Marine actinomycete Streptomyces variabilis S26 as biocontrol agent for vibriosis in shrimp larval rearing systems

Solly Solomon[1], Divya Babu[1], Bhavya Kachiprath[1], Deepthi Augustine[1], Dhanya Kesavan[1], Manomi Sarasan[1], and Rosamma Philip[1]

[1]Cochin University of Science and Technology

April 25, 2023

Abstract

Indiscriminate use of antibiotics has led to the emergence of antibiotic resistant microbes and the loss of natural flora in aquaculture systems ultimately necessitating the ban of many of the chemotherapeutants in aquaculture. Actinobacteria play a profound role in the biogeochemical cycling in the marine environment and they represent the principal source of secondary metabolites with antimicrobial property. In the present study, 98 marine derived actinomycete isolates were screened for antimicrobial activity against the common aquatic pathogens. A potent actinomycete isolate S26, identified as Streptomyces variabilis based on 16S rRNA gene sequencing was used for further study. Optimization of the fermentation medium for secondary metabolite production was carried out by response surface methodology (RSM) using DESIGN EXPERT. The ANOVA of the quadratic regression model demonstrated that the model was highly significant for the response concerned i.e., antimicrobial activity as evident from the Fisher's F- test with a very low probability value [(P model>F) = 0.0001]. Of the 10 different solutions suggested by the software, the most suitable composition was found to be starch, 1.38 %; soy powder, 0.88 %; ammonium sulphate, 0.16 % and salinity, 27.76 production medium was applied in the Penaeus monodon larval rearing system and the total Vibrio count and survival rate were estimated. S. variabilis S26 treatment showed a significant reduction in Vibrios and better survival in the Penaeus monodon culture system compared to the control.

Marine actinomycete *Streptomyces variabilis* S26 as a biocontrol agent for vibriosis in shrimp larval rearing systems

Solly Solomon[1,2], Divya T Babu[1], Bhavya Kachiprath[1], Deepthi Augustine[1], Dhanya Kesavan[1], Manomi Sarasan[1] and Rosamma Philip[1*] *[1]Department of Marine Biology, Microbiology and Biochemistry, School of MarineSciences, Cochin University of Science and Technology, Kerala, India - 682016.[2]Fisheires Survey of India, Cochin Zonal Base, Kochangadi Road, Kochi, Kerala, India - 682005.**Corresponding Author: rosammap@gmail.com**Abstract**Indiscriminate use of antibiotics has led to the emergence of antibiotic resistant microbes and the loss of natural flora in aquaculture systems ultimately necessitating the ban of many of the chemotherapeutants in aquaculture. Actinobacteria play a profound role in the biogeochemical cycling in the marine environment and they represent the principal source of secondary metabolites with antimicrobial property. In the present study, 98 marine derived actinomycete isolates were screened for antimicrobial activity against the common aquatic pathogens. A potent actinomycete isolate S26, identified as *Streptomyces variabilis*based on 16S rRNA gene sequencing was used for further study. Optimization of the fermentation medium for secondary metabolite production was carried out by response surface methodology (RSM) using DESIGN EXPERT. The ANOVA of the quadratic regression model demonstrated that the model was highly significant for the response concerned*i.e.*, antimicrobial activity as evident from the Fisher'sF- test with a very low probability value [(Pmodel>F) = 0.0001]. Of the 10 different solutions suggested by the software, the most suitable composition was found to be starch, 1.38 %; soy powder, 0.88 %; ammonium sulphate, 0.16 % and salinity, 27.76 variabilis S26 cultured in the op-

timized production medium was applied in the *Penaeus monodon* larval rearing system and the total *Vibrio* count and survival rate were estimated. *S. variabilis* S26 treatment showed a significant reduction in Vibrios and better survival in the *Penaeus monodon*culture system compared to the control.**Abbreviations:** RSM - Response Surface Methodology; SDS - Sodium Dodecyl Sulphate; OVAT - 'One Variable at A Time'; CCD - Central Composite Design; CFU - colony forming units; PL - post-larvae; TCBS - Thiosulfate-citrate-bile salts-sucrose agar; ANOVA - analysis of variance.**Keywords**: Probiotics; Vibriosis; Streptomyces; Response surface methodology; Larval rearing

