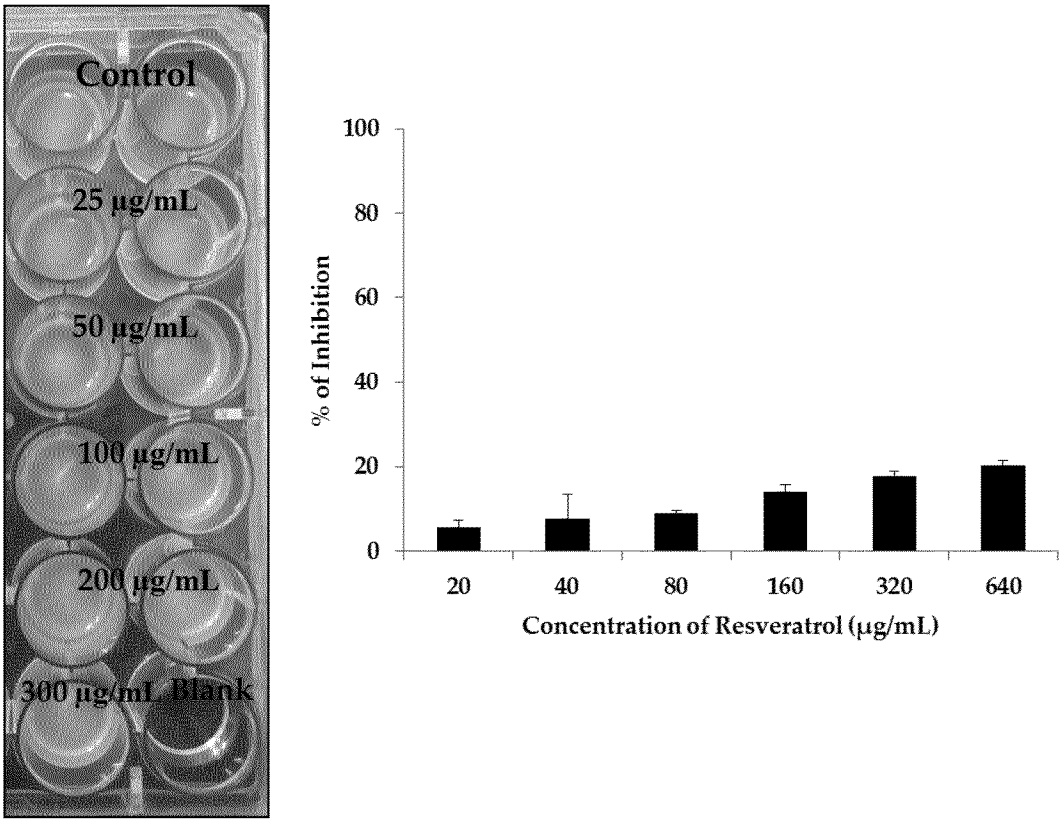


Figure 10d Inhibitory effect of resveratrol on growth of *V. vulnificus*

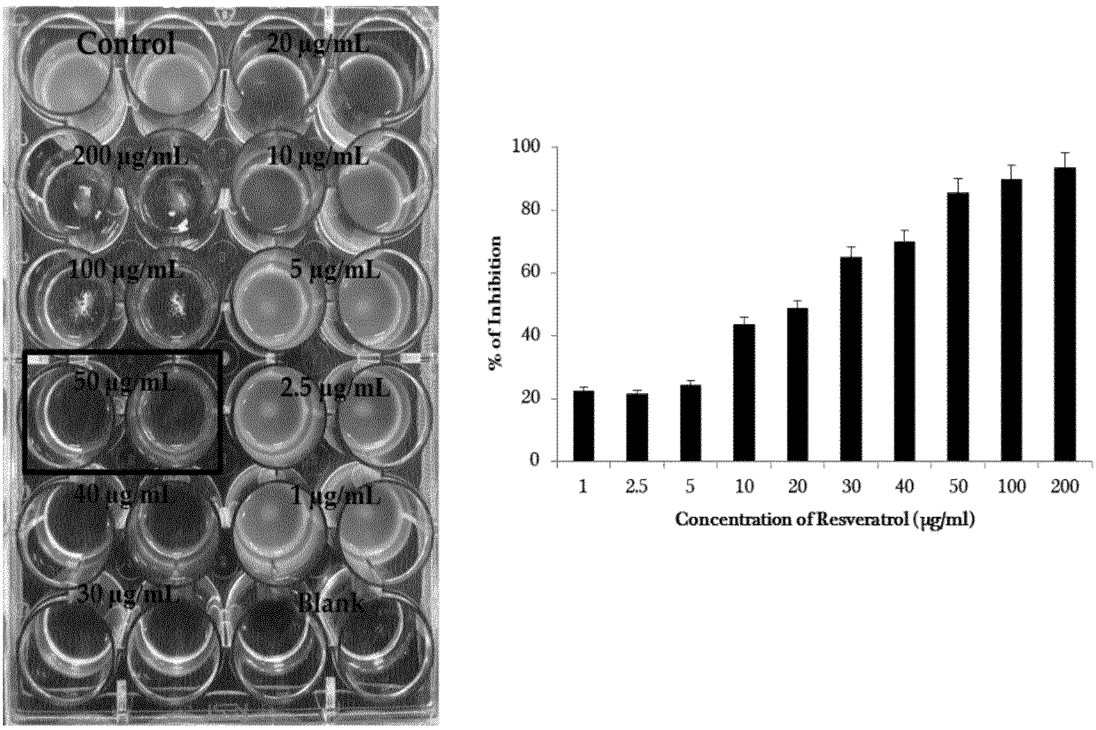


Figure 10e Inhibitory effect of resveratrol