Mosquito larvicidal and pupicidal action of biosurfactant produced by *Bacillus subtilis* A1 and *Pseudomonas stutzeri* NA3 against *Anopheles stephensi*

Punniyakotti Parthipan[1] · Raja Kumaresan Sarankumar[1] · Anitha Jaganathan[2] · Pandian Amuthavalli[2] · Ranganathan Babujanarthanam[3] · Pattanathu K.S.M. Rahman[4] · Kadarkarai Murugan[2,5] · Akon Higuchi[6] · Aruliah Rajasekar[1*]

[1]Environmental Molecular Microbiology Research Laboratory, Department of Biotechnology, Thiruvalluvar University, Serkkadu, Vellore, Tamilnadu, India- 632 115.

[2]Division of Entomology, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore, Tamilnadu, India-641046.

[3]Energy Bioscience Laboratory, Department of Biotechnology, Thiruvalluvar University, Serkkadu, Vellore, Tamilnadu, India- 632 115.

[4]Chemical and Bioprocess Engineering Group, School of Science and Technology, University of Teesside, Middlesbrough TS13BA Tees Valley, UK.

[5]Thiruvalluvar University, Serkkadu, Vellore, Tamilnadu, India- 632 115.

[6]Department of Chemical and Materials Engineering, National Central University, No. 300, Jhongli, Taoyuan 32001, Taiwan

[*]**Rajasekar Aruliah**

rajasekargood@gmail.com, rajasekar.aruliah@gmail.com

29

Abstract

30 *Anopheles stephensi* vector of a *Plasmodium parasite* which are responsible for spreading malaria in modern world. 31 Presently, malaria suppression is desirable one because of insecticide resistance nature of vector, specifically 32 improvement of *Plasmodium* species as highly resistant to a many antimalarial drugs. Present study focused as 33 biosurfactant production using two potential biosurfactant producing bacterial strains such as *Bacillus subtilis* A1 34 and *Pseudomonas stutzeri* NA3 for mosquitocidal application. Produced biosurfactant were characterized using 35 fourier transform infrared (FTIR) spectroscopy and gas chromatography and mass spectrometry (GCMS) and 36 confirmed that the produced biosurfactant were lipopeptide in nature. Different concentration of the biosurfactant 37 ranged between 2-10 ppm was tested against different stages of *A. stephensi* and both biosurfactant were found as 38 exterminating larval and pupal stages of mosquitoes. LC50 values were 3.58 (I), 4.92 (II), 5.73 (III), 7.10 (IV) and 39 7.99 (pupae) and 2.61 (I), 3.68 (II), 4.48 (III), 5.55 (IV) and 6.99 (pupa) for biosurfactant produced by *B. subtilis* A1 40 and *P. stutzeri* NA3 respectively. Biosurfactant are eco-friendly and easily producible using low cost material. The 41 toxic nature of these biosurfactant to the targeted organism like *A. stephensi* is lead to promising application in the 42 medical field.

43

44 **Keywords**: Biosurfactant · *Anopheles stephensi* · mosquitocidal · Gas Chromatography · *Bacillus subtilis* · 45 Lipopeptide

46

47 **Introduction**

48

49 Mosquitoes signify as an important menace for millions of animals and humans around the world, because they are 50 active as vectors for dangerous pathogens and parasites, also responsible for millions of fatality per yearly. 51 *Anopheles stephensi* vector of a *Plasmodium parasite* which are responsible for causing/spreading malaria. In 2013, 52 about 198 million cases of malaria were recorded and 584,000 deaths were estimated among them. After 2000, 53 malaria mortality rates have fallen by 47 % worldwide since specifically 54 % in the African region. Most fatality 54 was recorded among African children, where a child dies was counted every minute due to malaria. A malaria 55 mortality rate amongst brood in Africa has been abridged by an anticipated 58 % since 2000 (Mehlhorn, 2008).

56 However, the regenerations of malaria after suppression in many countries were still documented (Benelli et al.
57 2015a,b).

