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Antiviral viral compound from *Streptomyces ghanaensis* like strain against white spot syndrome virus (WSSV) of shrimp

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35 **Abstract**

36 Actinomycetes isolates collected from different environments were screened for
37 antiviral activity against WSSV. One isolate designated as CAHSH-2 showed antiviral
38 activity against WSSV at the concentration of 0.2 mg per shrimp. The laboratory trial of
39 determining antiviral activity of ethyl acetate extract (EtOAcE) of CAHSH-2 against WSSV
40 was carried out 21 times since 2014. CAHSH-2 isolate which showed antiviral activity was
41 characterized and identified as *Streptomyces ghanaensis* like strain. Among the five fractions
42 obtained from EtOAcE of potential actinomycetes isolate, F1 was found to have strong
43 antiviral activity. The F1A and F1B sub-fractions from F1 fraction were subjected to GC-MS,
44 FTIR, ^{1}H and ^{13}C NMR analyses and, the compounds identified were di-n-octyl phthalate and
45 bis (2-methylheptyl) phthalate, respectively. Among these compounds, di-n-octyl phthalate
46 showed strong antiviral activity against WSSV. Molecular docking studies revealed that di-n-
47 octyl phthalate was found to have high binding affinity with VP26 and VP28 proteins of
48 WSSV, whereas the bis (2-methylheptyl) phthalate showed low binding affinity with VP26
49 and VP28. The antiviral activity of EtOAcE of actinomycetes against WSSV was confirmed
50 by PCR, RT-PCR, Western blot and ELISA. The EA extract of active isolate was found to be
51 non-toxic to *Artemia*, post-larvae and adult *Litopenaeus vannamei*.

52 **Importance**

53

54 White spot syndrome virus (WSSV) is an important shrimp viral pathogen and
55 responsible for huge economic loss to shrimp culture industry worldwide including India. The
56 global loss due to WSSV has been estimated about USD 10 billion and the loss continues at
57 the same extent even now. Various strategies have been followed to prevent or control
58 diseases of aquatic animals. In spite of various preventive and control strategies, WSSV has
59 been still persisting for more than two decades. No control strategies have so far been evolved
60 to put a break to WSSV. In this situation, an attempt was made in the present work to screen
61 some actinomycetes isolates for antiviral activity against WSSV. Among these isolates, one
62 isolate identified as *Streptomyces ghanaensis* like isolate CAHSH-2 showed activity against
63 WSSV. This article gives the information about the antiviral compound against WSSV and
64 the mechanism of viral inhibition.

65

66

67 **Keywords:** Actinomycetes, *Litopenaeus vannamei*, *Artemia*, Antiviral activity, Molecular
68 docking.

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69 **1. Introduction**

70 Disease is now considered to be a major limiting factor in aquaculture production
71 worldwide. The economic loss due to diseases has been estimated about US$ 1 billion in
72 developing countries of Asia in 1990 alone. Since then, losses have increased. A 1995
73 estimate suggests that aquatic animal disease and environment-related problems may cause
74 annual losses to aquaculture production in Asian countries to the tune of more than US$ 2
75 billion per year. According to recent reports, global losses due to shrimp disease are more
76 than US$ 2.8 billion. In India, the loss due to white spot syndrome virus (WSSV) has been
77 estimated about USD 150 million per year, and the loss continues even now to the same
78 extent (1). Viral and bacterial diseases are very important since they are responsible for huge
79 economic loss in aquaculture systems worldwide. Although information on biology and
80 culture aspects of Indian cultivable species of fish, shrimp and prawn is available, studies on
81 diseases of aquatic animals in Indian aquaculture system and their control and prevention are
82 very few. The important viral and bacterial pathogens reported in Indian aquaculture system
83 are WSSV, infectious hypodermal and hematopoietic necrosis (IHHNV), monodon
84 baculovirus (MBV), hepatopancreatic necrosis virus (HPV), fish nodavirus, *Macrobrachium*
85 *rosenbergii* nodavirus (*Mr*NV), extra small virus, *Vibrio harveyi*, *V. anguillarum*, *Aeromonas*
86 *hydrophila*, *A. caviae* and *Edwardsiella tarda*. A surveillance program supported by National
87 Fisheries Development Board of ICAR, India is being carried out to monitor aquaculture
88 systems throughout the country for emerging diseases in shrimp, prawn and fish. As a result
89 of this program which is now into its third year, the occurrence of *Enterocytozoon*
90 *hepatopenaei* (2, 3) and infectious myonecrosis virus (IMNV) in pond-reared *L. vannamei*
91 (4), cyprinid herpesvirus-2 in goldfish (5) and tilapia lake virus in Nile tilapia (6) has been
92 reported. In the field condition, various control strategies such as improvement of
93 environmental conditions, stocking of specific pathogen-free shrimp post-larvae and
94 augmentation of disease resistance by oral immunostimulants, are being applied to control
95 WSSV infection. But none of these methods provided effective protection to shrimp against
96 WSSV infection.

97 Actinomycetes are potential microorganisms that are capable of producing a variety of
98 antiviral compounds working against viral pathogens of human, higher animals, aquatic
99 animals and plants. (7) have isolated bioactive compounds from actinomycetes possessing
100 high antiviral activity against acyclovir-resistant herpes simplex virus at non-cytotoxic
101 concentration. Promising non-plant derived glycan-targeting compounds such as the mannose-
102 specific pradimicin-A (PRMA) extracted from the actinomycete strain *Actinomadura hibisca*,

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103 showed strong binding capacity with human immunodeficiency virus (HIV) (8). Actinohivin
104 (AH) is a potent anti-HIV lectin that is produced by an actinomycete, *Longispora albida* gen.
105 nov, sp. nov (9, 10). (11) reported that Benzastatin C, a 3-chloro-tetrahydroquinolone alkaloid
106 from *Streptomyces nitrosporeus*, showed antiviral activity in a dose-dependant manner against
107 herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and vesicular
108 stomatitis virus (VSV). (12) have isolated a novel anti-influenza virus compound (JBIR-68)
109 from *Streptomyces* sp. RI18 and this compound inhibited influenza virus growth in plaque
110 assays. A bioactive compound, xiamycin D isolated from *Streptomyces* sp (#HK18) culture
111 inhabiting the topsoil in a Korean solar saltern was tested for antiviral activity against porcine
112 epidemic diarrhea virus (PEDV) and results showed that xiamycin D had the strongest
113 inhibitory effect on PEDV replication (13). Methylelaiophylin, an antiviral compound isolated
114 from *Streptomyces melanosporofaciens* showed antiviral activity against Newcastle disease
115 virus (NDV) (14).

116 Work on application of actinomycetes for aquatic animal health is very limited in
117 India. (15) have isolated twenty-five isolates of actinomycetes and tested them for their ability
118 to reduce WSSV-infection in shrimp. The antiviral activity of furan-2-yl acetate (C6H6O3)
119 extracted from Streptomyces VITSDK1 spp. was studied in cultured Sahul Indian Grouper
120 Eye (SIGE) cells infected with fish nodavirus (FNV). This compound (20 μg mL^{-1}) effectively
121 inhibited the replication of the FNV, and the viral titre was reduced from 4.3 to 2.45 log
122 TCID50 mL^{-1} on treatment (16). (17) reported the antiviral activity of crude extract of marine
123 *Streptomyces carpaticus* MK-01 isolated from seawater against viral hemorrhagic septicemia
124 virus of fish. Based on the above studies, an attempt was made in the present study to screen a
125 group of actinomycetes collected from different habitats for antiviral activity against WSSV of
126 shrimp.

127 **2. Materials and methods**

128 **2.1. Collection and maintenance of experimental animals**

129 Marine shrimp, *Litopenaeus vannamei* (10–15 g body weight) were collected from
130 grow-out ponds located at Nellore, AP or Ponneri, TN and maintained in 1000 l aquarium
131 tanks containing seawater with salinity between 15 and 20 ppt (50 shrimp per aquarium tank)
132 at room temperature which ranged from 27 to 30°C for 5 days prior to experiments. The
133 shrimp were fed with commercial pellet feed. Seawater was obtained from the Research
134 Centre of Central Institute of Brackishwater Aquaculture, Muttukadu near Chennai. Seawater
135 was pumped from the adjacent sea and allowed to sediment, thus removing sand and other

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136 particulate matter before use for shrimp. Five shrimps were randomly selected and screened
137 for WSSV by PCR with the primers designed by Yoganandhan *et al.* (18).

138 **2.2. White spot syndrome virus (WSSV)**

139 The WSSV suspension was prepared from WSSV-infected shrimp *L. vannamei* with
140 clinical signs of lethargy, reddish colouration and white spots. The infected shrimp were
141 collected from shrimp farms located near Nellore, India. Hemolymph was drawn directly
142 from the heart of infected shrimp using sterile syringes. The hemolymph was centrifuged at
143 3000 *g* for 20 min at 4ºC and the supernatant was collected. Then the collected supernatant
144 was recentrifuged at 8000 *g* for 30 min at 4ºC. The final supernatant fluid was then filtered
145 through a 0.4 µm filter before storage at -20ºC, until used in infectivity experiments. Prior to
146 storage, the presence of WSSV was confirmed by PCR using primers designed by
147 Yoganandhan *et al.* (18). Bioassay experiment was carried out to check the virulence of
148 WSSV by injecting the viral suspension intramuscularly in healthy shrimp. This viral
149 suspension was used for screening actinomycetes for antiviral activity against WSSV.

150 **2.3. Collection of Actinomycetes isolates**

151 In the present study, actinomycetes isolates were isolated from different
152 environments like marine sediment, plants, effluents from industrial areas and freshwater
153 samples using standard isolating procedures and recommended culture media (19, 20, 21).
154 *Streptomyces ghanaensis* and *S. viridosporus* used as reference strains were obtained from
155 IMTECH Culture Collection, Chandigarh, India and used for comparison to characterize the
156 isolates of actinomycetes.

157 **2.4. Extraction of metabolites**

158 Actinomycetes isolates were grown separately in different media such as starch casein
159 broth (SCB) and production medium-I (PM-I), PM-II, and PM-III to select the most suitable
160 medium. The cultures were incubated at 28ºC in a rotary shaker. The cultures were harvested
161 after 7 days and filtered through Whatman No.1 filter paper. The culture filtrates were
162 centrifuged at 10,000 rpm at 4°C and the cell free supernatants were used for the extractions of
163 secondary metabolites. Each culture supernatant was extracted three times by manual shaking
164 with equal volume of ethyl acetate (1:1) in a separating funnel. The solvent layer was
165 collected and concentrated using a rotary evaporator (Buchi, Switzerland) to obtain crude
166 metabolites. The antiviral activity of ethyl acetate actinomycetes extract against WSSV was
167 determined using standard protocol.

168 **2.5. Preliminary Screening of antiviral activity:**

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169 The antiviral activity of actinomycetes extract against WSSV was tested in shrimp
170 based on the procedures described by Balasubramanian et al. (22, 23). Extracts prepared from
171 different isolates of actinomyctes using ethyl acetate were tested to determine the antiviral
172 activity against WSSV. The inactivation of WSSV was confirmed by bioassay, PCR and
173 Western blot analyses. For screening antiviral activity, the shrimp (5 animals per tank in
174 triplicate) were injected intramuscularly with a mixture of viral suspension, extract of
175 actinomycetes and NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl and 0.02 M EDTA, pH 7.4) at
176 the volume of 30 µl per animal (5 µl of viral suspension, 10 µl of actinomyctes extract with
177 varying concentration {100 or 500 µg/animal} and 15 µl of NTE buffer). The positive control
178 consisted of a mixture of 25 µl NTE buffer and 5 µl viral suspension. For toxicity studies, the
179 animals were injected with the extracts of actinomycetes of various concentrations as
180 mentioned above. All these mixtures were incubated at 29 °C for 30 min to 1 h before
181 injection. After incubation, the mixture was injected into experimental animals
182 intramuscularly. Mortalities were recorded for each day of experimental period after post
183 infection with WSSV.

184 **2.6. Characteristics of potential isolate**

185 Morphological and cultural characteristics and pigment production of actinomycetes
186 isolate having antiviral activity were examined as described by Shirling and Gottlieb (24).
187 Morphological characters such as colony characteristics, pigment production, absence or
188 presence of aerial and substrate mycelium were observed on actinomycetes isolate grown on
189 International Streptomyces Project (ISP) media 1 to 8. The arrangement of spores and
190 sporulating structures were examined microscopically by using cover slip culture method (25,
191 26). The spore morphology was studied by scanning electron microscope using electron grid
192 at different magnifications. Cell wall amino acids and whole cell sugars of actinomycetes
193 isolate were analyzed as per the standard protocols (27, 28). The growth of the potential
194 isolate was studied under various conditions such as temperature which ranged from 15 to
195 40°C, pH from 4 to 10 and NaCl concentrations from 0 to 10% to optimize the growth
196 conditions using standard protocols. For starch utilization test, actinomycetes isolate was
197 streaked on starch agar plate and incubated at 28°C for 7 d. After incubation, iodine solution
198 was poured on the agar and examined for the hydrolysis of starch by the production of clear
199 zone around the bacterial growth. Potential actinomycetes isolate was streaked on gelatin agar
200 plates and incubated at 28°C for 7 d for testing gelatin hydrolysis. After incubation, plates
201 were flooded with 1 mL of mercuric chloride solution and observed for zone of hydrolysis.

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202 To test the casein hydrolysis, isolate was streaked on skimmed milk agar medium and
203 incubated at 28°C for 7 d and observed for zone of hydrolysis. In the urea hydrolysis test,
204 isolates were inoculated into sterile urea agar slants and incubated at 28°C for 7 d and change
205 in colour was observed. Isolates were inoculated into Simon's citrate slant agar and incubated
206 at 28°C for 7 d and a change in colour was observed. In addition to above tests, other
207 biochemical characters were studied using Ready prepared KB003 Hi 25 kit (HiMedia,
208 India). The tests were ONPG, Lysine, Ornithine, Urease, Phenylalanine, Nitrate reduction,
209 H_2S production, Citrate utilization, Voges Proskauer's, Methyl red test, Indole production,
210 Malonate, Esculin hydrolysis, Oxidase, Catalase, Crystal violet, Tween – 20, Sodium azade
211 (0.01%), Sodium azade (0.02%), growth in Macconkey agar and Casein utilization. All
212 reaction wells of the kit (Strip-I and Strip-II) were inoculated with a loop of actinomycetes
213 culture and incubated at 28°C for 14 days and the results were observed.

214 The ability of actinomycetes to utilize various amino acids as energy source was
215 tested by using basal medium with 0.1% of various amino acids such as L-Arginine, L-Phenyl
216 alanine, L-Tyrosine, L-Methionine, L-Histidine, DL-Tryptophane, L-Cysteine and L-
217 Glutamine. The ability of actinomycetes isolates to utilize various carbon compounds as
218 energy source was tested using ready prepared HiMedia kit (KB009 HiCarbo™ kit KB009A
219 /KB009B1 /KB009C). The kit has 35 sugars like Lactose, Xylose, Maltose, Fructose,
220 Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose,
221 Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol,
222 Arabitol, Erythritol, alpha-Methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, alpha-
223 Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Citrate, Malona and Sorbose,
224 and 1 control. The wells in kits (Part A, Part B1 and Part C) were inoculated with
225 actinomycetes isolates and incubated at 28°C for 14 days. After incubation, the wells in the
226 kits were observed for bacterial growth by comparing with negative and positive controls.