on growth of *A. hydrophila*

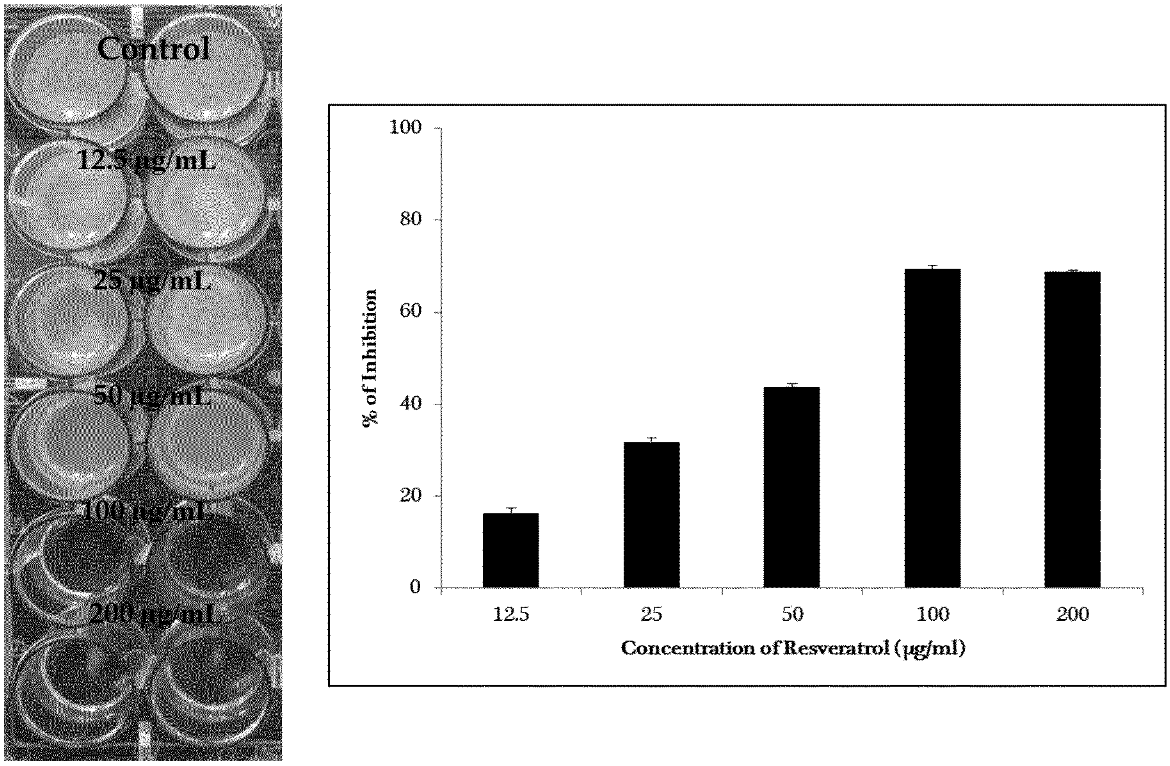


Figure 10f Inhibitory effect of resveratrol on growth of *P. fluorescens*

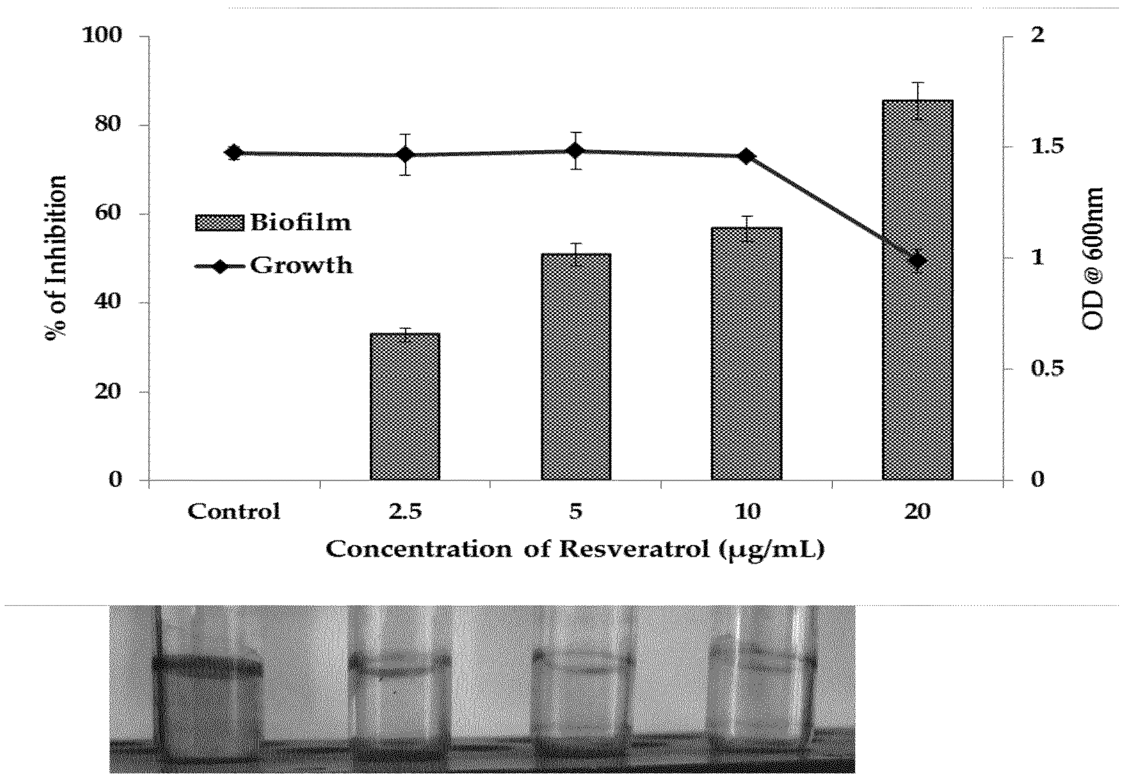