Introduction

Aquaculture is one of the fastest growing food sectors globally and provides the richest source of protein. Currently the culture systems are under the threat of various diseases caused by biological and non-biological agents. Among the bacterial diseases mainly the vibriosis causes huge economic loss in penaeid shrimp culture system especially in hatcheries. *Vibrio harveyi* causes mass mortality of *Penaeus monodon* larvae [1]. The use and abuse of antibiotics in aquaculture system has led to the emergence of antibiotic resistant microbes and loss of natural flora resulting in restrictions on the usage of chemotherapeutants in aquaculture. Hence the use of probiotics is gaining more importance as they improve the health of the animals without causing any deleterious effects in the culture system. Actinobacteria play a profound role in the marine environment by contributing to the breakdown and recycling of organic compounds [2]. They are gaining importance not only for their taxonomic and ecological perspectives, but also for their unique secondary metabolites notably antibiotics [3,4,5] and enzymes [6]. Despite being such a potential source of bioactive compounds, the probiotic role of actinomycetes mainly in aquaculture systems is comparatively less explored. You et al. [7] proposed the ability of actinomycetes to release antimicrobial compounds, degrade organic compounds and the formation of heat and desiccation resistant spores that can be explored for their possible use as a probiotic in aquaculture. You et al. [8] in 2007 reported the use of marine actinomycetes against biofilm formation by *Vibrio* spp. in aquaculture systems. Antibiotic production by the microbes is largely dependent on the nature and concentration of ingredients in fermentation medium [9,10]. Influence of particular nutrients on antibiotic biosynthesis is determined by the chemical structure of antibiotic substances [11]. Since the classical method of media optimization has been found inadequate for a full understanding of the response, optimization studies are done using Response Surface Methodology (RSM) or Box Wilson Methodology [12] which is a combination of statistical and mathematical techniques widely used to determine the effects of several variables that influence the responses by varying them simultaneously in limited number of experiments. The present work is aimed to study the anti-vibrio activity of a marine actinomycete S26 for the exclusion of Vibrios from the *Penaeus monodon* larval culture system.

2. Materials and methods

2.1. Microorganisms used for the study Marine actinomycetes (98 Nos.) already isolated from the continental shelf and slope sediments of the Arabian Sea and the Bay of Bengal and maintained in the Microbiology Laboratory of Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology (CUSAT), India were used for the study. 2.2. Screening of actinomycete isolates for antimicrobial activity The actinomycetes were screened for antibiotic production primarily by the cross-streak method against aquaculture pathogens *viz. Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, V. alginolyticus, V. cholerae, V. fluvialis, Vibrio harveyi, V. parahaemolyticus, Pseudomonas aeruginosa* and *Staphylococcus aureus.* These pathogens were obtained from National Centre for Aquatic Animal Health, Cochin University of Science and Technology. All the *Vibrio* spp. were streaked on prawn flesh agar medium to improve virulence [13]. Based on primary screening results, secondary screening (Kirby-Bauer disc diffusion) was done with five actinomycete strains (L25, M3, M16, SA14 and S26).**2.3. Selection of fermentation (production) medium for marine actinomycetes** Loop full of actinomycete spores from the 5 actinomycete isolates were inoculated into 50 ml each of the seed medium (nutrient broth) in 250 ml Erlenmeyer flask and incubated for 48 hours at room temperature (28±2°C) on a rotary shaker at 150 rpm. 10 ml each of the seed medium culture was inoculated into the five different production media (Table 1), incubated at 28±2 °C for 10 days and tested for activity against the patho-