227 The genomic DNA was extracted from actinomycetes isolate using the phenol-
228 chloroform method and 16S rDNA gene was amplified using the universal primer (5' AGA
229 GTT TGA TCM TGG CTC AG 3' and 5' TAC GGY TAC CTT GTT ACG ACT T 3') with
230 the standard PCR protocol. The PCR product was purified using QIAquick PCR Purification
231 Kit (Qiagen, India) and sequenced using an ABI3730xl sequencer. The nucleotides of the 16S
232 rDNA sequence was matched with the other microbes in the NCBI database using the
233 BLAST program. The sequence was deposited in GenBank. The construction of phylogenetic
234 tree was carried out by using Geneious Basic software and evolutionary history inferred using
235 the neighbor joining method (29).

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236 **2.7. Purification of bioactive compound**

237 The purification of the bioactive compound having strong antiviral activity against
238 WSSV was carried out using silica gel column chromatography as described by Atta et al.
239 (2009). Twenty gram of silica gel (60 – 120 mm mesh) was mixed with 5 g of ethyl acetate
240 extract of actinomycetes using absolute chloroform and kept for a few hours for drying. Then
241 the mixture was packed in 50 × 2.5 cm size column. The sample was eluted with the
242 petroleum ether and ethyl acetate solvents in the ratio of 60:40 at the flow rate of one drop
243 per minute. All eluted fractions were concentrated at room temperature and checked for
244 antiviral activity against WSSV using the protocol described above. The active fraction (F1)
245 was further fractioned using column chromatography using petroleum ether and methanol in
246 the ratio of 70:30 as a mobile phase. Sub-fractions (F1A and F1B) were collected as
247 described above and determined the antiviral activity.

248 **2.8. Identification of the active compound**

249 The compounds obtained from potential actinomycetes isolates were dissolved in
250 ethyl acetate at a concentration of 100 mg/ml and the ultraviolet spectra were recorded on
251 Shimadzu UV - Vis 2450 Spectrophotometer at 200 - 400 nm range using UV-Probe
252 software. A 13 mm Potassium bromide (KBr) pellet using active compounds was prepared
253 and subjected to FTIR analysis to obtain the spectrum between 400–4000 cm^{-1} at a resolution
254 of 4 cm–1 using Shimadzu IR affinity–1 FTIR spectrometer (Japan). The purified bioactive
255 compounds isolated from potential actinomycetes isolate were analyzed by using GC Clarus
256 500 (Perkin Elmer, Singapore) equipped with an Elite–5MS column (30.0 m x 250 µm× 0.25
257 µm composed of 5% diphenyl–and 95% dimethyl–polysiloxane). The detection of the
258 compound was based on 90% similarity between the MS spectra of the unknown compound
259 and reference compounds accessible in the MS spectra library of National Institute for
260 Standards and Technology (NIST). The ^{1}H and ^{13}C NMR spectra for the purified compounds
261 having antiviral activity against WSSV were recorded on spectrometer (Bruker, Germany,
262 400 MHz) using CDCl3 as the solvent and tetramethylsilane as the internal reference. The
263 structure of the antiviral compound extracted from *Streptomyces ghanaensis* CAHSH2 was
264 established with the help of spectral data obtained from spectroscopic analysis. The 2D
265 structure of the compound was obtained using Chem3D Draw Ultra software (Version 10)
266 (30).

267 **2.9. Molecular docking studies**

268 3-Dimensional (3D) structures of structural proteins VP26 (PDB id:2edm) and VP28
269 (PDB id: 2ed6) of WSSV were obtained from Protein Data Bank (PDB) (Berman, *et al.*,

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270 2000). The 3D coordinates of VP26 and VP28 were imported and visualized in PyMOL
271 viewer (31). From PDB coordinates, co-crystallized ligand atoms were identified and
272 removed. Then crystallographic water molecules eliminated from the viral protein coordinates
273 and hydrogen atoms were added to the 3D structure and energy minimizations were
274 performed using Swiss pdb viewer (32). The structure of antiviral compounds (di-n-octyl
275 phthalate and bis (2-methylheptyl] phthalate) were drawn in Chemsketch (33) and 3D
276 coordinates were generated using Corina online 3D conversion software (34). These two
277 compounds were then optimized using chimera for flexible conformations (35). The
278 molecular structure-based docking studies were carried out using Auto-Dock version 4.0 (36).
279 These two antiviral compounds were then docked to the two viral proteins, VP26 and VP28.
280 Lamarckian genetic docking algorithm was utilized to generate accurate binding
281 conformations of di-n-octyl phthalate and bis (2-methylheptyl) phthalate in VP26 and VP28
282 (37).

283 **2.10. Confirmation of antiviral activity of EtOAcE against WSSV**

284 The antiviral activity of ethyl acetate extract of actinomyctes isolate (EtOAcE) against
285 WSSV of shrimp was confirmed by polymerase chain reaction (PCR), reverse transcriptase –
286 PCR (RT-PCR), Western blot, ELISA and survival of shrimp in bioassay using standard
287 protocols (38, 39, 40, 41). Quantitative real-time PCR was performed to determine the WSSV
288 viral load in gill tissue of shrimp injected with EtOAcE-treated WSSV and other shrimp
289 groups. The viral copy number was estimated by Step One Plus Real-Time PCR System
290 (Applied Biosystems, USA) using Taqman assay (42).

291 **2.11. Toxicity studies**

292 The toxicity of ethyl acetate extract of potential isolate and pure compound having strong
293 antiviral activity against WSSV was evaluated in *Artemia* nauplii and post-larvae of *L.*
294 *vannamei* by bathing treatment and in adult by intramuscular injection using standard
295 protocols.
296

297 **3. Results**

298 **3.1. Preliminary screening of actinomycetes isolates against WSSV**

299 Sixty-four actinomycetes isolates collected from different environments were
300 screened for antiviral activity against WSSV. Ethyl acetate extracts of five isolates showed
301 antiviral activity against WSSV at the concentration of 1 mg per shrimp. One isolate
302 designated as CAHSH-2 showed strong antiviral activity and inactivated WSSV at the
303 concentration of 0.2 mg per shrimp and more than 90% survival was observed whereas other

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304 isolates failed to inactivate WSSV at lower concentrations. The CAHSH-2 isolate was
305 isolated from plant and selected for detailed studies. The laboratory trial of determining
306 antiviral activity of ethyl extract of CAHSH-2 against WSSV was carried out 21 times since
307 2014, and the results are given in Table 1. No survival of shrimp injected with WSSV was
308 observed at 48 h p.i. The survival percentage ranged from 88.8 to 98 in shrimp injected with
309 NTE buffer. In the case of shrimp injected with CAHSH-2-extarct treated WSSV, the survival
310 was found to be high and ranged from 88.8 to 100%. The shrimp injected with the extract of
311 CAHSH-2 only showed survival which ranged from 81.4 to 98.3%.

312 **3.2. Characteristics of potential Actinomycetes isolates**

313 The CAHSH-2 isolate having strong antiviral activity against WSSV was characterized
314 by colony morphology, spore structure, cultural characteristics, physiological tests,
315 biochemical tests, carbon utilization, nitrogen utilization and antibiotic sensitivity, and
316 molecular taxonomy by sequencing 16S rRNA gene. The results are presented in table 2 and
317 Fig.1. All these characteristics of isolates were studied along with reference strains,
318 *Streptomyces ghanaensis* and *S. viridosporus* obtained from IMTECH, Chandigarh, India. The
319 colony of CAHSH-2 contained both aerial and substrate mycelia. Microscopic observation of
320 actinobacterial isolate revealed the presence of well-developed short chain and branched
321 mycelium which consisted of verticillate type spores (Fig 2). The isolated CAHSH-2 strain is a
322 Gram positive and non-motile actinomycete. The optimal growth conditions of the isolate were
323 temperature at 28 ^{o}C, pH of 7 and NaCl concentration of 2%. This isolate utilized a wide
324 variety of carbohydrates as a carbon source. Histidine among various amino acids tested was
325 found to be the best source of nitrogen for the growth of CAHSH-2. The potential isolate
326 utilized lysine and ornithine but failed to deaminate phenylalanine. This isolate failed to
327 reduce the nitrate and did not produce H_2S and was found to be positive for urease and citrate
328 utilization showing their ability to hydrolyze urea and citrate. It was found to be positive for
329 VP test, ONPG test, oxidase and MR test, and negative for indole production and catalase.
330 Analysis of whole-cell components revealed LL-type of diaminopimelic acid and glycine, and
331 no characteristic sugar pattern. PCR amplification of 16S rDNA of CAHSH-2 yielded 1311
332 nucleotides and its sequence was deposited in GenBank under the accession number
333 KT006906.1. The BLAST analysis of the sequence revealed that the CAHSH-2 showed 99%
334 similarity with *Streptomyces ghanaensis* (NR-112460.1). Furthermore, phylogenetic tree was
335 constructed with CAHSH-2 isolate and neighbor joining phylogenetic tree using cluster W
336 software which revealed that it was *Streptomyces* sp. with high bootstrap value (Fig 3). Based

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337 on this, CAHSH-2 isolated from *Ocimum tenuiflorum* was identified as *Streptomyces*
338 *ghanaensis* like strain and designated as *Streptomyces ghanaensis* – CAHSH-2

339 **3.3. Extraction, purification and activity of antiviral compounds**

340 Five prominent bands were observed in TLC plate loaded with ethyl acetate extract
341 having antiviral activity against WSSV (Fig 4). The Rf value of five bands was determined as
342 0.49-0.62 (Band I); 0.62-0.72 (Band II); 0.72 – 0.87 (Band III) 0.72-0.87 (Band IV) and 0.87-
343 0.92 (Band V). The extract was fractionated by silica gel column and five fractions (F1, F2,
344 F3, F4 and F5) were obtained. Among the five fractions, F1 was found to have strong antiviral
345 activity. The mortality of shrimp injected with F1 treated WSSV at the concentration of 100 μg
346 per shrimp was estimated to be about 0% on 3 d p.i., 5.56% on 5 d p.i and 13.89% on 10 d p.i.
347 The F1 was further fractioned by column chromatography and two sub fractions, F1A and F1B
348 were obtained. These two sub-fractions were tested for their antiviral activity against WSSV
349 and both sub-fractions showed antiviral activity against WSSV (Table 3 a, b). The mortality of
350 shrimp injected with F1B treated WSSV at the concentration of 100 μg per shrimp was
351 estimated to be about 0% on 3 and 5 d p.i., and 11.1% on 10 d p.i. In the case of shrimp
352 injected with F1A treated WSSV, the mortality was found to be 66.7% and 81.5% on 5 and 10
353 d p.i., respectively at the concentration of 100 μg per shrimp. In the case of shrimp injected
354 with WSSV, 100% mortality was observed on 3 d p.i.

355 **3.4. Identification of antiviral compounds**

356 The F1A and F1B sub-fractions from active fraction exhibited a maximum UV
357 absorption at 287 nm and 279 nm, respectively in ethyl acetate (Fig 5a). The FTIR spectrum of
358 the F1B showed characteristic peaks corresponding to standard library spectra (Fig 5b). A
359 broad peak observed at 3076.47 cm^{-1} indicated the presence O–H stretching. Peak observed at
360 2966.38 cm^{-1} indicated aromatic C–H stretching frequency. Peak observed at 1736 cm^{-1}
361 indicated the presence of C=O stretch (ketone). Peak observed at 1639 cm^{-1} corresponded to
362 C=O stretching (esters). Peak at 1462.68 cm^{-1} represented C=C stretching. The FTIR spectrum
363 of F1A showed the absence of hydroxyl group but showed the presence of ester moiety
364 (1714.23 and 1282.90 cm−1) and aromatic system (1639.22, 1461.35, 908.70 and 722.32
365 cm−1) (Fig 5b). The results of Gas chromatography - mass spectrum (GC-MS) analysis of
366 antiviral compounds (F1A and F1B) from actinomycetes isolate CAHSH-2 revealed the
367 molecular formula of $C_{24}H_{38}O_4$ with molecular weight of 390 g/mol for F1A and $C_{24}H_{38}O_4$ and
368 388.54 g/mol for F1B (Fig 5c).

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369 The ^{1}H NMR of the compound (F1A) showed the symmetry of the molecule. The two-
370 terminal methyl and the two- secondary methyl at C-2^1 appeared at δ 0.88 integrating for 12
371 protons. The aromatic protons H-3 and H-4 appeared at δ 7.74 and 7.73 as AA1BB1 system.
372 The methylene protons at H-11 attached to the ester appeared at δ 4.09 as multiplet. The other
373 methylene protons appeared at δ 1.43-1.33 (Fig 5d).
374 The ^{13}C NMR spectrum also confirmed the structure. The ester carbonyl appeared at δ
375 167.7 while the methylene attached to ester oxygen appeared δ 68.1. The terminal methyl
376 appeared at δ 14.1 while the methyl attached to C-2^1 appeared at δ 10.9. The three aromatic
377 carbons C-2, C-3 and C-4 appeared at δ 130.9, 128.8 and 132.4, respectively. The other
378 carbons are also accounted for 167.70, 132.35, 130.89, 128.84, 65.88, 31.79, 29.25, 29.18,
379 28.55, 25.97, 22.63, 14.06 ppm (Fig 5e). The compound having the antiviral activity against
380 WSSV was identified as Di-N-Octyl phthalate. based on the interpretation of the results of
381 GC-MS, FTIR and NMR spectrum analyses.
382 The ^{1}H NMR of the compound (F1B) showed the symmetry of the molecule. The two-
383 terminal methyl and the two-secondary methyl at C-2^1 appeared at δ 0.88 integrating for 12
384 protons. The aromatic protons H-3 and H-4 appeared at δ 7.67 and 7.52 as AA1BB1 system.
385 The methylene protons at H-11 attached to the ester appeared at δ 4.09 as multiplet. The other
386 methylene protons appeared at δ 1.43-1.33 (Fig 5f).
387 The ^{13}C NMR spectrum also confirmed the structure. The ester carbonyl appeared at δ
388 167.7 while the methylene attached to ester oxygen appeared δ 68.1. The terminal methyl
389 appeared at δ 14.1 while the methyl attached to C-2^1 appeared at δ 10.9. The three aromatic
390 carbons C-2, C-3 and C-4 appeared at δ 130.9, 128.8 and 132.4, respectively. The other
391 carbons are also accounted for 167.7 (C-1), 68.1 (C-11), 38.7 (C-21), 23.7 (C-31), 28.9 (C-41),
392 30.3 (C-51), 22.9 (C-61), 14.1 (C-71), 10.9 (CH-CH3) (Fig 5g). The F1A having limited
393 antiviral activity against WSSV was identified as bis (2-methylheptyl) phthalate.