Figure 11a Effect of resveratrol on biofilm of *V. parahaemolyticus*

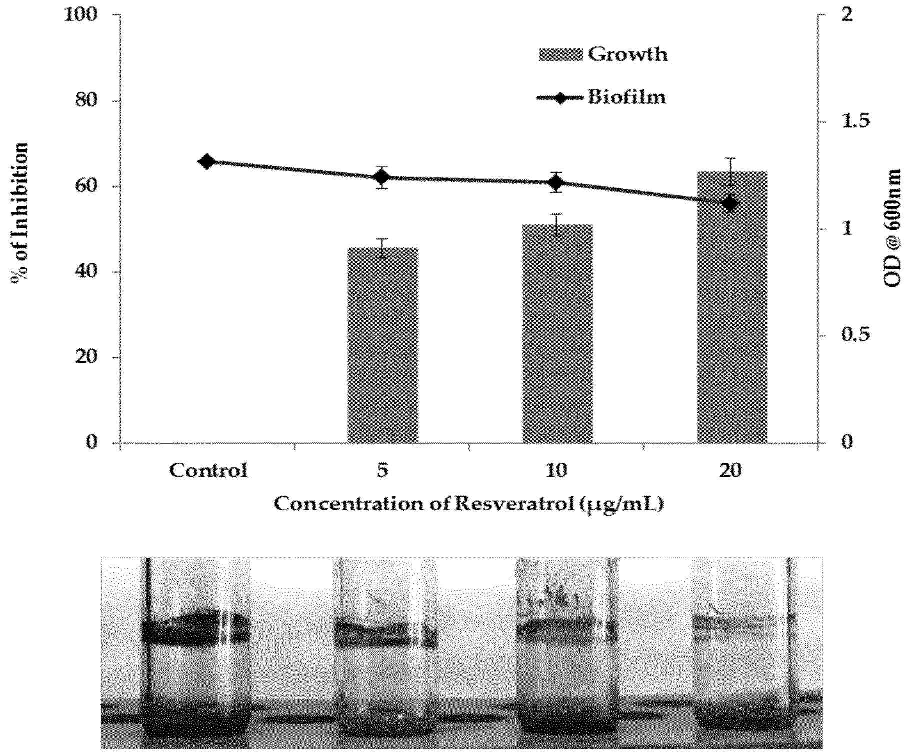


Figure 11b Effect of resveratrol on biofilm of *V. alginolyticus*

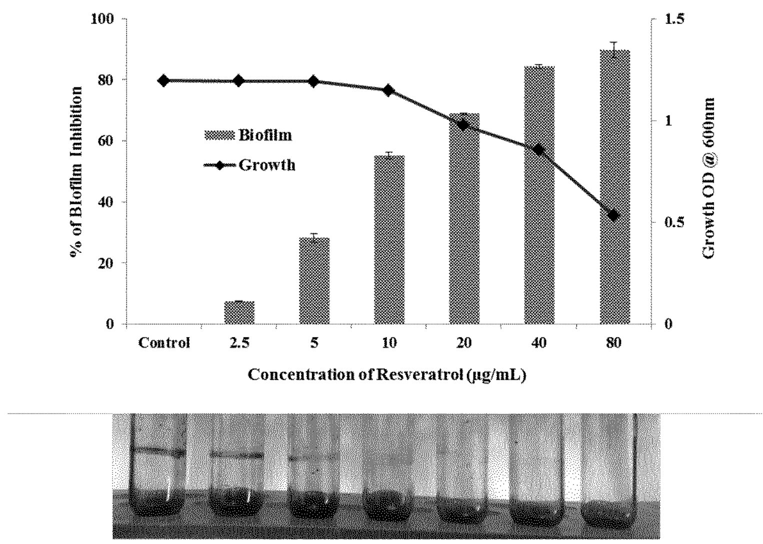