gens. Of the five isolates, S26 was selected for its marked bioactivity and selected for further studies.**2.4. Molecular identification of the selected marine actinomycete isolate (S26)** The spore suspension of actinomycete culture S26 was inoculated into nutrient broth and incubated in an orbital shaker at 28 °C, 120 rpm for 16-18 hrs. The cells were pelleted at 15000xg for 10 min and then suspended in TEN buffer (100 mM Tris-HCl; 100 mM EDTA, (pH 8.0); 100 mM sodium phosphate (pH 8.0) and 1.5 M NaCl) having 10 % sodium dodecyl sulphate (SDS). Proteinase K was then added to a final concentration of 0.1 µg/µl and mixed gently. The suspension was kept at 55 °C for 2 hours for incubation and proper cell lysis. The suspension of lysed cells was cooled to room temperature and an equal volume of phenol equilibrated with 0.5M Tris-HCl (pH: 8.0) was added and gently mixed by slowly inverting the tube for 10 min. The two phases were separated by centrifugation at 5000xg for 15 min at room temperature. The viscous aqueous phase was transferred to a clean centrifuge tube and the extraction with phenol was repeated twice. A third extraction with a 24:1 mixture of chloroform and iso-amyl alcohol was carried out and the aqueous phase was collected carefully. DNA dissolved in solution were precipitated after the addition of 0.1 volume of 3 M sodium acetate (pH: 5.2) and 0.6 volume of isopropanol or 3 volumes of absolute ethanol. Incubation at -20 °C for 12 hrs precipitated the DNA. The precipitated DNA was pelleted by centrifugation at 10,000xg for 15 min at room temperature. The excess salt was removed by washing the DNA pellets three times in 70 % ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volumes of TE buffer and stored at -20 °C. Agarose gel electrophoresis was done to check the purity of DNA. The 16S rDNA was amplified using universal eubacterial primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [14]. The primers were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used involves an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation (94 °C for 20 sec), annealing (58 °C for 20 sec) and extension (72 °C for 90 sec), and a final extension (72 °C for 10 min). The PCR products were electrophoresed on 1.5 % agarose gel, stained with ethidium bromide (10 µg/ml) to ensure that a fragment of the correct size had been amplified. The gel was visualized on a Gel documentation system (Bio-Rad, USA). The purified PCR product was then sequenced at SciGenom, Cochin, India using ABI PRISM 3700 Big Dye sequencer using the primers 27F, 1492R and 530F (5'-GTGCCAGCCGCCGCGC-3'). Sequences were analyzed and the Basic Local Alignment Search Tool (BLAST) algorithm [15] was used to search the GenBank database for homologous sequences (http://www.ncbi.nlm.nih.gov/). The sequences were multiple aligned and were used to construct a phylogenetic tree by the neighbor-joining (NJ) method [16], using the MEGA-11 package [17]. Bootstrap analysis was based on 1000 replicates. The obtained sequence was submitted to NCBI GenBank under the accession number OQ398385.**2.5. Optimization of the production medium (OVAT analysis)**Medium B was selected based on the inhibition zone against the bacterial pathogens. Optimization of the medium was done for maximum antibiotic production by the selected actinomycete *Streptomyces variabilis*(S26). One dimensional screening was done initially to find out the range that has to be selected for further optimization experiment. All experiments were carried out in triplicates to minimize the error. The various parameters selected for the study were salinity, pH and nutrients *viz.* starch, soy meal and ammonium sulphate. To find out the optimum range of the above variables a definite range of these parameters were selected and applied in medium B.**2.6. Inoculation of the media and test for bioactivity**Young culture of S26 in nutrient broth was inoculated into the experimental medium and after incubation for 48 hrs at 28 °C, the modified Kirby-Baur disc diffusion method was employed for testing bioactivity against *V. harveyi* and the zone of inhibition was measured.**2.7. Optimization of Physico-chemical parameters of the production medium**The initial test range of each ingredient for approaching the optimal conditions was selected based on the experimental results of the traditional 'One Variable at A Time' (OVAT) method and was further optimized by a full factorial Central Composite Design (CCD) of the Response Surface Methodology (RSM). The experimental design, data analysis and quadratic model building were carried out with the aid of the statistical software Design-Expert (version 6.0., Stat-Ease Inc., Minneapolis, USA). The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using the same software. The software suggested 30 combinations with 16 factorial points (2^n: n= no of factors), 8 axial points (2n) and 6 central points for the different ranges of the 4 different media components. The actual values of the variables and the combinations of the design are presented in Table 2 and 3. The

effects of media components on antibiotic activity were statistically analyzed with response surfaces and the medium composition was optimized using mathematical equations and response surface plots.**2.8. Marine actinomycete *Streptomyces variabilis* S26 as a biocontrol agent against vibriosis2.8.1. Experimental animals**A batch of apparently healthy post-larvae of *Penaeus monodon* (PL-18; mean body weight 0.04-0.05 g; PCR negative for White Spot Syndrome Virus) were brought from a commercial prawn hatchery in Kochi (India). They were transferred to aquarium tanks of 30 Liter (L) capacity and acclimatized for one week under laboratory conditions. These larvae were maintained on control diets for a period of one week.**2.8.2. Actinomycete culture**Spore suspension of the actinomycete *S. variabilis* S26 was inoculated into optimized medium and incubated for 3-4 days at 28 °C in an incubator shaker. The biomass was harvested by centrifugation at 10,000xg for 15 min.**2.8.3. Experimental design**Apparently healthy larvae were distributed, 50 each, to 30 L fibre glass tanks containing 20 L sea water. 1 g of the actinomycete biomass was introduced to the experimental tanks and the control tank was maintained without actinobacteria. The experiments were done in triplicate for each treatment group and the control group. Both the control group and the treatment group of animals were fed a commercial diet (Grobest feed 'smart' S1). Water exchange was not done to create a stressful environment. Total *Vibrio* count of rearing water was monitored at 0^{th}, 36^{th}, $72^{nd}$and 108^{th} hour intervals on (Thiosulfate–citrate–bile salts–sucrose agar) TCBS agar. The plates were incubated at 28 degC, and the colonies formed on TCBS were counted and expressed as CFU mL^{-1}. The experiment data was analyzed by student's*t*- test. Significance level for the analysis was set to $P < 0.05$.**3. Results3.1. Screening for the selection of actinomycetes as biocontrol agents**Out of 98 actinomycete isolates subjected for primary screening, only 49 % of the isolates showed antimicrobial activity against the test pathogens. Among the total 48 isolates, eight showed bioactivity against almost all the tested pathogens (Fig.1). About 85 % of the isolates showed marked inhibition against *Bacillus cereus.* Though a wide majority showed anti-vibrio activity, *V. harveyi* was inhibited by only 10 % of the tested isolates. With respect to the observation in the primary screening, five actinomycetes with broad spectrum bioactivity were subjected to secondary screening and its bioactivity was confirmed (Fig.2).