394 **3.5. Molecular docking studies**

395 In this study, the aim was to target VP26 and VP28 proteins of WSSV. FTsite
396 identified three ligand binding sites for these target proteins (Fig 6a). A significant variation
397 among these three ligand binding sites was observed. Among these three sites obtained from
398 FTsite, site 1 was conserved in VP 26 and VP28 and therefore site 1 was selected for
399 molecular docking study. The ligand binding sites in VP26 are TYR46, GLN48, MET49,
400 MET50, ARG51, ARG67, TYR69, ASN70, THR71, PRO72 and ligand binding sites in VP28
401 are GLU50, ASN51, LEU52, ARG53, PRO55, VAL70, PHE72, ASP73, SER74, ASP75,
402 THR76 and ILE82. The 3D structure of probable ligand binding site is shown in surface model

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403 and amino acid residues located at ligand binding site of VP 26 and VP28 are shown in (Fig
404 6b). The optimized di-n-octyl phthalate and bis (2-methylheptyl) phthalate structures were
405 docked into the ligand binding site of VP26 and VP28 (Fig 6c). The docking results were
406 analysed and results revealed that the lowest binding energy value represents stabled protein-
407 ligand complex compared to higher binding energy value. Table 4, reveals that a variation was
408 observed in the binding affinity of di-n-octyl phthalate and bis (2-methylheptyl) phthalate with
409 VP26 and VP28 viral proteins. The di-n-octyl phthalate showed the lowest binding energy
410 values with VP26 and VP28 (-5.54 Kcal/Mol and -4.7 Kcal/Mol) when compared to bis (2-
411 methylheptyl) phthalate. The di-n-octyl phthalate was found to have high binding affinity with
412 VP26 and VP28, whereas binding affinity of the bis (2-methylheptyl) phthalate with VP26 and
413 VP28 was low. The binding poses of di-n-octyl phthalate and bis (2-methylheptyl) phthalate
414 into VP26 and VP28 proteins of WSSV are shown in (Fig 6d-g)
415 Further investigation was carried out on docked complexes to check H-bond network
416 between ligands and viral proteins. From the interaction analysis, a difference was noticed in
417 the formation of H-bond between the compounds [di-n-octyl phthalate and bis (2-
418 methylheptyl) phthalate] and viral proteins (VP26 and VP28) (Fig 6h [A-D]). Upon analysis of
419 the interaction of di-n-octyl phthalate with VP26, it was observed that the amino acid residues
420 Arg67, Tyr69 and Arg51 contributed four H-bonds with di-n-octyl phthalate whereas in VP28,
421 the ligand binding site residues Met49 and Thr71 participate in the H-bond interaction. On
422 analyzing di-n-octyl phthalate - VP28 complex, it was seen that only one cationic amino acid
423 residue Arg53 was involved in the formation of seven H-bond interactions with di-n-octyl
424 phthalate. The interaction analysis of bis (2-methylheptyl) phthalate - VP28 complex reveals
425 that the cationic residue Arg53 contributed three H-bonds with bis (2-methylheptyl) phthalate.
426 Binding pose of di-n-octyl phthalate and bis (2-methylheptyl) phthalate with VP26 and VP28
427 viral proteins and close up view of each complex is shown in surface model. In the present
428 study, on analyzing VP26 and VP28 in complex with di-n-octyl phthalate and bis (2-
429 methylheptyl) phthalate, di-n-octyl phthalate showed higher binding affinity and multiple
430 hydrogen bonds with VP26 and VP28 viral proteins.
431

432 **3.6. Confirmation of antiviral activity of potential actinomycetes isolates**

433 The PCR results showed WSSV-positive in shrimp injected with EtOAcE of CAHSH-
434 2 treated WSSV till 10 d p.i. and became negative on 15 d p.i (Fig 7a). The RT-PCR analysis
435 revealed that the expression of VP28 gene of WSSV was gradually decreased and totally
436 disappeared after 5 d p.i. in shrimp injected CAHSH-2 treated WSSV (Fig 7b). The shrimp

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437 injected with EtOAcE of CAHSH-2 treated WSSV showed negative to WSSV after 5 d p.i. by
438 Western blot (Fig 7c-d). In ELISA, the OD value corresponding to the presence of WSSV
439 (VP28 protein) in shrimp injected with WSSV treated with EtOAcE of CAHSH-2 was found to
440 decrease gradually and became negligible after 5 d p.i (Fig 7e).

441 The load of WSSV was quantified in gill and head tissues of shrimp injected with
442 EtOAcE of CAHSH-2 treated WSSV by real time PCR at different time intervals. In the gill
443 tissue, the WSSV load was estimated to be about $2.7 X 10^5$, $5.4 X 10^3$, 150 and 0.97 copies per
444 mg of tissue at 2, 5, 10 and 15 d p.i., respectively. In the head tissue, the WSSV load was
445 estimated to be about $3.1 X 10^4$, $2.7 X 10^3$, 19 and 0.96 copies per mg of tissue at 2, 5, 10 and
446 15 d p.i., respectively. In the shrimp injected with WSSV, the WSSV was estimated to be
447 about $6.2 X 10^9$ in gill tissue $4.3 X 10^7$ in head tissue at moribund stage (48 h p.i.) (Table 5).

448 **3.7. Toxicity studies**

449 The toxicity of the ethyl acetate extract of actinomycetes isolate (CAHSH-2) and
450 antiviral compound (Di-N-Octyl phthalate) purified from CAHSH-2 isolate was studied *in vivo*
451 using *Artemia* nauplii, and shrimp post-larvae by immersion method and sub-adult and adult
452 shrimp by intramuscular injection method. The EtOAcE of CAHSH-2 and pure compound
453 purified from the extract of CAHSH-2 isolate were found to be non-toxic to the *Artemia*
454 nauplii, post-larvae and adult *L. vannamei* (Table 6a). The mortality of experimental animals
455 was found to be insignificant and similar to the negative control group of animals. In *Artemia*
456 nauplii treated with EtOAcE of CAHSH-2 at the concentration of 10 mg or 50 mg per litre, the
457 survival percentage ranged from 97.3 to 100% while the survival ranged from 98.73 to 99.46%
458 in *Artemia* treated with pure compound during 48 hours of exposure. In shrimp post-larvae, the
459 survival percentage ranged from 94.67 to 100 in the case of EtOAcE- CAHSH-2 treatment at
460 the concentration of 10 mg or 50 mg per litre and 98.73 to 99.46 in the case of pure compound
461 treatment at the concentration of 1 or 2 mg per litre for 48 hours of exposure (Table 6b). The
462 toxicity of extract and pure compound was also tested in adult *L. vannamei* by intramuscular
463 injection. The survival percent was found to be 96.67 and 93.33 in shrimp injected with
464 EtOAcE- CAHSH-2 at the concentration of 1 mg/shrimp and 2 mg/shrimp, respectively after
465 10 days of treatment. In the case of pure compound, the percent survival was found to be 96.67
466 in shrimp injected with 2 mg of pure compound per shrimp after 10 days of treatment.

467 **4. Discussion**

468 Disease outbreaks caused by viral and bacterial pathogens have been recognized as a
469 major constraint to aquaculture production, trade and are responsible for severe economic loss
470 in aquaculture industry worldwide. Various strategies (efficient diagnostics, good nutrition,

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471 efficient vaccines, immunostimulants, non-specific immune-enhancers and probiotics) have
472 been followed to prevent or control diseases of aquatic animals in the culture systems. In spite
473 of these strategies, some of the diseases (WSS of shrimp and EUS in fish) have been persisting
474 for more than a decade. No control strategies have so far been developed to prevent these
475 diseases. Hence, novel strategies need to be developed to control or prevent WSSV of shrimp
476 at field level since it has tremendous value in shrimp culture industry worldwide. In the present
477 study, an attempt was made to screen actinomycetes isolates for possible antiviral compounds
478 especially for WSSV.

479 Several reports revealed that the actinomycetes from different habitats were found to be
480 a good source of antiviral and antibacterial compounds against viruses and bacteria of plants,
481 animals and humans (43, 44, 45, 46, 47, 48, 49, 50, 51, 52). A bioactive compound from a
482 marine actinomycete isolate, *S. kaviengensis* showed strong antiviral activity against equine
483 encephalitis virus (53). Padilla *et al*. (54) reported the antiviral activity of the extracts from
484 termite associated actinobacteria against bovine viral diarrhoea virus. Lee *et al*. (11) isolated an
485 antiviral compound namely benzatatin C from *S. nitrosporeus* and reported antiviral activity
486 against herpes simplex virus type 1 and type 2, and vesicular stomatitis virus, and that this
487 antiviral activity was found to be mediated due to the chlorine moiety in its molecular
488 structure. Strand *et al*. (55) isolated a bioactive compound from marine *Streptomyces* sp. with
489 antiviral activity against adenovirus. Hasobe *et al*. (56) reported that guanine-7-N-oxide
490 isolated from *Streptococcus* sp. was found to inhibit *in vitro* replication of fish herpes virus,
491 rhabdovirus, and infectious pancreatic necrovirus. Furan 2-yl acetate produced by a marine
492 *Streptomyces* spp. VITSDK1 was found to be efficient to control fish nodavirus in seabass cell
493 line (57). All the above-mentioned studies carried out by various workers indicate that
494 actinomycetes isolates are a potential source of antiviral compounds. In the light of these
495 studies was carried out to screen actinomycetes isolates collected from different habitats
496 against WSSV.

497 Totally 64 actinomycetes isolates collected from different environments were screened
498 for antiviral activity against WSSV since this virus is a serious viral pathogen responsible for
499 severe mortality and economic loss in shrimp culture industry not only in India but also in all
500 shrimp growing countries. One among 64 isolates the isolate designated as CAHSH-2 was
501 found to be very effective in inactivating the WSSV at the low concentration of 0.2 mg per
502 shrimp. As observed in the present study, actinomycetes isolates were found to reduce WSSV
503 infection in shrimp (15, 49, 50). All studies related to antiviral activity of actinomycetes

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504 isolates against WSSV currently are at a preliminary level only. A detailed study as carried out
505 in the present study has so far not been carried out.
506 The ethyl acetate extract of CAHSH-2 showed strong antiviral activity against WSSV
507 (no mortality and no signs of WSSV) at the concentration of 0.2 mg per shrimp. Different
508 methods such as *in vitro* assay using cell lines and animal models are being followed in
509 different laboratories to screen the natural products for antiviral activity. In the present study,
510 the antiviral activity of ethyl acetate extract of actinomycetes isolates against WSSV was
511 assessed in shrimp due to lack of shrimp cell lines as followed by different workers (58, 22,
512 23). Testing the antiviral compounds in animal models has relatively maximum predictive
513 value and more advantages when compared to *in vitro* assay using cell lines or chicken eggs.
514 Testing in an animal model system would help to identify both antiviral activity and antiviral
515 agents, and also help to determine the toxicity of compounds instantaneously while screening
516 antiviral activity. The use of an animal model for antiviral screening of natural products should
517 have some essential features such as use of virus with minimal alteration by adaptation, use of
518 the natural route of infection and size of inoculum, and similarity of infection, pathogenesis,
519 host response and compound toxicity (59).
520 The ethyl acetate extract of CAHSH-2 was found to be highly efficient to inactivate
521 WSSV at the concentration of 200 μg per shrimp and this concentration was found to be non-
522 lethal to the shrimp. The potential ethyl acetate extract was subjected to silica gel column and
523 five fractions were obtained. Among the five fractions, fraction-F1 was found to be active
524 against WSSV and further analysis of this active fraction resulted in the isolation of two active
525 sub-fractions. Two different phthalate esters, namely di-n-octyl phthalate and bis (2-
526 methylheptyl) phthalate were purified from active fraction and the structural elucidation of
527 these compounds was confirmed by FT-IR, GC-MS and NMR spectra analyses. Among these
528 compounds, di-n-octyl phthalate was found to more efficiency to inactive WSSV at the
529 concentration of 50 μg per shrimp and 78% survival was found in WSSV challenged shrimp at
530 10 d p.i. whereas 89% survival was found in WSSV-challenged shrimp at the concentration of
531 200 μg per shrimp. As observed in the present study, different antiviral compounds against the
532 viruses of human, animals and plants were reported by many workers. Nakagawa *et al*. (60)
533 isolated and identified pentalactones from *Streptomyces* sp. M-2718 and reported that they
534 were active against several DNA viruses. The antiviral activity of guanine-7-N-oxide produced
535 by *Streptomyces* sp. against fish herpes virus, rhabdovirus and IPNV was tested and it was
536 found to inhibit the replication of these viruses (56). The results obtained in the present study

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537 on potential antiviral compounds against WSSV from actinomycetes isolate agree with
538 previous works on antiviral compounds against viruses of human, animals and fish. Hence, this
539 potential actinomycetes isolate may be useful when incorporated in the feed to control WSSV
540 infection in the culture system.

541 Two compounds, di-n-octyl phthalate and bis (2-methylheptyl) phthalate were isolated
542 from an endophytic actinomycetes isolate and were found to be effective against WSSV of
543 shrimp. As observed in the present study, the antiviral activity of these compounds was also
544 reported by many workers. Elnaby *et al*. (61) tested the antiviral activity of di-n-octyl phthalate
545 isolated from a marine actinomycetes isolate (*S. parvus*) against hepatitis C virus (HCV).
546 Compounds produced by *Pseudoalteromonas piscida* contained benzene 1-pentyloctyl,
547 benzene 1-butylheptyl and di-n-octyl phthalate, and these compounds were found to be active
548 against HCV (62). Yin *et al*. (63) identified di-n-octyl phthalate in the crude extract from the
549 endophytic fungus *Aspergillus terreus* MP15 of *Swietenia macrophylla* leaf and reported
550 antibacterial activity against food-borne bacteria. In the present study, antiviral activity of di-n-
551 octyl phthalate isolated from endophytic actinomycetes isolate against WSSV was reported for
552 the first time. The anti-WSSV compound namely bis (2-methylheptyl) phthalate isolated from
553 actinomycetes isolate in the present study was also reported to be present in the leaves of
554 *Pongamia pinnata* and effective against WSSV (64).

555 The present study provides a suitable 3D model for di-n-octyl phthalate with VP26 or
556 VP28 and bis (2-methylheptyl) phthalate with VP26 or VP28 complex formation. The ligand
557 binding site on target proteins VP26 and VP28 were predicted using FTsite. We predicted
558 three ligand binding sites for these target proteins. A significant variation in these three-ligand
559 binding sites was observed (65). VP26 and VP28 are the major structural proteins of the outer
560 membrane of WSSV of shrimp. Based on *in vivo* neutralization experiments on WSSV in *P.*
561 *monodon* with antibodies raised against VP28, it is suggested that VP28 is located in the
562 "spikes" of the WSSV envelope and this protein may thus be involved in the systemic infection
563 of WSSV in shrimp (66, 67) and also play an important role in the process of infection in
564 shrimp. Neutralizing the VP28 protein using anti-VP28 or silencing the genes of VP28 and
565 VP26 using gene specific dsRNA increased the survival of WSSV-challenged shrimp (66, 67,
566 68, 69). These studies clearly indicate that the blocking of VP28 or VP26 would help to protect
567 the shrimp from WSSV infection. The high survival of shrimp injected with CAHSH-2-treated
568 WSSV might be due to the blocking of VP28 and VP26 proteins by the compounds [di-n-octyl
569 phthalate and bis (2-methylheptyl) phthalate] present in the actinomycetes isolate (CAHSH-2)
570 as evidenced from the docking studies. Among these two compounds, di-n-octyl phthalate was

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571 found to be more efficient to inactive WSSV in *in vivo* experiments when compared to bis (2-
572 methylheptyl) phthalate. This observation is further confirmed by the docking studies which
573 showed that di-n-octyl phthalate has high binding stability with VP26 and VP28 whereas the
574 bis (2-methylheptyl) phthalate has low binding affinity with VP26 and VP28. High binding
575 stability with VP28 and VP26 might be the reason for high efficiency of di-n-octyl phthalate to
576 inactivate the WSSV when compared to bis (2-methylheptyl) phthalate. Similar observation
577 was made by Sundharsana *et al*. (70) in *in silico* molecular docking and simulation analysis of
578 VP26 and VP28 proteins of WSSV with the ligand 3-(1-chloropiperidin-4-yl)-6-fluoro
579 benzisoxazole 2 and the results revealed the ligand binding with polar amino acids in the pore
580 region of viral proteins.