Figure 11c Effect of resveratrol on biofilm of *V. harveyi*

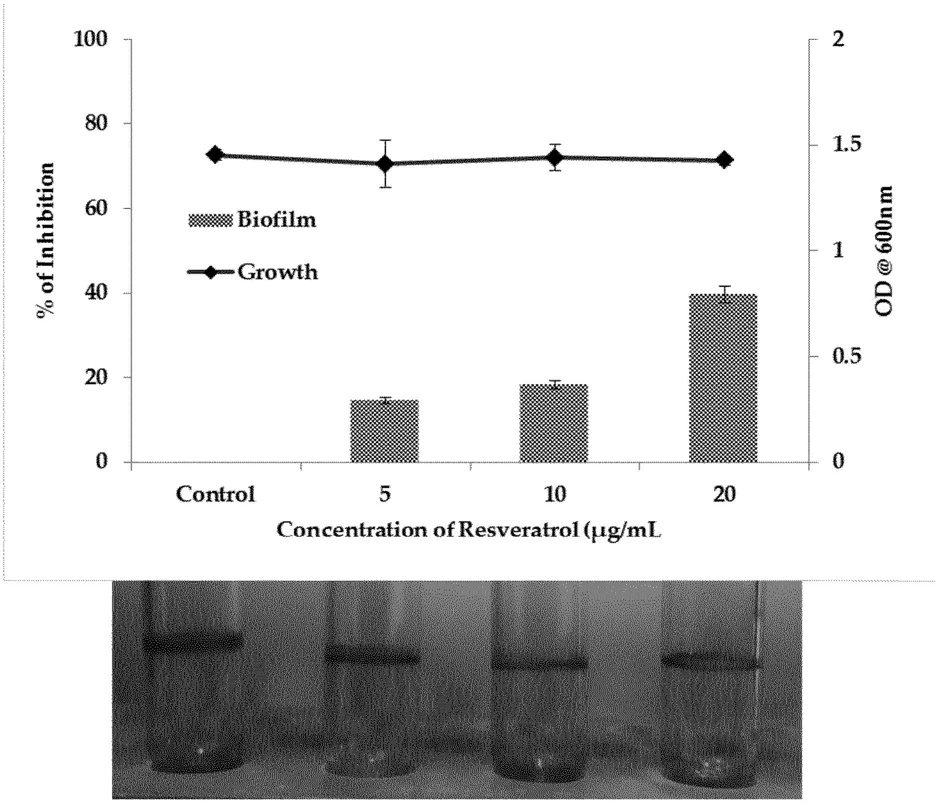


Figure 11d Effect of resveratrol on biofilm of *A. hydrophila*

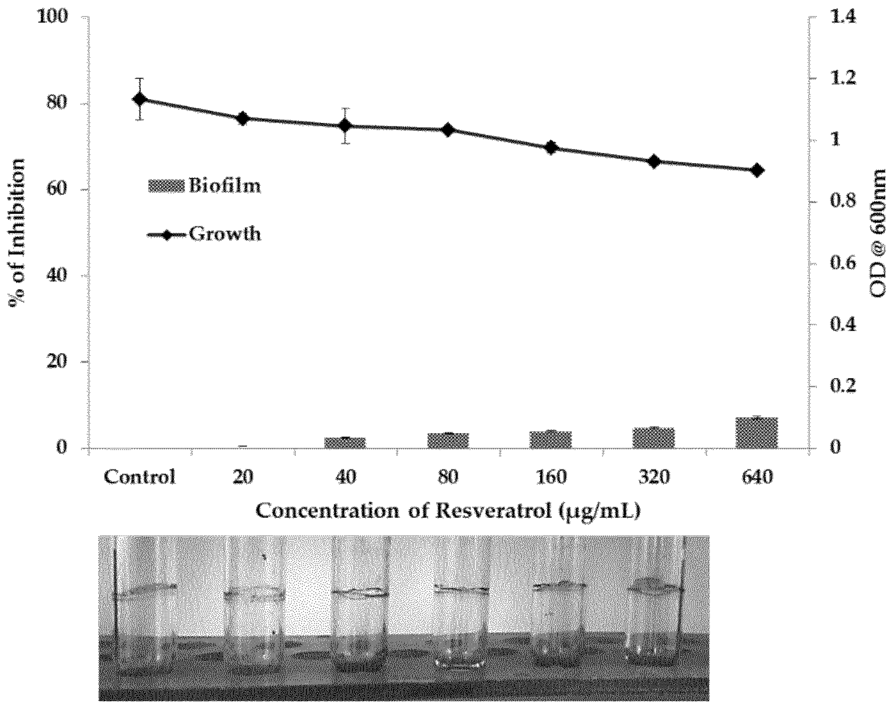