58 Presently, malaria eradication is needed one due to insecticide resistance in vector, along with improvement
59 of *Plasmodium* species as resistant to a many antimalarial drugs. The most proficient way to managing of the vector
60 could be achieved at undeveloped stage of their life cycle (Mahesh Kumar et al. 2012). Herein position, eco-friendly
61 efficient controlling tools were urgently needed (Benelli et al. 2015a,b). Plant derivatives were used by many
62 researchers to control the vector growth, such as *Polygonum hydropiper* L., *Origanum scabrum, Clausena anisata*
63 and etc., (Maheswaran and Ignacimuthu, 2014; Govindarajan et al. 2016; Mukandiwa et al. 2016). Recently some
64 researchers used silver nanoparticles synthesized using plant material to control the mosquitoes (Poopathi et al.
65 2014; Murugan et al. 2015; Subramaniam et al. 2015; Subramaniam et al. 2016; Murugan et al. 2016). Ultimately,
66 bio-control of vectors is a suitable hopeful substitute to synthetic chemical pesticides. In this esteem, plentiful
67 biological materials have been tested to evaluate their probable to manage the mosquitos (Knight et al. 2003).
68 Toxins from bacterial strains *Bacillus sphaericus* and *B. thuringenesis var.israelensis* were revealed to be useful
69 against mosquito larvae at very low dosage and harmless to non-targeted organisms (Das and Mukherjee, 2006).
70 Nonetheless, the biolarvicide product extracted from *B. sphaericus* strain is noted to be lesser effective against
71 *Anopheles culicifacies* and barely competent against *Aedes aegypti* (Mittal, 2003).

72 Some of bacterial strains and their metabolites were used to control mosquitos, such as *Bacillus subtilis*
73 (Das and Mukherjee, 2006; Geetha et al. 2010) and *Bacillus circulans* (Darriet and Hougard, 2002). Rhamnolipid a
74 biosurfactant produced by *Pseudomonas aeruginosa* was potentially used to control the *Aedes aegypti* (Silva et al.
75 2015). Another biosurfactant 'Di-rhamnolipid' produced by bacterium *Pseudomonas fluorescens* was active against
76 the pupae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* (Prabakaran et al. 2015). However,
77 many of these activities by bacterial strains and their products have not been completely studied for the
78 characteristic of their bio-control potential. *Bacillus subtilis* synthesis a wide range of biologically active compounds
79 such as fatty acids, lipopeptide whish has immense prospective for biopharmaceutical importance for example their
80 use as antibacterial, antiviral, and antitumor agents (Cameotra and Makkar, 2004). Lipopeptides shows insecticide
81 potential against *Drosophila melanogaster* (Assie et al. 2002), their larvicidal properties besides mosquito vectors
82 has not much tested.