581 In the present study, the antiviral activity of potential actinomycetes isolate, active
582 fractions and pure antiviral compounds against WSSV was confirmed by various methods such
583 as bioassay test, PCR, RT-PCR, Western blot, indirect ELISA and real time PCR. In the
584 previous works, the antiviral activity of actinomycetes isolates against WSSV was confirmed
585 by either bioassay test, PCR assay or both (15, 49, 50). The results of all confirmatory assays
586 indicate the potentiality of actinomycetes isolates to inactivate the WSSV as observed by
587 Kumar *et al*. (15) and Jenifer *et al*. (49).

588 Before the application of antiviral drugs from actinomycetes or any other organism,
589 they should be tested for toxicity to prove that it is non-toxic to the host and the environment
590 (71, 72). In the present study, the toxicity of ethyl acetate extract of actinomycetes isolate
591 (CAHSH-2) and antiviral compound (di-n-octyl phthalate) was tested *in vivo* using *Artemia*
592 nauplii, post-larvae and adult shrimp, and *in vitro* assay using fish cell lines. The results of *in*
593 *vivo* assays revealed that high doses of extract or active compound did not produce mortality in
594 *Artemia*, post-larvae and adult shrimp, and this indicates that the extract of potential
595 actinomycetes isolate and active compound are safe for use in shrimp culture system as
596 observed by other workers (73, 74, 74). Das *et al*. (73) reported that *Streptomyces* sp. is not
597 toxic to both nauplii and adults of *A. salina*. More studies revealed that *Streptomyces* strains
598 from different environments were harmless and did not result in any sign of abnormal behavior
599 or mortality in *P. monodon* (74) and *L. vannamei* (75).

600 Various characters like morphological, physiological and biochemical characters are
601 used in the classical approach to identify the actinomycetes isolates. The classical method of
602 classification described in the identification key by Nonomura (77) and Bergey's Manual of
603 Determinative Bacteriology (78) was found to be useful to classify the actinomycetes isolates
604 in the present study. All the characteristics studied for CAHSH-2 in the present study were

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605 compared with that of reference strain, *Streptomyces ghanaensis* obtained from MTCC,
606 Chandigarh, India, and showed 94% similarity. In addition to these characteristics, molecular tools
607 were applied to bacterial taxonomy like other organisms (78, 79). In the present study, the 16S
608 RNA method was followed to identify the CAHSH-2 isolate. Nucleotide sequences for the 16S
609 ribosomal RNA (rRNA) genes are available for almost all actinomycete species and reference to
610 these sequences allow for easy identification of actinomycetes isolates. The 16S RNA gene
611 sequence of CAHSH-2 isolate exhibited sequence similarity of 99% with *S. ghanaensis*. Based on
612 morphological, physiological, biochemical and molecular taxonomy, the CAHSH-2 isolate was
613 identified as *Streptomyces ghanaensis*-like strain and deposited in the repository of C. Abdul
614 Hakeem College with the designation *Streptomyces ghanaensis*-CHASH-2 for scientific purpose.

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Fig.1

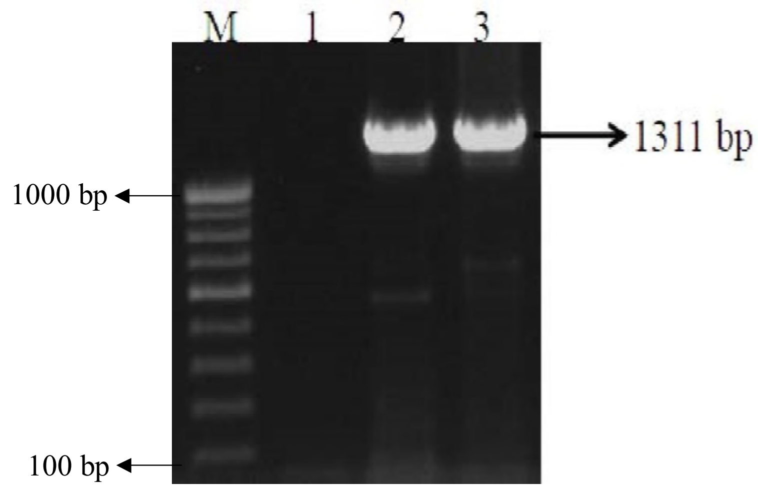
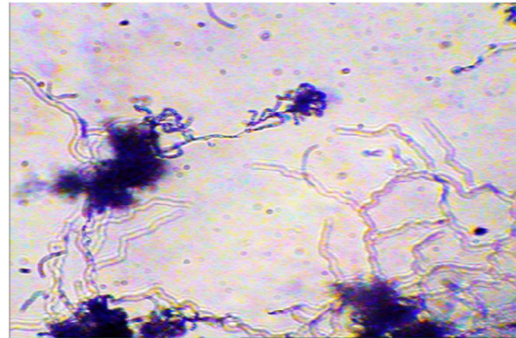


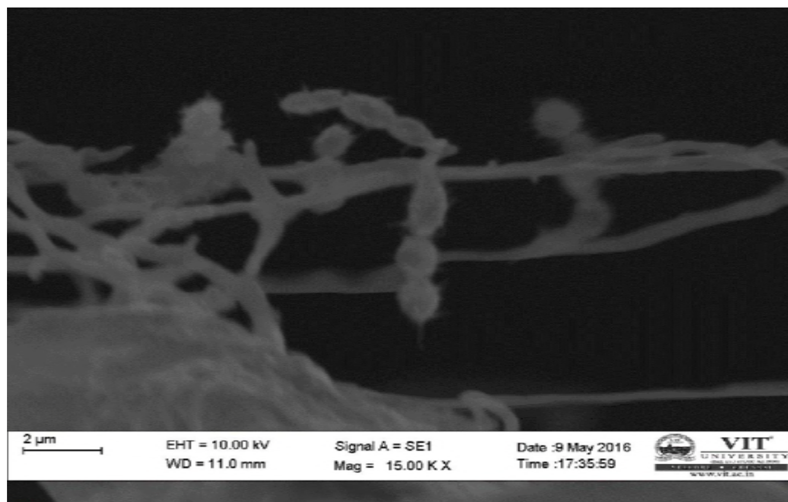
Fig.1. Amplification of 16S rRNA gene of Actinomycetes isolate CAHSH-2 and reference strain of *Streptomyces ghanaensis* obtained from IMTECH. Lane M- 100 bp Marker; Lane 1- Negative Control; Lane 2- CAHSH-2; Lane 3- *Streptomyces ghanaensis*

Fig. 2

Colony morphology with grey coloured aerial mycelium



Retinaculum apertum spore chains (400 X)



Scanning Electron Micrograph of spore morphology of CAHSH-2

Fig. 2 Morphological characteristics of CAHSH-2 after 14 days of growth at 28 ± 2ºC on ISP medium-3

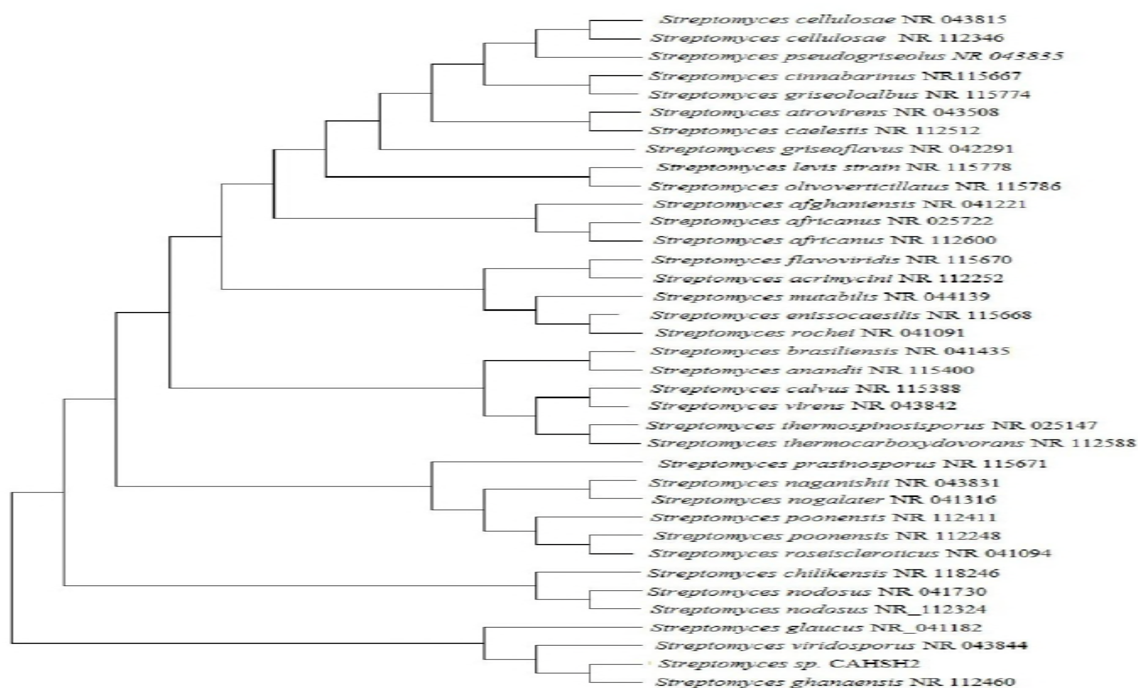


Fig. 3 Rooted neighbour joining phylogenetic tree of actinomycetes CAHSH-2 based on 16S rRNA gene sequences, showing the relationship between actinomycetes CAHSH-2 and related representative species of the *Streptomye ghanaensis*.

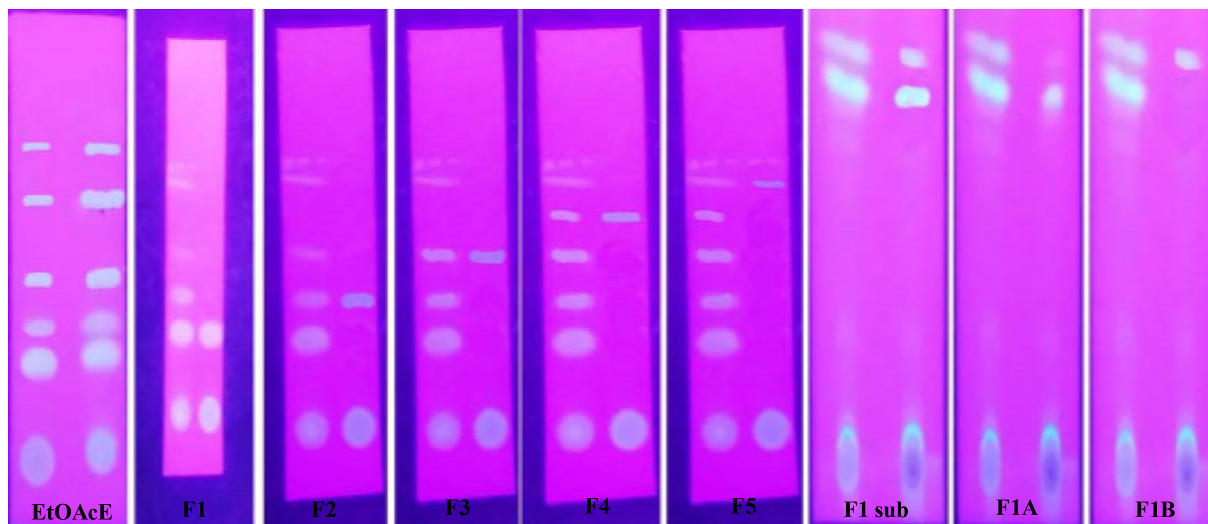


Fig. 4. Thin layer chromatographic analysis of EtOAcE of CAHSH-2 and their homogeneity, and TLC analysis of active fraction and its homogeneity.

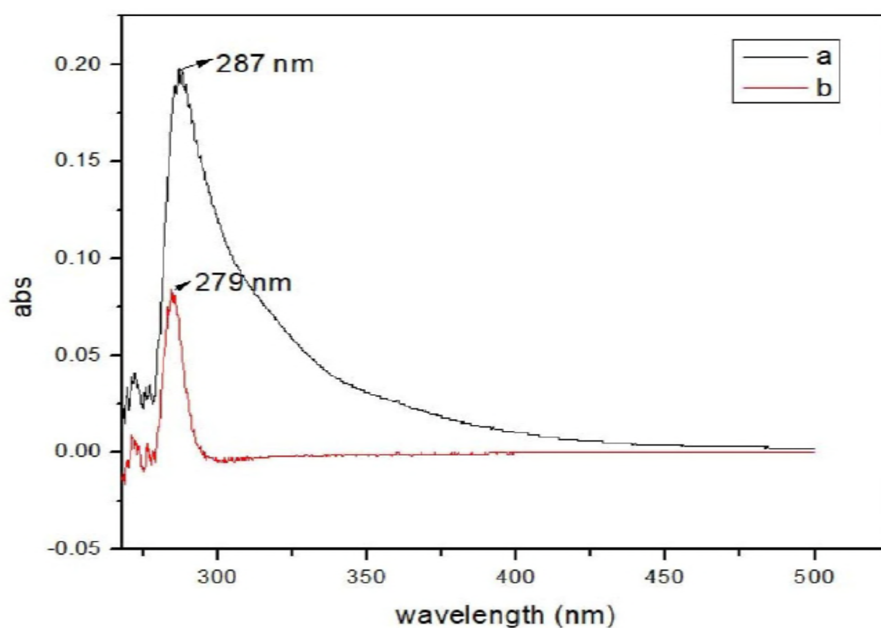


Fig. 5a UV Spectroscopy analysis of purified bioactive compounds of F1A (a) and F1B (b) from active fraction of EtOAcE of actinomycetes CAHSH-2.

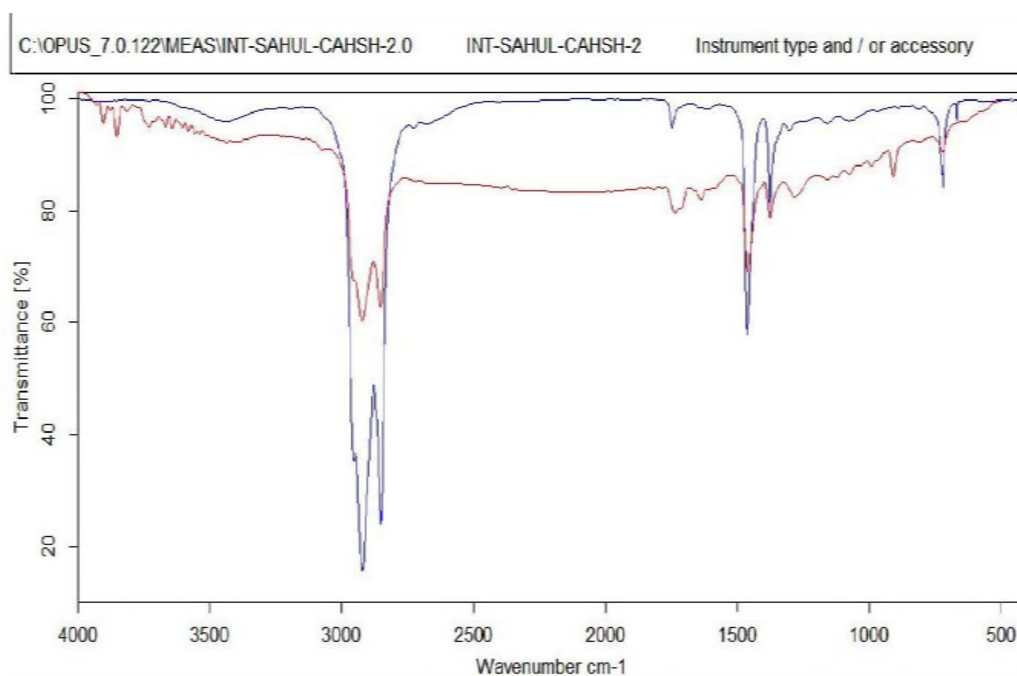


Fig. 5b. FT-IR Spectroscopy analysis of the purified bioactive compounds, F1A (Blue line) and F1B (Red line) purified from the EtOAcE of actinomycetes CAHSH-2.