Figure 11e Effect of resveratrol on biofilm of *V. vulnificus*

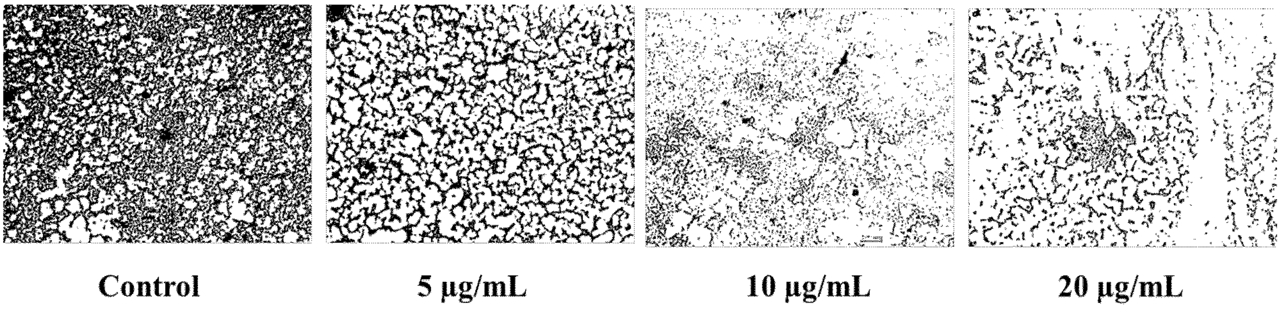


Figure 12a Light microscopic analysis of *V. parahaemolyticus* biofilm grown in