83 Biosurfactants are biologically active compounds produced by groups of microorganisms that utilize their
84 energy sources such as oils, hydrocarbons and simple sugars (Parthipan et al. 2017). They have the capabilities to
85 reduce surface/interface tension with liquid and solid substances (Das and Mukherjee, 2007). Biosurfactants are
86 extensively utilized for numerous intentions in different sectors like, oil recovery process, food processing industry,
87 cleaning purpose, crude oil drilling lubricants, bioremediation of oil contaminated sites and pharmaceutical industry
88 (Makkar et al. 2011; Freitas de Oliveira et al. 2013; Parthipan et al. 2017). Biosurfactants have many advantages,
89 i.e., they are less toxic, eco-friendly, easily degradable, very stable/active in high temperature/salinity regions and
90 can easily producible using cheap organic sources (Rienzo et al. 2016). The present research is deal with
91 biosynthesis and characterization of biosurfactant using two bacterial strains such as *Bacillus subtilis* A1 and
92 *Pseudomonas stutzeri* NA3 for mosquito control in laboratory conditions.
93
94 **Materials and methods**
95
96 **Bacterial strain and culture conditions**
97
98 In this study, two bacterial strains were used, *Bacillus subtilis* A1 (KP895564) and *Pseudomonas stutzeri* NA3
99 (KU708859), which are isolated in sample (A1 from crude oil and NA3 from injection water) collected at Indian
100 crude oil reservoir. These bacterial strains were sub-cultured in Luria–Bertani (LB) medium (g/l 10.0 tryptone, 5.0
101 yeast extract, 10.0 sodium chloride with 15.0 agar (Himedia, Mumbai, India)) and incubated for 24 hrs at 40°C for
102 *B. subtilis* A1 and 30°C for *P. stutzeri* NA3 respectively. Further inoculums was prepared by picking single colonies
103 and inoculated in LB broth (pH: 7.0) and incubated in orbital shaker incubator (150 rpm) at 37°C for 24 hrs
104 (Parthipan et al. 2017).
105
106 **Biosurfactant production**
107
108 Biosurfactant production was carried as described in Parthipan et al. (2017). In brief, sterile minimal salt medium
109 (MSM) (g/l: 0.5 $FeCl_3$, 0.2 $MgSo_4$, 1.0 NH_4NO_3, 1.0 KH_2PO_4, 1.0 K_2HPO_4 and 0.02 $CaCl_2$ (Himedia, Mumbai,
110 India)), supplemented with 2% sucrose as carbon source. Pre-cultured bacterial strains *B. subtilis* A1 and

111 *Pseudomonas stutzeri* NA3 were inoculated (initial load: 1.6×10^4 CFU ml^{-1}) and incubated for 120 hrs at 40°C for
112 *B. subtilis* A1 and 30°C for *P. stutzeri* NA3 respectively in an orbital shaker (150 rpm). After incubation period,
113 bacterial biomass was separated by centrifugation with 3400 x *g* at 4°C for 20 min (refrigerated centrifuge, Remi-
114 India: R-248). Further biosurfactant containing solutions were acidified (pH 2.0) by help of 6 M HCl. The acidified
115 solutions were kept at 4 °C for overnight to complete precipitation. Further precipitated biosurfactants were
116 collected by centrifugation at 8000 g for 20 min at 4 °C and dissolved in deionized sterile water (pH 7.0), followed
117 by extraction using 65:15 ratio of chloroform:methanol. These solvents were removed using a rotary evaporator, and
118 the biosurfactant phase was sluiced with three volumes of hexane to eliminate free fatty acids, alcohols and alkanes.
119 This procedure was repeated three times. The biosurfactant was further characterized using FT-IR and GC-MS
120 spectroscopy. Both biosurfactant obtained in this method were checked for oil displacement test (Hassanshahian,
121 2014)
122
123 **Characterization of biosurfactant**
124
125 The obtained biosurfactant was characterized by Fourier transform infrared spectrum (FT-IR) and gas
126 chromatographic mass spectrum (GC-MS). The functional groups of the biosurfactant recovered from both bacterial
127 strains *B. subtilis* A1 and *P. stutzeri* NA3 were characterized using FT-IR (Perkin–Elmer, Nicolet Nexus - 470)
128 (Parthipan et al. 2017). In brief, biosurfactant was mixed with KBr in the ratio of 1:100 to make pellet. Further
129 prepared pellet was set aside in the sample holder and analysed in the IR region ranged between 400- 4000 cm^{-1}.
130 Further biosurfactants were characterised by Gas chromatography as described by Parthipan et al (2017). Briefly 1
131 µl of methanol diluted samples was injected into a gas chromatograph (Shimadzu QP2010 Ultra, Rtx-5Sil MS (30 m
132 × 0.25 mm ID × 0.25 µm). Helium was used as carrier gas with the flow rate of 1.5 ml min^{-1} and the temperature of
133 the GC injector was set as 260°C. The gradient temperature was set between 60 to 260°C at a rate of 5°C min^{-1},
134 through an isothermal phase 10 min at the end of the run. The electron impact ion basis was constant at 200°C. Mass
135 spectra were observed at 70 keV. The mass spectra were acquired with a m/z range: 40–600 ultra-high resolution
136 approach with an acquisition speed of 6 spectra/sec. The detection of components was made in scan mode by using
137 NIST11 and Wiley8 library.
138