Fig. 5c. ^{1}H NMR spectrum analysis of the purified bioactive compound, F1A from the active fraction of EtOAcE of actinomycetes CAHSH-2.

Fig. 5d. ^{13}C NMR spectrum analysis of the purified bioactive compound, F1A from the active fraction of EtOAcE of actinomycetes CAHSH-2.

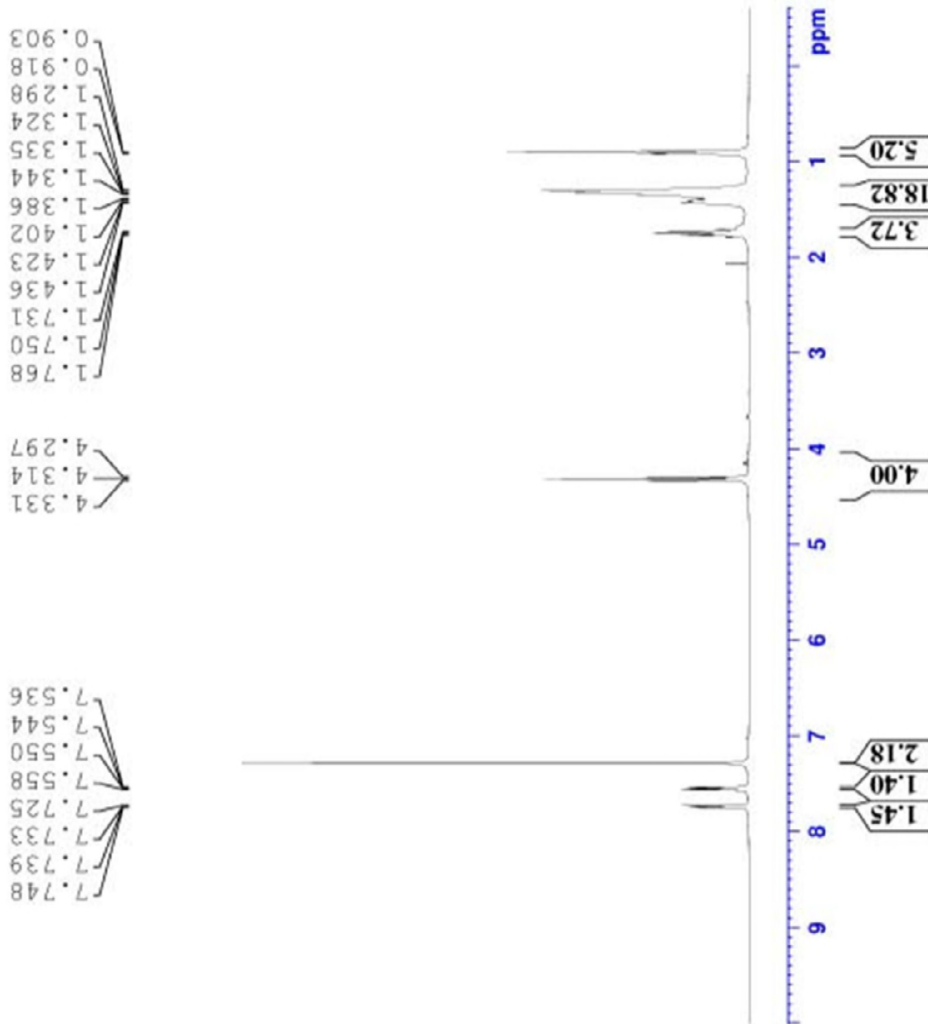


Fig 5e. ^{1}H NMR spectrum analysis of the purified bioactive compound, F1B from the active fraction of EtOAcE of actinomycetes CAHSH-2.

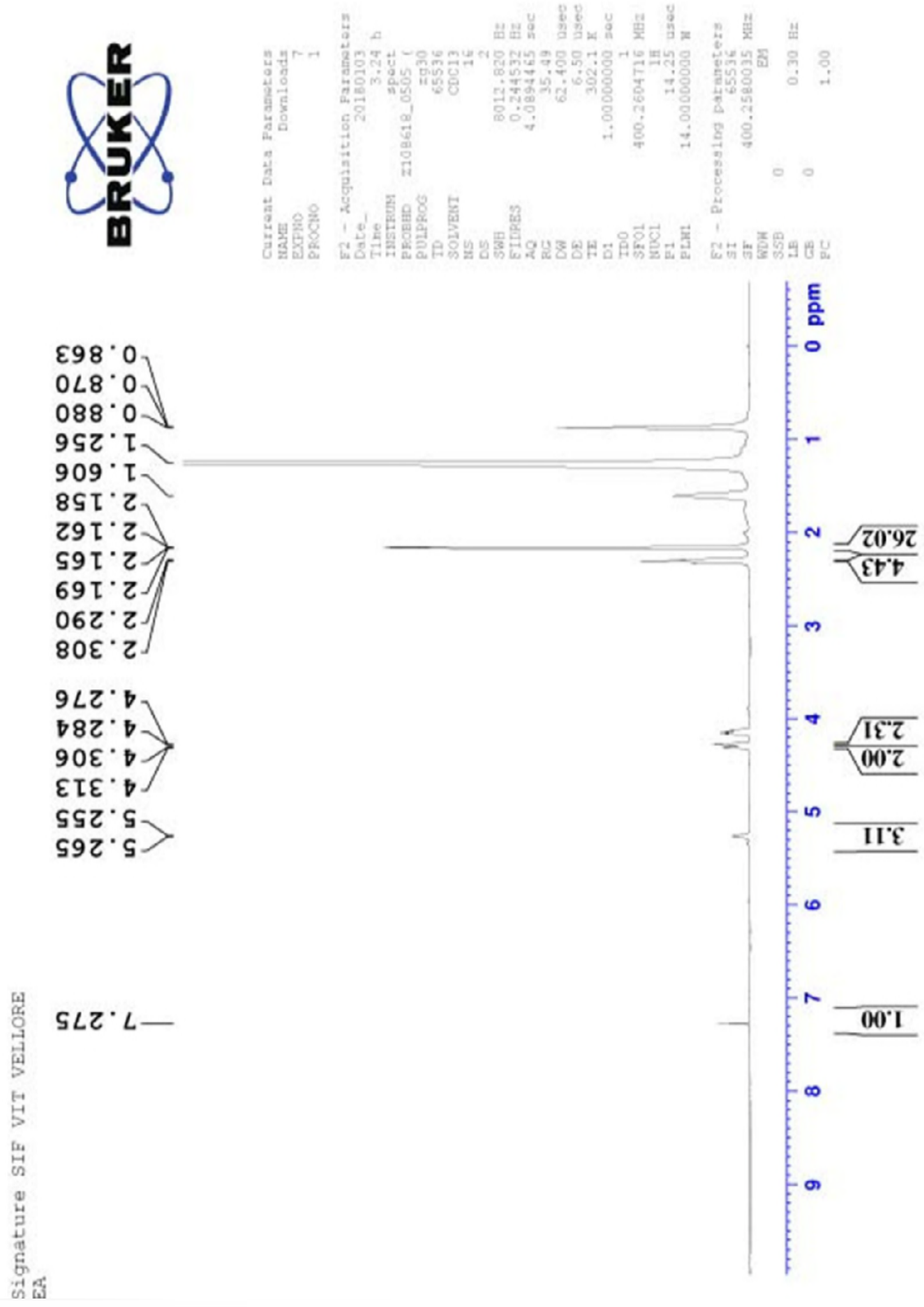
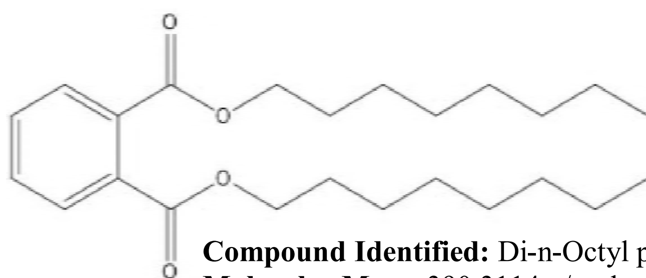


Fig. 5f. ^{13}C NMR spectrum analysis of the purified bioactive compound, F1B from the active fraction of EtOAcE of actinomycetes CAHSH-2.



Compound Identified: Di-n-Octyl phthalate
Molecular Mass: 390.3114 g/mol
Empirical Formula: $C_{24}H_{38}O_4$

Fig. 5g Molecular structure of the purified bioactive compound, Di-n-Octyl phthalate isolated from active fraction of EtOAcE of *Streptomyces* sp. CAHSH-2.

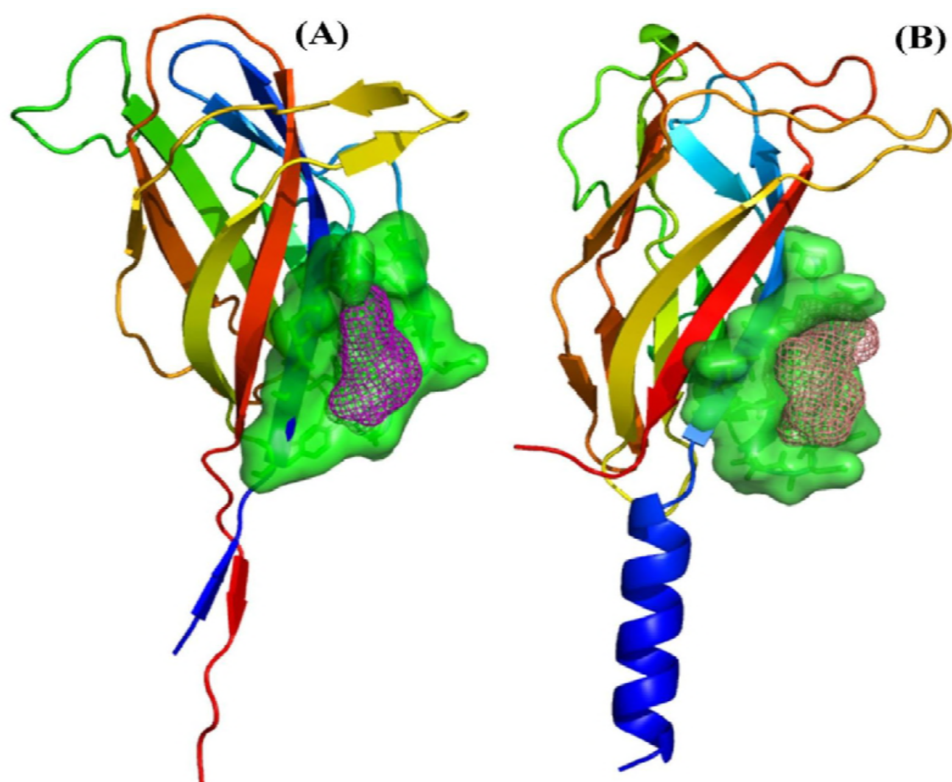


Fig. 6a. 3D Structure of VP26 (A) and VP28 (B). Ligand binding site is obtained by FTSite and active site residues are shown in green colour surface model. Ligand binding pocket are show in mesh model.

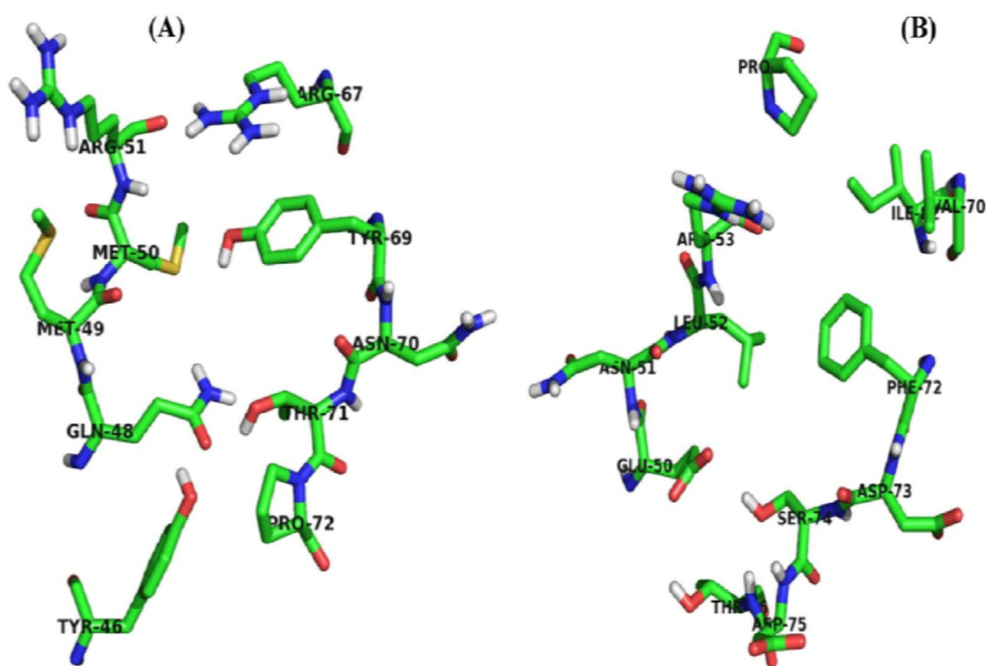


Fig. 6b. Amino acid residues located at active site of VP26 (A) and VP28 (B).