the absence and presence of resveratrol.

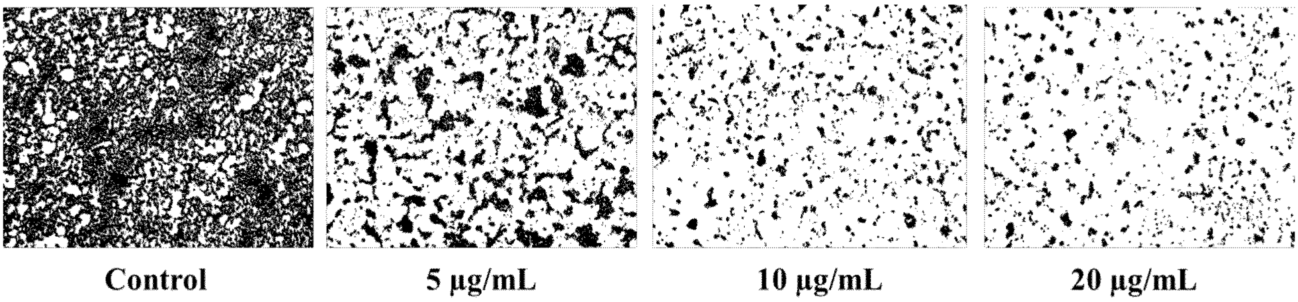


Figure 12b Light microscopic analysis of *V. alginolyticus* biofilm grown in the absence and presence of resveratrol