3.2. Selection of production

It was observed that the antibiotic production varied significantly depending on the media constituents. Medium B supported marked antibiotic production and hence chosen as the basal medium for optimization. Among the selected actinobacterial strains, S26 showed pronounced antibacterial activity to all the tested pathogens and maximum inhibition was against *V. harveyi*.**3.3. Molecular identification and phylogenetic analysis of the antagonistic actinomycete S26**BLASTn analysis of the 16S rRNA nucleotide sequence of S26 at NCBI, showed 99.77 % similarity to *Streptomyces variabilis.* Phylogenetic tree was constructed to study the taxonomic position of S26 with that of 16S rDNA sequences of various *Streptomyces* spp. (Fig.3).**3.4. Optimization of the medium**Estimation of antibacterial activity with respect to OVAT method revealed the range of the media components that controlled the antibiotic production significantly (Fig.4). It was observed that the bioactivity increased with the increase in concentration of starch up to 1.5 g, beyond which the activity was found to decrease. Similar results were obtained with the ingredient soy powder, where maximum activity was obtained at a concentration of 1 g. With increase in concentration of ammonium sulphate, bactericidal activity increased up to a level of 0.15 g and above that no considerable increase was observed. Absence of bioactivity was noticed in the range 0-15 ppt salinity and steadily increased from 15 to 30 the four media components *viz.* starch, soy powder, ammonium sulphate and salinity were selected based on the results of optimization of one variable at a time approach (Fig.4). Using CCD method, a total of 30 experiments with appropriate combination of starch, soy powder, ammonium sulphate and salinity were conducted (Table 3). The matrix was analyzed by standard analysis of variance (ANOVA) as approximate to the experimental design used. The ANOVA of the quadratic regression model demonstrated that the model was highly significant (Table 4) for the response concerned *i.e.*, antimicrobial compound production as evident from the Fisher's F- test with a very low probability value [$(P\text{model}>F) = 0.0001$]. In this case, linear coefficient B and C along with quadratic coefficients such as B^2, C^2 and D^2 were significant model terms, where 'B' is soy powder, 'C' is ammonium sulphate and 'D' is salinity (Fig.5). The OVAT analysis of the media components showed the range of each media component and condition.