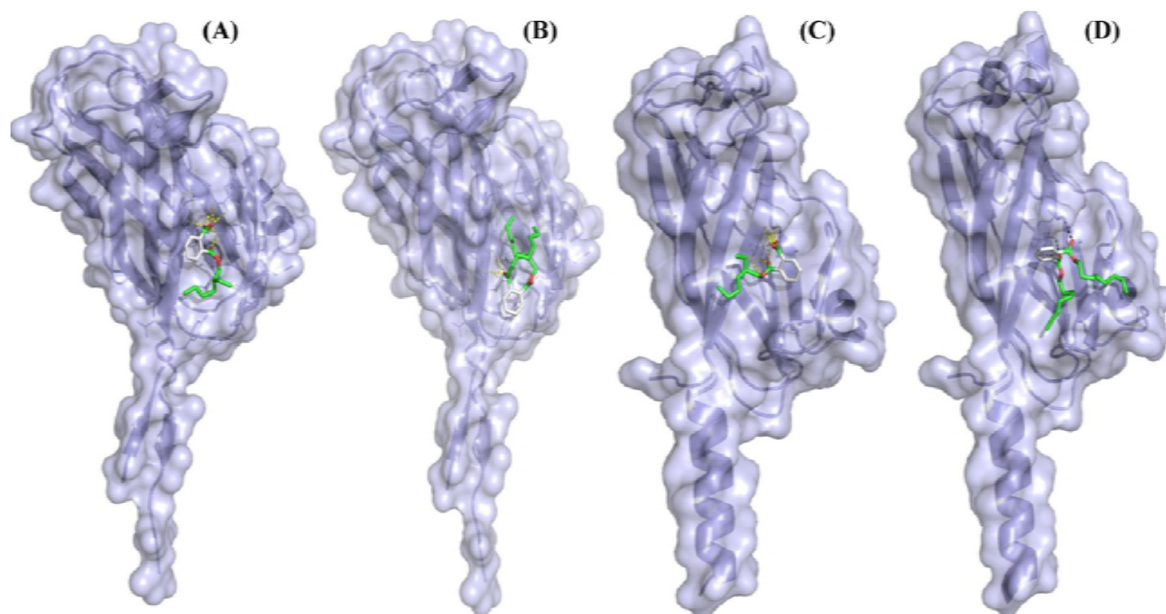


Fig. 6c. Molecular docking of di-n-octyl phthalate and bis(2-methylheptyl) phthalate with VP26 and VP28. Binding pattern of di-n-octyl phthalate with VP26 (A), Binding pattern of bis (2-methylheptyl) phthalate with VP26 (B), Binding pattern of di-n-octyl phthalate with VP28 (C) and Binding pattern of bis (2-methylheptyl) phthalate with VP28 (D).

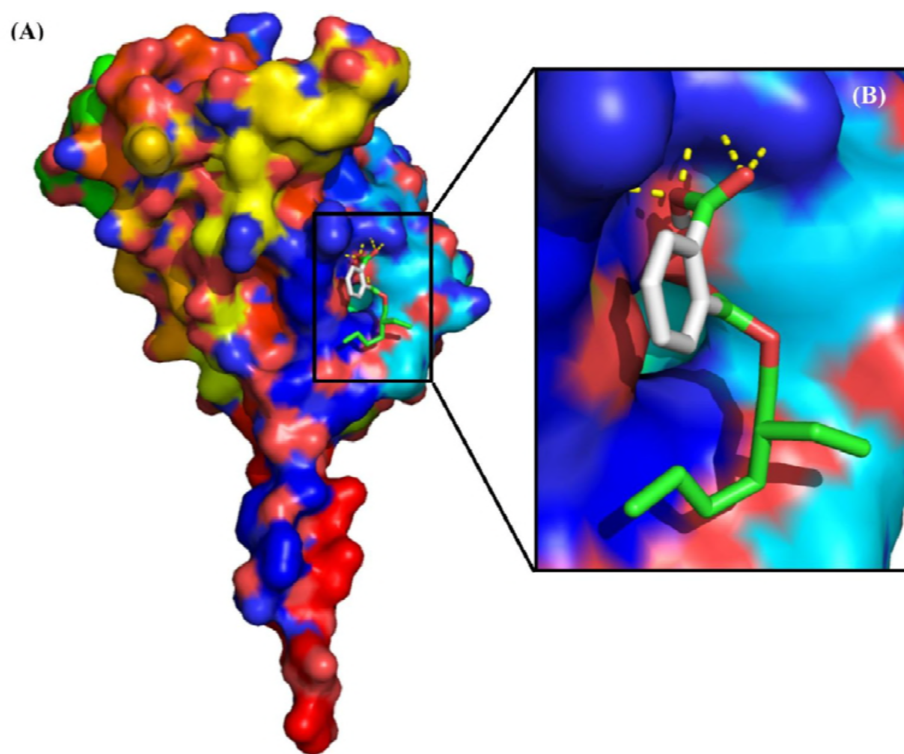


Fig. 6d. Binding pose of di-n-octyl phthalate in the binding site of VP26 (A). A close-up view of the binding pose of di-n-octyl phthalate (B).

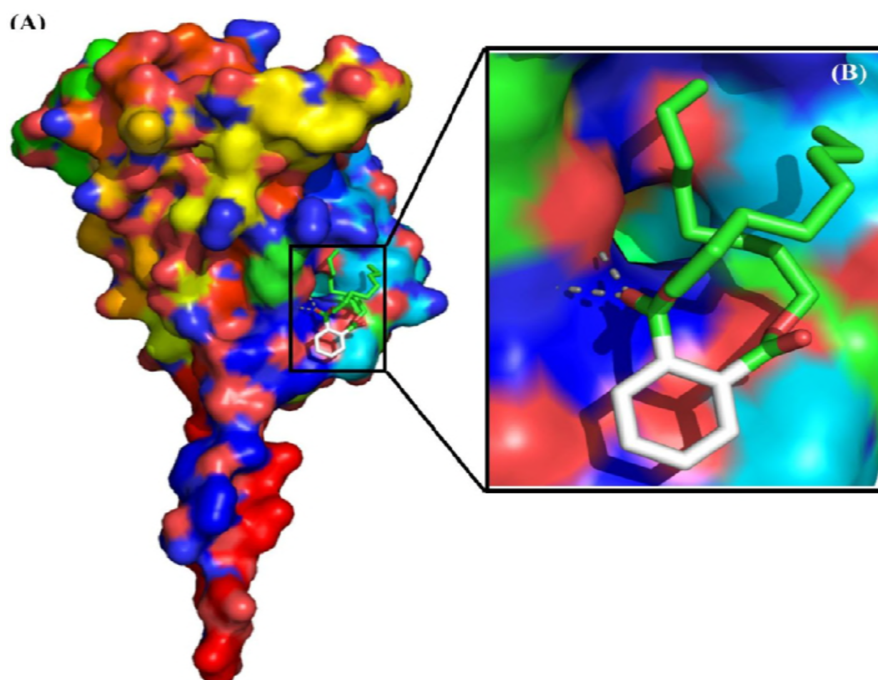


Fig. 6e. Binding pose of bis (2-methylheptyl) phthalate in the binding site of VP26 (A) and A close-up view of the binding pose of bis (2-methylheptyl) phthalate (B).

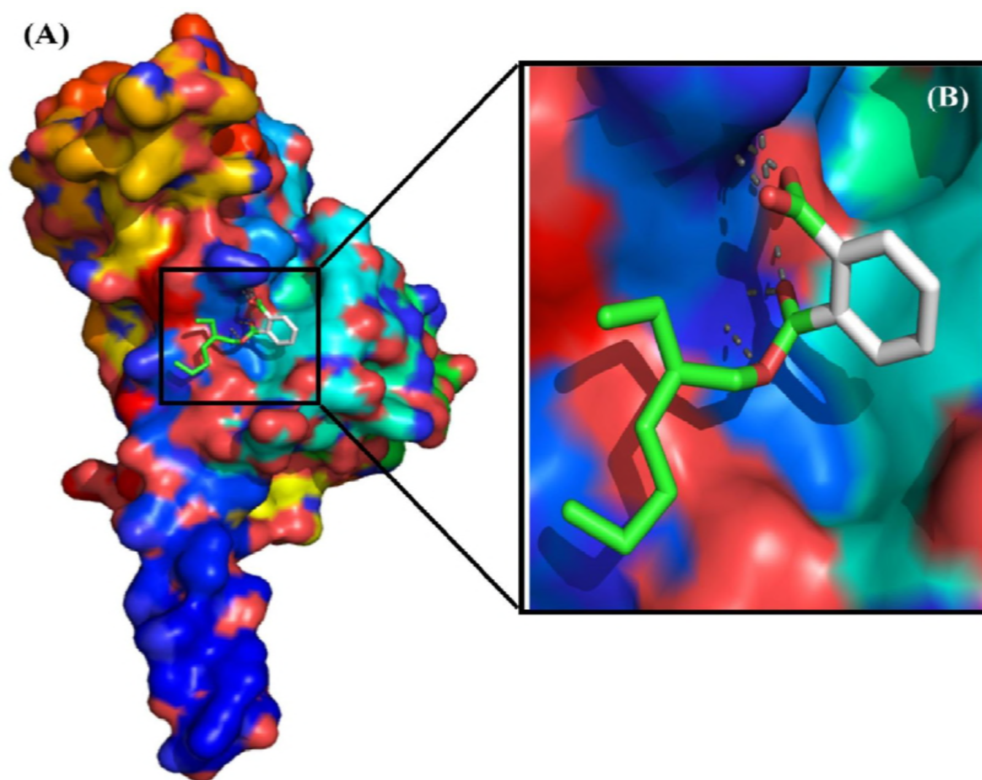


Fig.6f. Binding pose of di-n-octyl phthalate in the binding site of VP28 (A) and a close-up view of the binding pose of di-n-octyl phthalate (B).

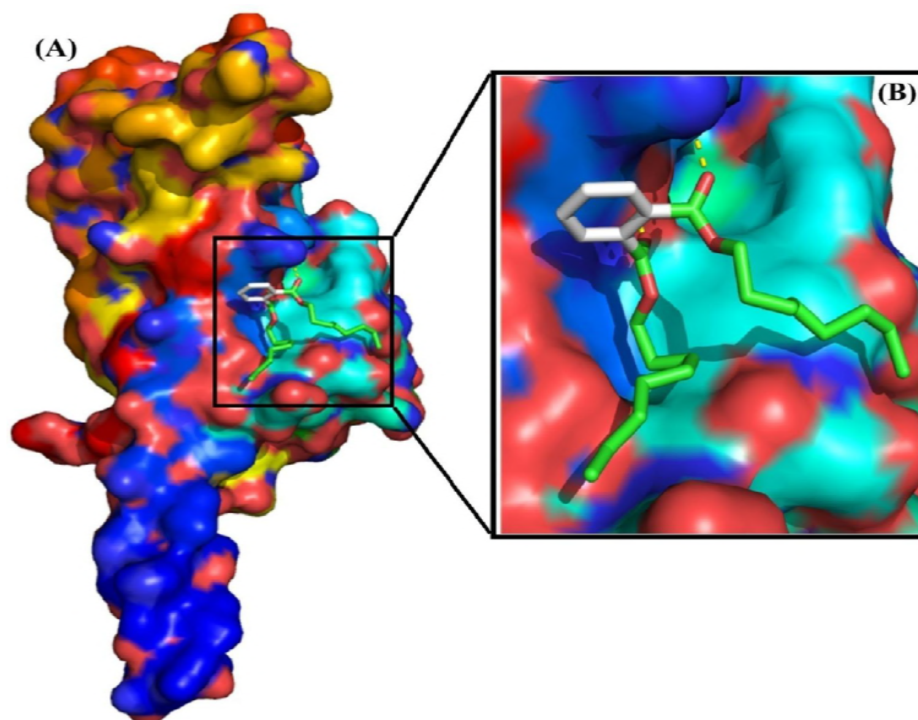


Fig. 6g. Binding pose of bis (2-methylheptyl) phthalate in the binding site of VP28 (A) and a close-up view of the binding pose of bis (2-methylheptyl) phthalate (B).

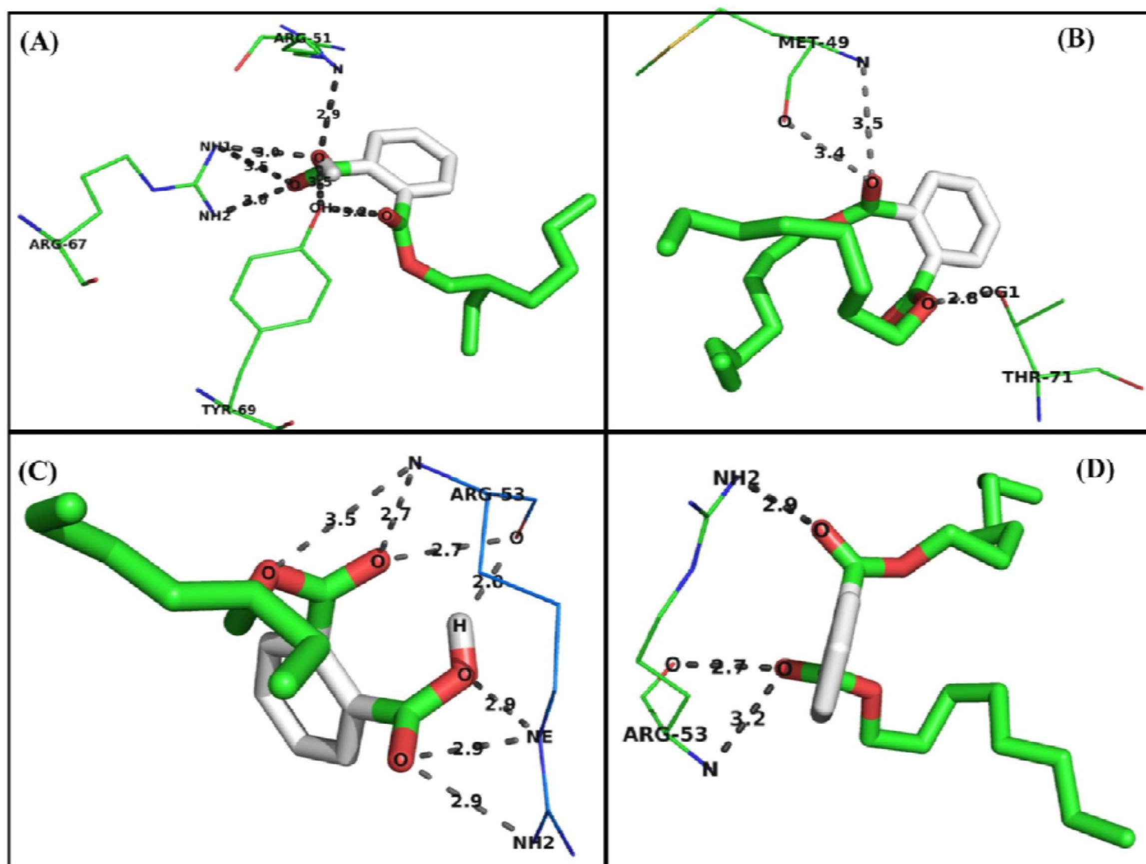


Fig. 6h. Molecular interaction of di-n-octyl phthalate and bis (2-methylheptyl) phthalate with VP26 and VP28. (A). Interaction of di-n-octyl phthalate with VP26. (B) Interaction of bis (2-methylheptyl) phthalate with VP26. (C) Interaction of di-n-octyl phthalate with VP28. (D) H-bond network between bis (2-methylheptyl) phthalate and VP28.

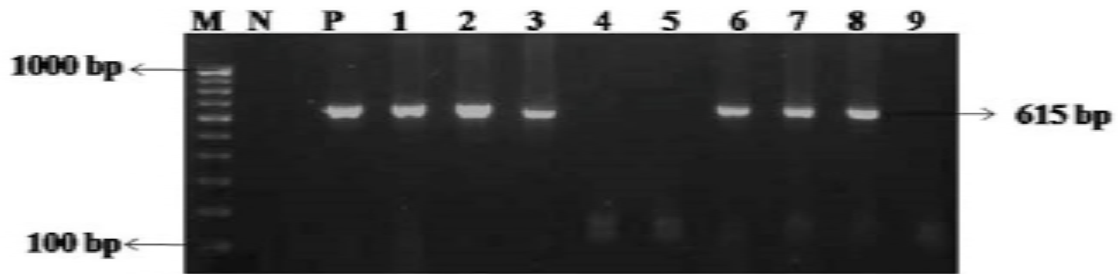


Fig.7a. PCR detection of WSSV in the gill and head tissues of *L. vannamei* injected with EtOAcE of CAHSH-2 treated WSSV at different time intervals. Lane M –DNA 100 bp Marker, Lane N – Negative Control, Lane P – Positive Control, Lane 1 – 3rd day gill, Lane 2– 5th day gill, Lane 3 –10th day gill, Lane 4 –15th day gill, Lane 5 – Negative control, Lane 6 – 3rd day head tissue, Lane 7 –5th day head tissue, Lane 8 - 10th day head tissue, Lane 9 –15th day head tissue.