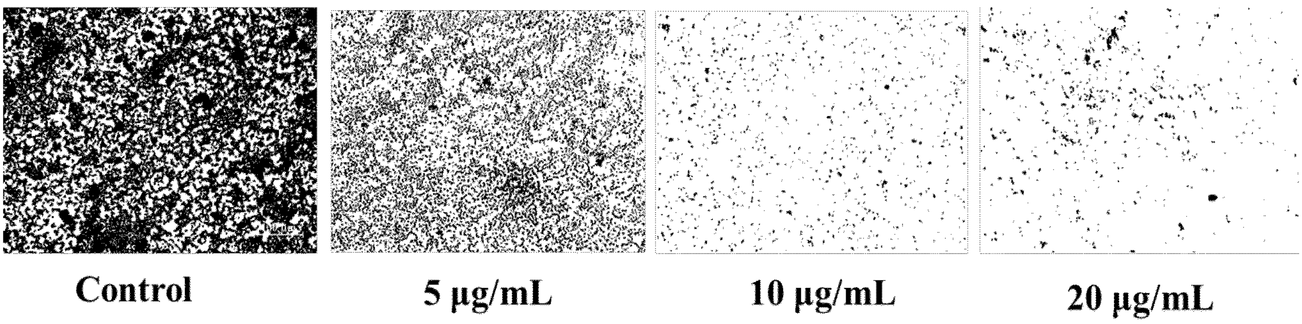


Figure 12c Light microscopic analysis of *V. harveyi* biofilm grown in the absence and presence of resveratrol

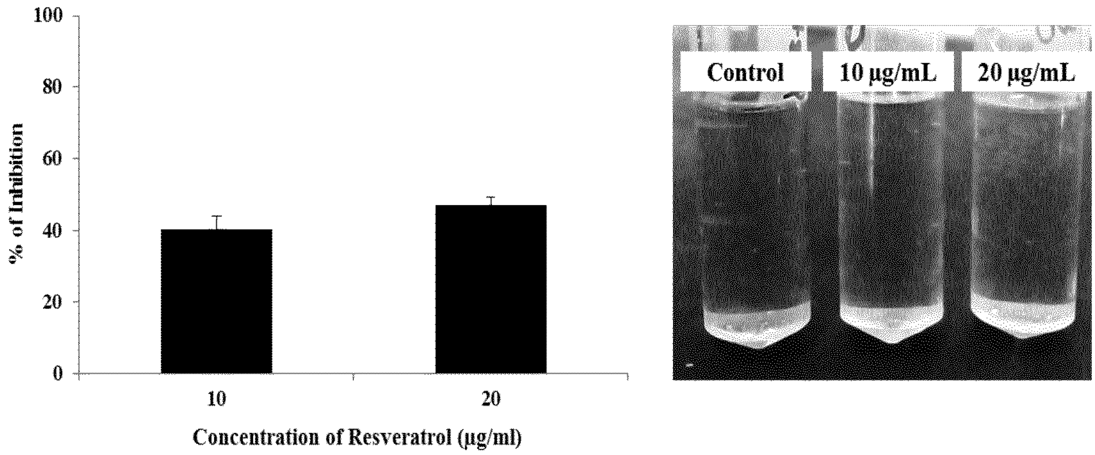
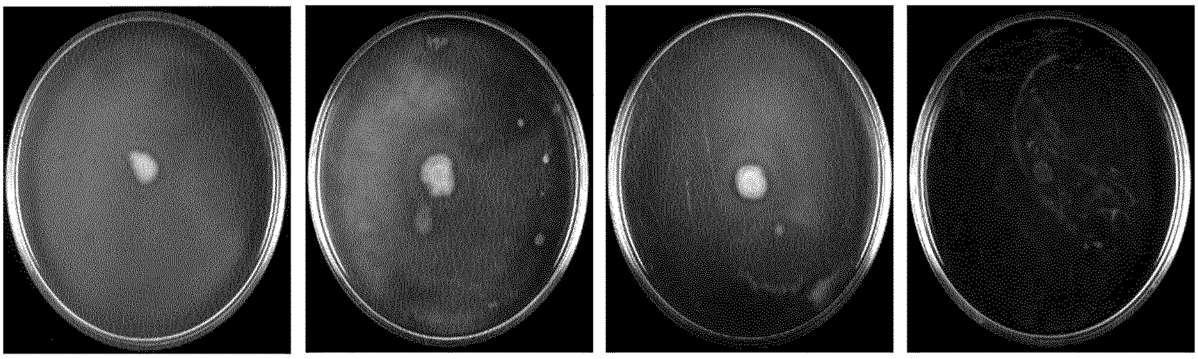
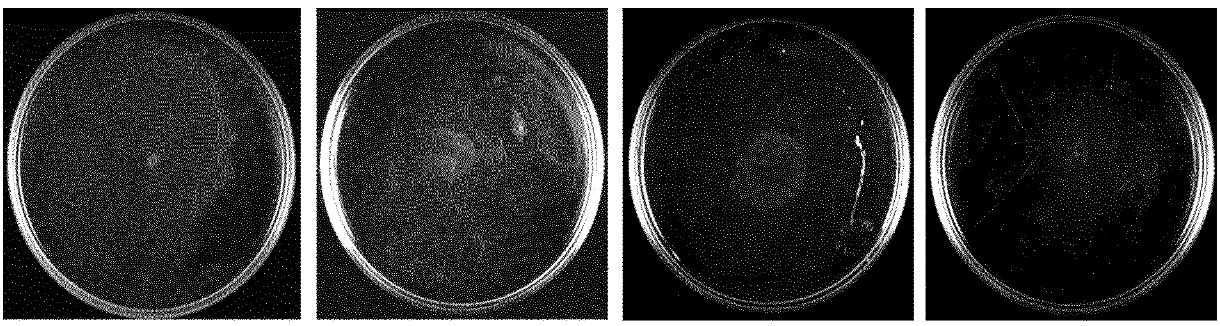