It gave an optimum activity when starch was at a concentration of 1 g/100ml; soy powder at 0.5 g/100ml; ammonium sulphate at 0.15 g/100ml; salinity at 30 ppt and pH at 7.**3.5.** *Streptomyces variabilis* **S26 as a biocontrol agent**Generally, the *Vibrio* count showed an increasing trend over the culture period in both the treated group and control group. But the actinomycete (S26) treatment could significantly suppress the proliferation of these Vibrios (Fig.6). The student t test for the observed data showed a P value of 0.007. The percentage survival was significantly higher in the actinomycete treated tanks compared to the control (Fig.7).**4. Discussion**Screening for novel antimicrobial agents is a continuous process to meet the increasing demand for therapeutants and overcome the phenomenon of drug resistance. Actinomycetes being one of the most potential candidates for the same have been intensively screened from various ecological niches. Only very few reports are available on the antagonistic*Streptomyces* from the marine environment and their use as biocontrol agents in aquaculture systems [18]. Antibiotics belonging to the classes' *viz.,* aminoglycosides, ansamycins, anthracyclines, glycopeptides, macrolides, peptides, tetracyclines, etc. had been developed from *Streptomyces* spp. [19, 20]. The discovery of new bioactive compounds hinges upon selective and sensitive screening methods. In the present study, of the 98 isolates screened, 49 % showed marked activity against the pathogens. Similar observations were made by other workers also [21, 22]. Significant antibacterial activity by actinomycetes against the pathogens*viz., S. aureus, P. aeruginosa* and *B. subtilis* and*Vibrio* species could be observed [23, 24, 25]. The performance of the actinomycete isolates was found to be different in different media in terms of its inhibitory action against the pathogens. The composition of the media influenced the production of the active principle. The importance of the media composition for the production of antibiotic by marine microorganisms was demonstrated by Okazaki and Okami [26]. Previous reports by Sujatha et al. [27] states that the optimization of fermentation conditions can increase the production of secondary metabolites. The production of secondary metabolites in actinobacteria is greatly influenced by various fermentation parameters such as available nutrients [28], pH and temperature [27] partial pressure of oxygen (pO_2) [29], agitation [30], mineral salts [31], metal ions [32], precursors and inducers [10], and inhibitors [33]. Actinobacteria that produce secondary metabolites often have the potential to produce various compounds from a single strain [34]. In the present study, we could clearly observe significant increase in activity, with medium B supporting the best production. The induction might be due to the complementary interaction of various media ingredients. The strains seemed to produce antibiotic substances after 7 days in significant amounts. This might be due to the enhanced secondary metabolite production during the sporulating stage or due to the rapid utilization of the initial nutrients to increase the cell number to the threshold level that is necessary for the production of the antibiotics. The release of antibiotics by *Streptomyces* is in a defensive mode to compete microorganisms in the environment. These are gyrase interfering small molecules which hinders the growth and replication in competing microbes. *Streptomyces* protect themselves with the help of efflux pumps, ribosomal protection proteins and modifying enzymes. The pH of the medium at a range of 6-8 was observed to be critical for the growth of the organisms and the higher and lower level drastically reduced the antibiotic production which is in agreement with the work of Sujatha et al. [27]. In the present study, it was observed that higher concentrations of starch and soy powder inhibited the antibiotic production by the strain S26, though the growth was enhanced. The presence of ready to use carbon sources and nutrients usually promote the growth of actinomycetes but not the antibiotic production. The effect of ammonium sulphate is due to the presence of NH_4^+ that favors the formation of glutamate in the carbohydrate metabolism, whose one or more hydroxylation led to antibacterial activity. The absence of growth at lower salinity was due to the test organism being marine in origin. The growth pattern in varying pH reconfirmed the optimum pH level as neutral for the growth of actinomycetes. ANOVA showed that soy powder, ammonium sulphate and salinity were controlling antibiotic production significantly. Though the interactive models seemed to be insignificant, it cannot be avoided as it is a hierarchical model. The response graphs showed that there is no increase in response for starch and soy powder above 1 % in the media. It was also evident that the suggested combinations remained within the observed range from the 10 solutions generated by the software. As per the experimental results, the optimum value of the medium components that gave maximum production of antimicrobial compounds was starch, 1.38 %; soy powder, 0.88 %; ammonium sulphate, 0.16 % and salinity, 27.76 the concentration of the medium components could be reduced considerably yielding better production at a lower price. Vibrios are opportunistic pathogens