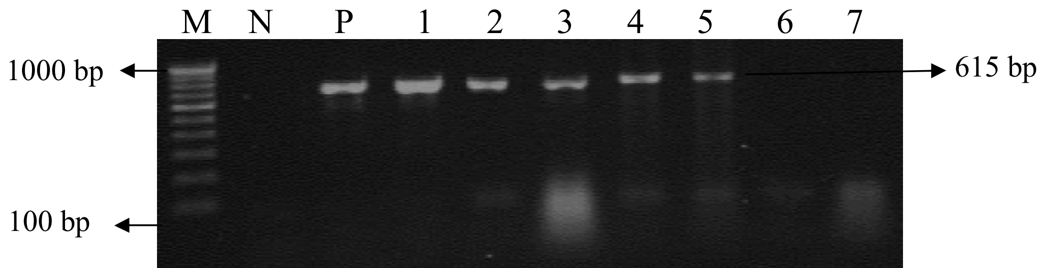


Fig.7b. RT-PCR detection of VP28 transcript in the gill tissue of *L. vannamei* injected with EtOAcE of CAHSH-2 treated WSSV at different time intervals. Lane M –DNA 100 bp Marker, Lane N – Negative Control, Lane P – Positive Control, Lane 1- 1st day, Lane 2 – 2nd day, Lane 3 – 3rd day, Lane 4– 5th day, Lane 5 –10th day, Lane 6 –13th day, Lane7- 15th day.

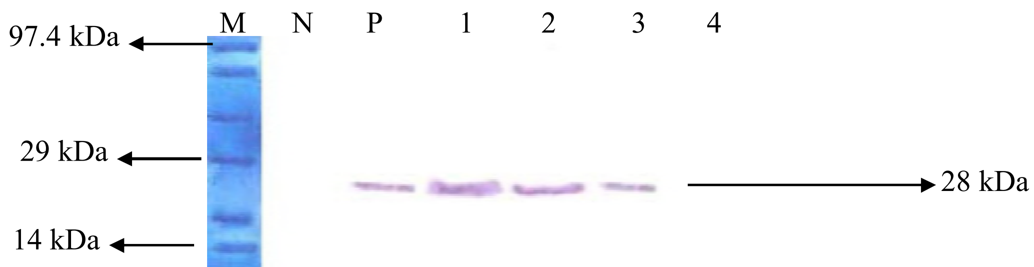


Fig. 7c. Detection of WSSV by Western blot analysis using anti–rVP28 of WSSV in the gill tissue of *L. vannamei* injected with EtOAcE of CAHSH-2 treated WSSV at different time intervals. Lane M – Protein marker; Lane N – negative control; Lane P – positive control; Lane 1 – 2nd day, Lane 2 – 5th day, Lane 3 – 10th day, Lane 4 –15th day.

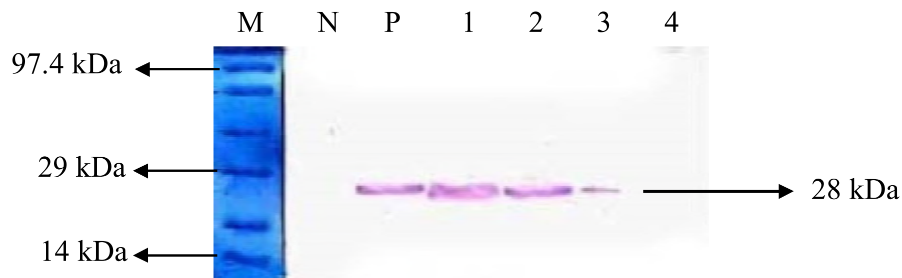


Fig. 7d. Detection of WSSV by Western blot analysis using anti–rVP28 of WSSV in the head tissue of *L. vannamei* injected with EtOAcE of CAHSH-3 treated WSSV at different time intervals. Lane M – Protein marker; Lane N – negative control; Lane P – positive control; Lane 1 – 2nd day, Lane 2 – 5th day, Lane 3 – 10th day, Lane 4 –15th day

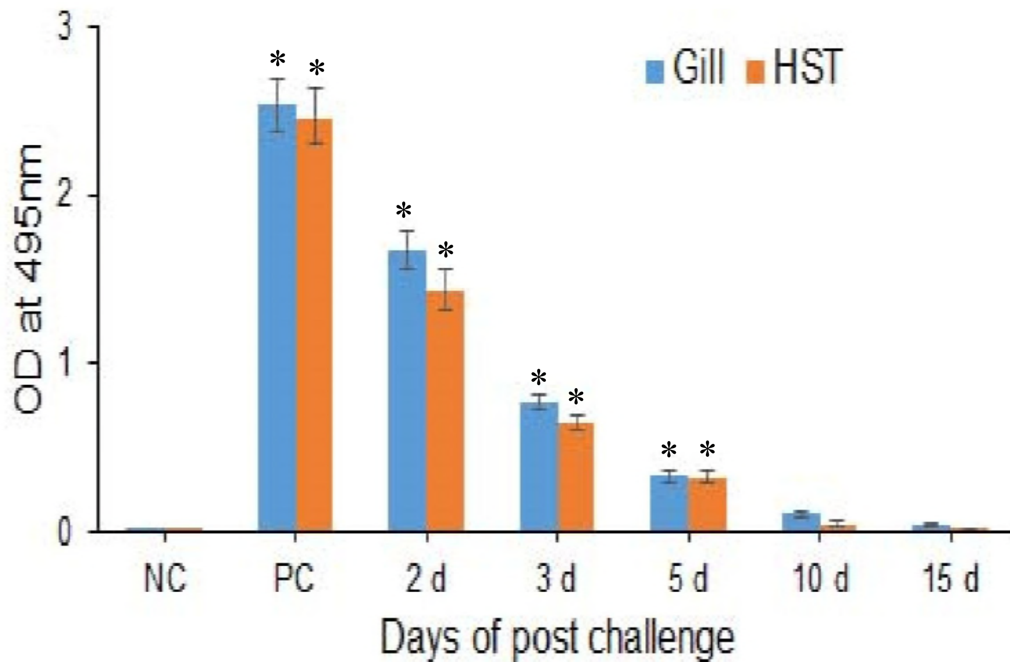


Fig. 7e. Detection of WSSV by ELISA using anti–rVP28 of WSSV in the gill and head tissues of *L. vannamei* injected with EtOAcE of CAHSH-2 treated WSSV at different time intervals.

Table 1. Date wise laboratory trials carried out to assess the antiviral activity of EtOAcE of Actinomycetes isolate (CAHSH-2) against WSSV since 2014.

Date of experiment[&]	No of animals used	Average survival of negative control[*]	Average survival of positive control[@]	Average survival of shrimp injected with WSSV treated with EtOAcE[$]	Average survival of shrimp injected with EtOAcE only[#]
12.07.2014	60	98.3±2.14	0	96.6±3.16	98.3±3.42
13.08.2014	24	96.0±2.06	0	96.0±3.05	92.0±2.08
15.08.2014	33	97.1±3.15	0	94.2±2.26	97.1±1.96
19.08.2014	21	96.6±2.48	0	95.0±2.57	90.0±2.88
10.09.2014	30	96.6±3.33	0	93.3±3.33	96.6±3.66
01.10.2014	42	97.5±2.29	0	95.0±2.19	92.5±2.49
26.02.2015	24	96.0±2.61	0	96.0±2.99	92.0±4.15
01.03.2015	27	96.2±3.32	0	92.5±1.87	96.2±3.84
04.04.2015	15	93.3±3.38	0	100	86.6±6.66
08.04.2015	18	94.4±2.28	0	88.8±2.15	94.4±2.16
25.07.2015	48	98.0±2.50	0	96.0±3.26	94.0±2.95
06.08.2015	30	96.6±3.33	0	93.3±5.12	96.6±3.33
04.09.2015	24	96.0±4.61	0	96.0±3.30	92.0±2.49
21.09.2015	21	96.6±3.33	0	95.0±2.14	90.0±2.09
21.11.2015	33	97.1±2.19	0	94.2±2.55	97.1±3.19
08.01.2016	48	98.0±2.49	0	96.0±3.66	94.0±4.08
04.02.2016	15	93.3±3.33	0	93.3±3.33	86.6±3.33
10.02.2016	27	96.2±216	0	100	96.2±2.49
03.03.2016	18	88.8±2.28	0	94.4±2.27	88.8±2.26
14.03.2016	30	93.3±3.33	0	96.3±2.28	93.3±3.34
25.05.2016	27	96.2±2.13	0	92.5±3.19	81.4±2.83

*Shrimp injected with NTE buffer; [@]Shrimp injected with WSSV (5 µl of haemolymph from moribund WSSV-infected shrimp per shrimp) intramuscularly; [$]Shrimp injected with mixture of WSSV (5 µlof WSSV inoculum) and Actinomycetes extract at the concentration of 200 µg per shrimp; [#]Shrimp injected with actinomycetes extract only. [&]Experimental period was 20 days.

Table 2a. Morphological characteristics of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH

Characteristics	Actinomycetes CAHSH-2			Actinomycetes CAHSH -3			Reference strain	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
Spore morphological characteristics	+++	+++	+++	+++	+++	+++	+++	+++
Presence of Aerial mycelium	+++	+++	+++	+++	+++	+++	+++	+++
Presence of Substrate mycelium	Dark grey	Dark grey	Dark grey	Green	Green	Green	Dark grey	Green
Colour of aerial mycelium	Yellow	Yellow	Yellow	Cream	Cream	Cream	Yellow	Cream
Colour of substrate mycelium	Branched	Branched	Branched	Branched & twisted	Branched & twisted	Branched & twisted	Branched	Branched & twisted
Spore type	Retinaculum apertum	Retinaculum apertum	Retinaculum apertum	Spiral	Spiral	Spiral	Retinaculum apertum	Spiral
Spore shape	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval
Spore Surface	Thorny	Thorny	Thorny	Spiny	Spiny	Spiny	Thorny	Spiny
Spore chain	Long chain	Long chain	Long chain	Long chain	Long chain	Long chain	Long chain	Long chain
Spore motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Acid fast	+	+	+	+	+	+	+	+
Sporangia	-	-	-	-	-	-	-	-
Melanin production	Green	Green	Green	-	-	-	Green	-
Diffusible pigments	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

IS – in triplicate

Table 2b. Cultural characteristics of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH

Media used/ Characteristics	Actinomycetes CAHSH-2			Actinomycetes CAHSH -3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
Malt extract and yeast extract agar (ISP-2, Pridham *et al.*, 1956-57)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Grey	Grey	Grey	Greenish red	Greenish red	Greenish red	Grey	Greenish red
Substrate mycelium	Yellow	Yellow	Yellow	Olive	Olive	Olive	Yellow	Olive
Soluble pigment	-	-	-	-	-	-	-	-
melanin pigment	-	-	-	-	-	-	-	-
Oat meal agar (ISP-3, Kuster, 1959a)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Grey	Grey	Grey	Green	Green	Green	Grey	Green
Substrate mycelium	Green	Green	Green	Olive	Olive	Olive	Green	Olive
Soluble pigment	-	-	-	-	-	-	-	-
melanin pigment	-	-	-	-	-	-	-	-
Inorganic Salt Starch agar (ISP-4, Kuster, 1959a)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Green	Green	Green	Dark green	Dark green	Dark green	Green	Dark green
Substrate mycelium	White Grey	White Grey	White Grey	Green	Green	Green	White Grey	Green
Soluble pigment	-	-	-	-	-	-	-	-
Melanine pigment	-	-	-	-	-	-	-	-
Glycerol - asparagines agar (ISP-5, Pridham and Lyons, 1961)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Green	Green	Green	Brownish grey	Brownish grey	Brownish grey	Green	Brownish grey
Substrate mycelium	Green	Green	Green	Greenish white	Greenish white	Greenish white	Green	Greenish white
Soluble pigment	-	-	-	-	-	-	-	-

Continue…,

melanin pigment	-	-	-	-	-	-	-	-
Peptone - yeast extract Ion agar (ISP-6, Tresner and Danga, 1958)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Green	Green	Green	White	White	White	Green	White
Substrate mycelium	Green	Green	Green	Yellow	Yellow	Yellow	Green	Yellow
Soluble pigment	-	-	-	-	-	-	-	-
melanin pigment	-	-	-	-	-	-	-	-
Tyrosine agar (ISP-7, Shinobu, 1958)								
Growth	+++	+++	+++	+++	+++	+++	+++	+++
Aerial mycelium	Green	Green	Green	Whitish yellow	Whitish yellow	Whitish yellow	Green	Whitish yellow
Substrate mycelium	Whitish yellow	Whitish yellow	Whitish yellow	Brown	Brown	Brown	Whitish yellow	Brown
Soluble pigment	-	-	-	-	-	-	-	-
Melanin pigment	-	-	-	-	-	-	-	-

IS – in triplicate

Table 2c. Physiological characteristics of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH

Characteristics	Actinomycetes CAHSH2			Actinomycetes CAHSH 3			Reference strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomycs ghanaensis*	*Streptomyces viridosporus*
pH range								
pH 4	+	+	+	+	+	+	+	+
pH 5	+	+	+	+	+	+	+	+
pH 6	++	++	++	++	++	++	++	++
pH 7	+++	+++	+++	+++	+++	+++	+++	+++
pH 8	++	++	++	+++	+++	+++	++	+++
pH 9	+	+	+	++	++	++	+	++
pH 10	-	-	-	-	-	-	-	-
Temperature (°C)								
10	+	+	+	++	++	++	+	++
20	++	++	++	+++	+++	+++	++	+++
25	+++	+++	+++	+++	+++	+++	+++	+++
30	+++	+++	+++	++	++	++	+++	++
35	++	++	++	++	++	++	++	++
40	-	-	-	++	++	++	-	++
Sodium chloride (% in v/w)								
0	+/-	+/-	+/-	-	-	-	+/-	-
1								
2	+++	+++	+++	+++	+++	+++	+++	+++
3	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++
6	+	+	+	+	+	+	+	+
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-

IS – in triplicate

Table 2d Carbohydrate utilization test forCAHSH-2 and CAHSH-3 along with reference strains from IMTECH.