Figure 13 Effect of resveratrol on EPS production in *V. alginolyticus*



Control 5 µg/mL 10 µg/mL 20 µg/mL

Figure 14a Effect of resveratrol on swarming motility of *V. alginolyticus*



Control 5 µg/mL 10 µg/mL 20 µg/mL

Figure 14b Effect of resveratrol on swimming motility of *V. alginolyticus*

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/061864

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K10/30 A23K20/111 A23K50/80
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DARIO R. VALENZANO ET AL: "Resveratrol Prolongs Lifespan and Retards the Onset of Age-Related Markers in a Short-Lived Vertebrate", CURRENT BIOLOGY, vol. 16, no. 3, 1 February 2006 (2006-02-01), pages 296-300, XP055483790, GB ISSN: 0960-9822, DOI: 10.1016/j.cub.2005.12.038 the whole document -----	1,2,5,6, 8,9, 12-14

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[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2018	21/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Heirbaut, Marc

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILSON WHITNEY N ET AL: "Effects of resveratrol on growth and skeletal muscle physiology of juvenile southern flounder", COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY. PART A, MOLECULAR AND INTEGRATIVE PHYSIOLOGY, ELSEVIER SCIENCE, NEW YORK, NY, US, vol. 183, 16 December 2014 (2014-12-16), pages 27-35, XP029124373, ISSN: 1095-6433, DOI: 10.1016/J.CBPA.2014.12.014 the whole document -----	1,2,5,6, 8,9, 12-14
X	KOWALSKA AGATA ET AL: "Dietary resveratrol improves immunity but reduces reproduction of broodstock medakaOryzias latipes(Temminck & Schlegel)", FISH PHYSIOLOGY AND BIOCHEMISTRY, KUGLER PUBLICATIONS, AMSTERDAM, NL, vol. 43, no. 1, 18 July 2016 (2016-07-18), pages 27-37, XP036151436, ISSN: 0920-1742, DOI: 10.1007/S10695-016-0265-8 [retrieved on 2016-07-18] the whole document -----	1,2,5,6, 8,9, 12-14

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/061864

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 6, 9(completely); 5, 8, 12-14(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 6, 9(completely); 5, 8, 12-14(partially)

   Composition for feeding a fish or crustacean (claims 1-2; claims 5, 12-14 (partially)); method of increasing productivity of aquacultures of fish or decapod crustaceans (claim 6; claims 5, 8, 12-14 (partially)); use of a stilbene in aquaculture to increase the productivity of fish or decapod crustacean stock (claim 9; claims 5, 12-14 (partially)).

   ---

2. claims: 3, 4, 7, 10, 11(completely); 5, 8, 12-14(partially)

   Composition for the treatment of Vibrio biofilm formation in and/or on fish or decapod crustaceans (claims 3-4; claims 5, 8, 12-14 (partially)); method of reducing or preventing Vibrio biofilm formation (claim 7; claims 10-11; claims 5, 8, 12-14 (partially)).