found in the marine environment and hence under stressful conditions, the chances of infection in shrimps are more. Since the indiscriminate use of antibiotics has led to the development of resistance, the use of antibiotics in culture systems is restricted, and therefore alternate methods for exclusion of diseases especially Vibrios are essential. Application of indigenous microflora with potential antimicrobial activity in aquaculture systems would be a promising alternative. Defoirdt et al. [35] recommended isolating candidate probiotics from the culture system(s), which will facilitate their growth and establishment in the host. In the present study,*Streptomyces* spp. of marine origin was used. Exclusion of luminescent Vibrios using probiotic *Bacillus* and*Streptomyces* has been already reported [36, 37, 38, 39, 40]. Kumar et al. [41] observed the antiviral effect of actinomycetes while applied in feeds of *P. monodon*. Aguilera-Rivera et al. [42] had clearly stated the application of formulated probiotic feed that effectively reduces the load of Vibrios found in hepatopancreas and intestine of white shrimp. The exclusion of *V. parahaemolyticus*by probiotic feed by white shrimp maintained in a biofloc technology was reported by other studies also [43]. Another study by Sridevi and Dhevendaran [44] has also brought forward 6 actinomycete candidates isolated from seaweeds that has got probiotic potential, hence ascertaining the potential of marine ecosystem for novel probiotics. Studies proven that the probiotic consortium can act as an effective way for the reduction of pathogenic *Vibrio* species and prevention of mortality during *Vibrio* challenges [45, 46]. In the present work, stressful immune-compromising condition was created without water exchange and removal of excess feed or fecal matter, deteriorating the water quality. There was significantly high survival rate in penaeid post larvae compared to the control. The actinomycetes inhibited the growth of Vibrios by about 63 % in comparison to that of the control. Hence, *S. variabilis* S26 can be used as an anti-vibrio agent in penaeid larval rearing systems. In the present scenario, use of antibiotics in aquaculture practices is highly limited, necessitating the application of antagonistic microbes in aquaculture system. Extraction, purification and characterization of the bioactive principle might lead to the identification of novel antimicrobial compounds which can find potential application in medicine.**5. Acknowledgement**The authors are thankful to the Department of Biotechnology (DBT), Govt. of India for the research grant (BT/PR 13761/AAQ/03/514/2010) with which the work was carried out. We are also grateful to Department of Marine Biology, Microbiology and Biochemistry and National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology for providing necessary facilities to carry out the work.**6. Authors' contribution**SS carried out the experiment of the present work with support from DTB and DA. The work was carried out under the supervision of RP. SS wrote the manuscript. BK, MS, DTB and DK reviewed and edited the manuscript. All authors have read and approved the manuscript.**7. Compliance with ethical standards** This article does not contain any studies with human participants or animals performed by any of the authors.

8. Declaration of authors

The authors declare that there is no conflict of interest.

9. References

1. Musa N, Wei LS, Wee W (2008). Phenotypic and genotypic characteristics of Vibrio harveyi isolated from black tiger shimp (Penaeus monodon). World Appl. Sci. J. 3(6), 885-902.
2. Goodfellow M. and Haynes J.A. (1984). Actinomycetes in marine sediments. Ortiz-Ortiz L., Bojalil L. F., Yakoleff V. (Eds) Biological, Biochemical and Biomedical Aspects of Actinomycetes Academic Press Orlando. 453–472.
3. Strohl W (2004). Antimicrobials. Microbial Diversity and Bioprospecting (Bull AT, ed), pp. 288–313. ASM Press, Washington, DC. https://doi.org/10.1128/9781555817770.ch31.
4. Berdy J. Bioactive microbial metabolites. J Antibiot (Tokyo). 2005 Jan;58(1):1-26. doi: 10.1038/ja.2005.1. Erratum in: J Antibiot (Tokyo). 2005; 58(4):C-1.
5. Alam K, Mazumder A, Sikdar S, Zhao Y-M, Hao J, Song C, Wang Y, Sarkar R, Islam S, Zhang Y and Li A (2022). Streptomyces: The biofactory of secondary metabolites. Front. Microbiol. 13:968053. doi: 10.3389/fmicb.2022.968053.
6. Oldfield C, Wood NT, Gilbert SC, Murray FD, Faure FR. Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus Rhodococcus, and related taxa.

Antonie Van Leeuwenhoek. 1998; 74(1-3):119-132. doi: 10.1023/a:1001724516342.