Carbohydrate	Actinomycetes CAHSH-2			Actinomycetes CAHSH - 3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
Lactose	-	-	-	+++	+++	+++	+++	+++
Xylose	-	-	-	+++	+++	+++	+++	+++
D-Maltose	+++	+++	+++	+++	+++	+++	+++	+++
D-Fructose	+++	+++	+++	+++	+++	+++	+++	+++
Dextrose	+++	+++	+++	+++	+++	+++	+++	+++
Galactose	-	-	-	+++	+++	+++	+++	+++
Raffinose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	+++	+++	+++	+++	+++
Melibiose	-	-	-	+++	+++	+++	+++	+++
Sucrose	+++	+++	+++	-	-	-	-	-
L-Arabinose	-	-	-	++	++	++	++	++
Mannose	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-
Sodium gluconate	-	-	-	-	-	-	-	-
Glycerol	++	++	++	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-
Arabitol	-	-	-	++	++	++	++	++
Erythritol	-	-	-	-	-	-	-	-
alpha-Methyl-D-glucoside	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-
alpha-Methyl-D-Mannoside	-	-	-	-	-	-	-	-
Xylitol	+++	+++	+++	+++	+++	+++	+++	+++
ONPG	-	-	-					
Esculin	++	++	++	-	-	-	-	-
D- Arabinose	-	-	-	-	-	-	-	-
Citrate utilization	++	++	++	-	-	-	-	-
Malonate	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-
Glucose	+++	+++	+++	+	+	+	+	+
saccharose	-	-	-	-	-	-	-	-

IS – in triplicate

Table 2e. Amino acid and nitrogen utilization tests for CAHSH-2 and CAHSH-3 along with reference strains from IMTECH.

Nitrogen source	Actinomycetes CAHSH-2			Actinomycetes CAHSH - 3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
L-Arginine	+	+	+	+	+	+	+	+
L-Phenyl alanine	++	++	++	++	++	++	++	++
L-Tyrosine	+	+	+	++	++	++	+	++
L-Methionine	+	+	+	++	++	++	+	++
L-Histidine	+	+	+	++	++	++	+	++
DL-Tryptophane	++	++	++	+++	+++	+++	++	+++
L-Cysteine	+	+	+	++	++	++	+	++
L-Glutamine	++	++	++	++	++	++	++	++
Di-ammonium sulphate ($(NH_4)_2 SO_4$)	++	++	++	+++	+++	+++	++	+++
Ammonium nitrate ($NH_4 NO_3$)	++	++	++	+++	+++	+++	++	+++
Ammonium chloride (NH_4Cl)	++	++	++	-	-	-	++	-
Sodium nitrate ($NaNO_3$)	++	++	++	-	-	-	++	-
Urea	++	++	++	++	++	++	++	++
Yeast extract	+++	+++	+++	+++	+++	+++	+++	+++
Soybean Meal	+++	+++	+++	+++	+++	+++	+++	+++
$(NH_4)_2 SO_4$ +Yeast extract	+++	+++	+++	+/-	+/-	+/-	+++	+/-
Potassium nitrate	+	+	+	++	++	++	+	++

IS – in triplicate

Table 2f. Biochemical characterizations of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH.

Characteristics	Actinomycetes CAHSH-2			Actinomycetes CAHSH - 3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
ONPG	++	++	++	+++	+++	+++	++	+++
Lysine utilization	+++	+++	+++	+++	+++	+++	+++	+++
Ornithine utilization	+++	+++	+++	+++	+++	+++	+++	+++
Urease	+++	+++	+++	+++	+++	+++	+++	+++
Phenylalanine deamination	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Citrate utilization	+++	+++	+++	+++	+++	+++	+++	+++
Voges Proskauer's	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-
Malonate utilization	++	++	++	+++	+++	+++	++	+++
Esculin hydrolysis	++	++	++	-	-	-	++	-
Motility test	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-
Crystal violet utilization	+	+	+	+	+	+	+	+
Tween - 20 utilization	++	++	++	++	++	++	++	++
Sodium azade (0.01%) utilization	-	-	-	-	-	-	-	-
Sodium azade (0.02%) utilization	-	-	-	-	-	-	-	-
Macconkey agar utilization	++	++	++	+++	+++	+++	++	+++
Casein utilization	+++	+++	+++	++	++	++	+++	++

IS – in triplicate

Table 2g. Chemotaxonomy characterizations of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH (IS – in triplicate; I - Pattern I)**.**

Characteristics	Actinomycetes CAHSH-2			Actinomycetes CAHSH - 3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
Cell wall amino acid	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine	LL-DAP with glycine
Whole cell sugar	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern	No characteristic sugar pattern
Cell wall type	I	I	I	I	I	I	I	I

Table 2h
Degradation activities of CAHSH-2 and CAHSH-3 along with reference strains from IMTECH

Characteristics	Results							
	CAHSH-2			CAHSH-3			*Streptomyces ghanaensis*	*Streptomyces viridosporus*
	IS1	IS2	IS3	IS1	IS2	IS3		
Starch	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+
L-tyrosine	+	+	+	+	+	+	+	+
xylene	-	-	-	+	+	+	-	+
xanthine	-	-	-	+	+	+	-	+

Table 2i
Antibiotics Susceptibility of CAHSH-2 and CAHSH-3 along with reference strain

Antibiotics	Actinomycetes CAHSH-2			Actinomycetes CAHSH - 3			Reference Strains	
	IS1	IS2	IS3	IS1	IS2	IS3	*Streptomyces ghanaensis*	*Streptomyces viridosporus*
Ampicillin (AMP-10 mg)	-	-	-	-	-	-	-	-
Ciprofloxacin (CIP-5 mg)	-	-	-	-	-	-	-	-
Gentamycin (GEN -10 mg)	++	++	++	-	-	-	++	-
Linezolid (LZ -30 mg)	+++	+++	+++	-	-	-	+++	-
Streptomycin (S-10 mg)	-	-	-	-	-	-	-	-
Vancomycin (VA -30 - mg)	+++	+++	+++	-	-	-	+++	-
Ceftriaxone (CTR - 30 mg)	-	-	-	-	-	-	-	-
Chloramphenicol (C- 30 mg)	+	+	+	-	-	-	+	-
Clindamycin (CD -2 mg)	-	-	-	-	-	-	-	-
Erythromycin (E- 15 mg)	++	++	++	-	-	-	++	-
Tetracycline (TE -30 mg)	++	++	++	-	-	-	++	-

Table 3a. Antiviral activity of different fractions (F1, F2, F3, F4 and F5) from EtOAcE of CAHSH-2 isolate against WSSV in *Litopenaeus vannamei.*

Experimental Groups	No of shrimp used in each trail[$] 4 trials (n = 117)	Cumulative percentage mortality at different time intervals (days post injection)			
		2 d	3 d	5 d	10 d
Positive control[@]	3 X 3 = 9	83.33±3.33	100	100	100
Negative control*	3 X 3 = 9	0	0	5.56±0.88	11.11±1.15
Extract 200 μg per shrimp[#]	3 X 3 = 9	0	0	8.33±1.02	16.67±1.64
F1 - 100 μg per shrimp**	3 X 3 = 9	0	0	5.56±0.76	13.89±1.25
F1 - 50 μg per shrimp**	3 X 3 = 9	0	11.11±0.98	19.44±1.16	41.67±2.29
F2 - 100 μg per shrimp**	3 X 3 = 9	5.56±0.66	16.67±1.15	36.11±2.85	77.78±3.42
F2 - 50 μg per shrimp**	3 X 3 = 9	11.11±1.05	27.78±1.09	72.22±2.96	94.44±2.77
F3 - 100 μg per shrimp**	3 X 3 = 9	13.89±1.12	41.67±2.28	80.56±3.51	91.67±3.49
F3 - 50 μg per shrimp**	3 X 3 = 9	27.78±1.82	58.33±1.94	86.11±4.19	100
F4 - 100 μg per shrimp**	3 X 3 = 9	36.11±2.82	47.22±1.63	91.67±3.72	100
F4 - 50 μg per shrimp**	3 X 3 = 9	52.78±2.19	88.89±2.54	100	100
F5 - 100 μg per shrimp**	3 X 3 = 9	44.44±2.27	86.11±.14	100	100
F5 - 50 μg per shrimp**	3 X 3 = 9	55.56±2.73	88.89±3.29	100	100

*Shrimp injected with NTE buffer; [@]Shrimp injected with WSSV (5 μl of haemolymph from moribund WSSV-infected shrimp per shrimp) intramuscularly; **Shrimp injected with mixture of WSSV (5 μl of WSSV inoculum) and different fractions (F1, F2, F3, F4 and F5) from ethyl acetate extract extracted from Actinomycetes isolate (CAHSH-2) at different concentrations; [#]Shrimp injected with mixture of WSSV (5 μl of WSSV inoculum) and ethyl acetate extract extracted from Actinomycetes isolate (CAHSH-2) at the concentration of 200 μg per shrimp. [$]This trial was carried out for 4 times (21.11.2015, 08.01.2016, 30.01.2016 and 10.02.2016).

Table 3b. Antiviral activity of different sub-fractions (F1A and F1B) from separated from Fraction1 of EtOAcE of CAHSH-2 isolate against WSSV in *Litopenaeus vannamei.*

Experimental Groups	No of shrimp used in each trail$ 3 trials (n = 81)	Cumulative percentage mortality at different time intervals (days post injection)			
		2 d	3 d	5 d	10 d
Positive control@	3 X 3 = 9	74.07±2.47	100	100	100
Negative control*	3 X 3 = 9	0	0	7.41±0.68	11.11±0.99
Extract 200 µg per shrimp#	3 X 3 = 9	0	0	3.70±0.94	11.11±0.85
F1A - 100 µg per shrimp**	3 X 3 = 9	11.11±1.07	51.85±1.55	66.67±3.14	81.48±2.33
F1A - 50 µg per shrimp**	3 X 3 = 9	37.03±1.92	77.78±2.18	85.18±3.36	100
F1A - 25 µg per shrimp**	3 X 3 = 9	70.37±3.35	88.89±3.67	100	100
F1B - 100 µg per shrimp**	3 X 3 = 9	0	0	0	11.11±0.86
F1B - 50 µg per shrimp**	3 X 3 = 9	0	11.11±0.75	18.51±1.12	22.22±1.27
F1B - 25 µg per shrimp**	3 X 3 = 9	0	25.92±1.15	37.03±1.62	66.67±2.37

*Shrimp injected with NTE buffer; @Shrimp injected with WSSV (5 µl of haemolymph from moribund WSSV-infected shrimp per shrimp) intramuscularly; **Shrimp injected with mixture of WSSV (5 µl of WSSV inoculum) and different sub-fractions (F1A and F1B) separated from Fraction 1 of ethyl acetate extract extracted from Actinomycetes isolate (CAHSH-2) at different concentrations; #Shrimp injected with mixture of WSSV (5 µl of WSSV inoculum) and ethyl acetate extract extracted from Actinomycetes isolate (CAHSH-2) at the concentration of 200 µg per shrimp. $This trial was carried out for 3 times (14.05.2016, 21.05.2016 and 25.06.2016).

Table.4. **Auto dock results of di-n-octyl phthalate and bis(2-methylhepty) phthalate against VP26 and VP28**

Parameters	di-n-octyl phthalate - VP26	bis(2-methylhepty) phthalate VP26	di-n-octyl phthalate - VP28	bis(2-methylhepty) phthalate VP28
Binding energy Kcal/Mol	-5.54	-3.57	-4.7	-3.72
Ligand efficiency	-0.28	-0.13	-0.24	-0.13
Inhibit constant	87.47	2.43	360.06	1.87
Intermol_energy	-7.31	-5.92	-6.34	-6.93
Vdw_hb_desolv_ energy	-5.73	-5.86	-5.65	-6.83
Electrostatic energy	-1.57	-0.07	-0.69	-0.1

Table 5. WSSV load in different organs of shrimp injected with EtOAcE of CAHSH-2 treated WSSV by quantitative real-time PCR at different time intervals

Experimental Samples	WSSV load [copies per mg tissue with (Ct value)] in different organs at different time intervals (day)			
	2	5	10	15
Shrimp with WSSV - G	6.2×10^9 (16.2)	-	-	-
Shrimp with WSSV - H	4.3×10^7 (17.6)	-	-	-
Shrimp with WSSV + CAHSH-2 - G	2.7×10^5 (19.8)	5.4×10^3 (20.64)	1.5×10^2 (24.86)	0.97 (30.8)
Shrimp with WSSV + CAHSH-2 - H	3.1×10^4 (19.1)	2.8×10^3 (21.43)	19.4 (27.28)	0.96 (30.8)

Table 6.a. Toxicity of ethyl acetate extract (EtOAcE) of actinomycetes isolate (CAHSH-2) and di-n-octyl phthalate having antiviral activity against WSSV tested in *Artemia* nauplii, post-larvae and adult of *Litopenaeus vannamei*.

Experimental period (hours)	Survival of experimental animals (%) at different time intervals (h)														
	Artemia nauplii (n = 1500) *					Post-larvae of *L. vannamei* (n = 150) *					Adult *L. vannamei* (n = 30) @				
	Negative Control	EtOAcE of CAHSH-2		di-n-octyl phthalate		Negative Control	EtOAcE of CAHSH-2		di-n-octyl phthalate		Negative Control	EtOAcE of CAHSH-2		di-n-octyl phthalate	
		10 mg	50 mg	1 mg	2 mg		10 mg	50 mg	1 mg	2 mg		1 mg	2 mg	1 mg	2 mg
24 hrs	100±0.0	100±0.0	99.46±0.02	99.33±0.03	99.46±0.05	99.3±0.02	100±0.0	97.33±0.05	98.67±0.02	96.67±0.12	100±0.0	100±0.0	96.67±0.15	100±0.0	100±0.0
48 hrs	97.1±0.3	99.13±0.2	97.73±0.03	98.73±0.15	98.8±0.06	96.67±0.14	98.67±0.08	94.67±0.25	95.33±0.18	94.67±0.22	100±0.0	100±0.0	96.67±0.14	96.67±0.12	100±0.0
120 hrs	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	100±0.0	100±0.0	93.33±0.3	96.67±0.16	100±0.0
240 hrs	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	96.67±	96.67±0.4	93.33±0.21	96.67±0.20	96.67±0.19

*Animals exposed to the extract and pure compound by immersion method (mg per litre)

@Adult shrimp were intramuscularly injected with EtOAcE of CAHSH-3 or di-n-octyl phthalate (mg per shrimp)

Table 6.b. Toxicity of ethyl acetate extract (EtOAcE) of actinomycetes isolate (CAHSH-3) having antiviral activity against WSSV tested in *Artemia* nauplii, post-larvae and adult of *Litopenaeus vannamei*

Experimental period (hours)	Survival of experimental animals (%) at different time intervals (h)								
	Artemia nauplii (n = 1500)*			Post-larvae of *L. vannamei* (n = 150)*			Adult *L. vannamei* (n = 30)[@]		
	Negative Control	EtOAcE		Negative Control	EtOAcE		Negative Control	EtOAcE	
		10 mg	50 mg		10 mg	50 mg		1 mg	2 mg
24 hrs	99.67±0.32	99.73±0.22	99.27±0.25	100±0.0	99.13±0.18	98.47±0.29	100±0.0	96.67±0.44	100±0.0
48 hrs	98.47±0.58	98.53±0.02	97.67±0.37	98.93±0.24	97.73±0.11	97.4±0.31	96.67±0.42	96.67±0.58	96.67±0.36
120 hrs	ND	ND	ND	ND	ND	ND	96.67±0.53	93.33±0.61	93.33±0.03
240 hrs	ND	ND	ND	ND	ND	ND	93.33±3.33	93.33±0.74	86.67±0.92

*Animals exposed to the extract by immersion method (mg per litre)
[@]Adult shrimp were intramuscularly injected with EtOAcE of CAHSH-3 (mg per shrimp)