7. You, J. L., Cao, L. X., Liu, G. F. et al. Isolation and characterization of actinomycetes antagonistic to pathogenic Vibrio spp. from nearshore marine sediments. World J Microbiol Biotechnol 21(5), 679-682 (2005). https://doi.org/10.1007/s11274-004-3851-3.
8. You J, Xue X, Cao L, Lu X, Wang J, Zhang L, Zhou S. Inhibition of Vibrio biofilm formation by a marine actinomycete strain A66. Appl Microbiol Biotechnol. 2007; 76(5):1137-1144. doi: 10.1007/s00253-007-1074-x.
9. Aharonowitz Y, Demain AL. Carbon catabolite regulation of cephalosporin production in Streptomyces clavuligerus. Antimicrob Agents Chemother. 1978; 14(2):159-64. doi: 10.1128/AAC.14.2.159.
10. Martin JF, Demain AL. Control of antibiotic biosynthesis. Microbiol Rev. 1980; 44(2):230-251. doi: 10.1128/mr.44.2.230-251.1980.
11. Pereda A, Summers RG, Stassi DL, Ruan X, Katz L. The loading domain of the erythromycin polyketide synthase is not essential for erythromycin biosynthesis in Saccharopolyspora erythraea. Microbiology. 1998; 144 (Pt 2):543-553. doi: 10.1099/00221287-144-2-543.
12. Box GEP, Wilson KB (1951). On the experimental attainment of optimum conditions. J. Roy. Stat. Soc. B. 13, 1-45.
13. Singh I.S.B. and Philip R. (1993). Comparative efficiency of three media in isolating heterotrophic bacteria from the larvae and larval rearing water of Macrobrachium rosenbergii. Indian Journal of Microbiology, 33, 67–69.
14. Lane, D.J. (1991) 16S/23S rRNA Sequencing. In: Stackebrandt, E. and Goodfellow, M., Eds., Nucleic Acid Techniques in Bacterial Systematics, John Wiley and Sons, Chichester, 115-175.
15. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3):403-10. doi: 10.1016/S0022-2836(05)80360-2.
16. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4(4):406-425. doi: 10.1093/oxfordjournals.molbev.a040454.
17. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021; 38(7):3022-3027. doi: 10.1093/molbev/msab120.
18. Augustine D, Jacob JC, Philip R (2015). Exclusion of Vibrio spp. by an antogonestic marine actinomycete Streptomyces rubrolavendulae M56, Aquaculture Research, 1–10. https://doi.org/10.1111/are.12746.
19. Baltz RH. Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? J Ind Microbiol Biotechnol. 2006 Jul;33(7):507-13. doi: 10.1007/s10295-005-0077-9.
20. Robertsen HL, Musiol-Kroll EM. Actinomycete-derived polyketides as a source of antibiotics and lead structures for the development of new antimicrobial drugs. Antibiotics (Basel). 2019; 8(4):157. doi: 10.3390/antibiotics8040157.
21. Thakur DA, Yadav BK, Gogoi TC, Bora TC (2007). Isolation and screening of Streptomyces in soil of protected forest areas from the states of Assam and Tirupura, India, for antimicrobial metabolites. J Mycol Med. 17(4), 242-249. https://doi.org/10.1016/j.mycmed.2007.08.001.
22. Abo-Shadi M. A., Sidkey N. M. and Al-Mutrafy A. M. (2010). Antimicrobial agent producing microbes from some soils' rhizosphere in Al-Madinah Al-Munawwarah, KSA. Journal of American Science 6 (10) 915- 925.
23. Devi NKA, Jeyarani M, Balakrishnan K (2006). Isolation and identification of marine actinomycetes and their potential in antimicrobial activity. Pak. J. Biol. Sci. 9, 470-472.
24. Dhanasekaran D, Thauddin N, Panneerselvam A (2009). Distribution and ecobiology of antagonistic Streptomyces from agriculture and coastal soil in Tamilnadu, India. Journal of Culture Collections. 6:10–20.
25. Valli S, Suvathi SS, Aysha OS, Nirmala P, Vinoth KP, Reena A. Antimicrobial potential of Actinomycetes species isolated from marine environment. Asian Pac J Trop Biomed. 2012; 2(6):469-473. doi: 10.1016/S2221-1691(12)60078-1.
26. Okazaki T, Okami Y. Studies on marine microorganisms. II. Actinomycetes in Sagami Bay and their

antibiotic substances. J Antibiot (Tokyo). 1972; 25(8):461-466.
27. Sujatha P, Bapi Raju KV, Ramana T. Studies on a new marine streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant Staphylococcus aureus. Microbiol Res. 2005; 160(2):119-126. doi: 10.1016/j.micres.2004.10.006.
28. Fguira LFB, Fotso S, Ameur-Mehdi RB, Mellouli L, Laatsch H. Purification and structure elucidation of antifungal and antibacterial activities of newly isolated Streptomyces sp. strain US80. Res Microbiol. 2005; 156(3):341-7. doi: 10.1016/j.resmic.2004.10.006.
29. Iwai Y, Omura S. Culture conditions for screening of new antibiotics. J Antibiot (Tokyo). 1982; 35(2):123-41. doi: 10.7164/antibiotics.35.123.
30. Bode HB, Bethe B, Hofs R, Zeeck A. Big effects from small changes: possible ways to explore nature's chemical diversity. Chembiochem. 2002; 3(7):619-27. doi: 10.1002/1439-7633(20020703)3:7<619::AID-CBIC619>3.0.CO;2-9.
31. Wang YH, Yang B, Ren J, Dong ML, Liang D, Xu AL (2003). Optimization of medium composition for the production of clavulanic acid by Streptomyces clavuligerus. Process Biochem. 40(3-4), 1116-1166. https://doi.org/10.1016/j.procbio.2004.04.010.
32. Gesheva V, Ivanova V, Gesheva R. Effects of nutrients on the production of AK-111-81 macrolide antibiotic by Streptomyces hygroscopicus. Microbiol Res. 2005;160(3):243-8. doi: 10.1016/j.micres.2004.06.005.
33. Bibb MJ. Regulation of secondary metabolism in Streptomycetes. Curr Opin Microbiol. 2005; 8(2):208-15. doi: 10.1016/j.mib.2005.02.016.
34. Schiewe HJ, Zeeck A. Cineromycins, gamma-butyrolactones and ansamycins by analysis of the secondary metabolite pattern created by a single strain of Streptomyces. J Antibiot (Tokyo). 1999; 52(7):635-42. doi: 10.7164/antibiotics.52.635.
35. Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol. 2007; (10):472-9. doi: 10.1016/j.tibtech.2007.08.001.
36. Moriarty DJW (1998). Control of luminous Vibrio species in penaeid aquaculture ponds. Aquaculture. 164(1-4), 351-358. https://doi.org/10.1016/S0044-8486(98)00199-9.
37. Rengpipat S, Phianphak W, Piyatiratitivoraku S, Menasveta P (1998). Effects of a probiotic bacterium on black tiger shrimp Penaeus monodon survival and growth. Aquaculture. 167(3-4), 301–313. https://doi.org/10.1016/S0044-8486(98)00305-6.
38. Vaseeharan B, Ramasamy P. Control of pathogenic Vibrio spp. by Bacillus subtilis BT23, a possible probiotic treatment for black tiger shrimp Penaeus monodon. Lett Appl Microbiol. 2003; 36(2):83-87. doi: 10.1046/j.1472-765x.2003.01255.x.
39. Decamp, O; Moriarty, DJW; Lavens P (2008). Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Research, 39(4), 334–338. doi:10.1111/j.1365-2109.2007.01664.x.
40. Mazon-Suastegui JM, Salas-Leiva JS, Medina-Marrero R, Medina-Garcia R, Garcia-Bernal M. Effect of Streptomyces probiotics on the gut microbiota of Litopenaeus vannamei challenged with Vibrio parahaemolyticus. Microbiologyopen. 2020; 9(2):e967. doi: 10.1002/mbo3.967.
41. Kumar SS, Philip R, Achuthankutty C (2006). Antiviral property of marine actinomycetes against white spot syndrome virus in penaeid shrimps, Current Science, 91(6), 807-811.
42. Aguilera-Rivera, D., Prieto-Davo, A., Escalante, K., Chavez, C., Cuzon, G., Gaxiola, G., 2014. Probiotic effect of floc on Vibrios in the Pacific white shrimp Litopenaeus vannamei. Aquaculture 424–425, 215–219. https://doi.org/10.1016/j.aquaculture. 2014.01.008.
43. Krummenauer, D., Poersch, L., Romano, L. A., Lara, G. R., Encarnacao, P. and Wasielesky Jr. W. (2014). The Effect of Probiotics in a Litopenaeus vannamei Biofloc Culture System Infected with Vibrio parahaemolyticus, Journal of Applied Aquaculture, 26:4, 370-379, DOI: 10.1080/10454438.2014.965575.
44. Sridevi K, Dhevendaran K. Streptomycetes from marine seaweeds: Their antimicrobial and antibiotic potential. Int J Appl Biol Pharm. 2014; 5(4); ISSN: 0976-4550, 74-79.

45. Quiroz-Guzman E, Vazquez-Juarez R, Luna-Gonzalez A, Balcazar JL, Barajas-Sandoval DR, Martinez-Diaz SF. Administration of probiotics improves the brine shrimp production and prevents detrimental effects of pathogenic Vibrio species. Mar Biotechnol (NY). 2018; 20(4):512-519. doi: 10.1007/s10126-018-9822-8.
46. Du S, Chen W, Yao Z, Huang X, Chen C, Guo H, Zhang D. Enterococcus faecium are associated with the modification of gut microbiota and shrimp post-larvae survival. Anim Microbiome. 2021; 3(1):88. doi: 10.1186/s42523-021-00152-x.

Hosted file

`Figures.docx` available at https://authorea.com/users/611025/articles/639731-marine-actinomycete-streptomyces-variabilis-s26-as-biocontrol-agent-for-vibriosis-in-shrimp-larval-rearing-systems

Hosted file

`Tables.docx` available at https://authorea.com/users/611025/articles/639731-marine-actinomycete-streptomyces-variabilis-s26-as-biocontrol-agent-for-vibriosis-in-shrimp-larval-rearing-systems