139 **Mosquito rearing**

140

141 Eggs of *Anopheles stephensi* were collected from water reservoirs within Coimbatore (Tamil Nadu, India) using an
142 "O"-type brush. Batches of 100–110 eggs were moved to 18×13×4 cm^3 enamel trays with 500mL of water. Here
143 eggs were permissible to hatch in lab setup (75–85% relative humidity (RH); 27±2 °C; 14:10 (L/D) photoperiod). 5g
144 of ground dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma-Aldrich, Germany) in ratio of 3:1 were
145 provided as feed for *A. stephensi* larvae. Freshly brewed larvae and pupae were carefully collected and will be used
146 in the toxicity experiments (Anitha et al. 2016).

147

148 **In-vitro larvicidal and pupicidal toxicity assay**

149

150 About 25 *A. stephensi* larvae (I, II, III or IV instar) and pupae were positioned for 24 h in a glass beaker containing
151 250 ml of dechlorinated water with desired concentration of the both biosurfactant separately. 0.5 mg of larval food
152 was supplied for each concentration of biosurfactant (Kovendan et al. 2012). Each concentration was repeated 5
153 times against all instars. Control mosquitoes were bared for 24 h to the solvent, mortality percentage was calculated
154 using following formula:

155 Percentage mortality = (number of dead individuals/ number of treated individuals)*100

156 Data analysis

157 In mosquito controlling experiments, lethal concentration (LC) 50 and LC90 were calculated by probit
158 analysis, as described by Finney (1971). Mosquitocidal efficiency was analyzed using analysis of variance
159 (ANOVA,) means were separated using Tukey's HSD test. $P<0.05$ was considered as significance of differences
160 among means.

161

162 **Results**

163

164 **Biosurfactant characterization**

165

166 Biosurfactants were produced effectively using both A1 and NA3 strains with the optimized production conditions
167 as reported in the Parthipan et al. (2017). Biosurfactant produced *B. subtilis* gave considerably level of
168 emulsification activity 84% (as reported in Parthipan et al. (2017)). Both strains showed the effective oil-
169 displacement activity as shown in Fig. 1a and 1b. Higher amount of biosurfactant was produced by the *B. subtilis*
170 A1 (4.85 g l^{-1}) as reported earlier (Parthipan et al. 2017), similarly strain NA3 was produced 3.81 g l^{-1} of
171 biosurfactant. FT-IR was analysed and observed that biosurfactant produced by A1 contains following functional
172 groups of –OH, $P\text{-}H_2$, C=O, $-CH_3$, $-CH_2-$, –COOH, O–H, CH_2 and C–I (Parthipan et al. 2017).

173 Similarly biosurfactant produced by NA3 also characterized by FT-IR and predicted the numerous peaks at
174 different positions were shown in Fig. 2. Peak at 1442cm^{-1} was due to the presence of N-H; peaks at 2923, 2853,
175 1461 and 1391cm^{-1} indicates the presence of aliphatic chains (-CH3 and -CH2-). A strong peak at 1639 specified
176 that occurrence of CO-N bond. Presences of peaks at 1091 and 722cm^{-1} may match to the C–N stretching vibrations.
177 With the previous literature about lipopeptide biosurfactant, these FT-IR descriptions confirmed that presence of the
178 aliphatic groups joined with a peptide moiety as distinguishing properties of lipopeptide biosurfactant (Zou et al.
179 2014).

180 The gas chromatography and mass spectrum characterization further revealed that the biosurfactant
181 extracted from both strains were lipopeptide in nature. Compounds obtained from strain A1 were reported as fatty
182 acids, such as hexadecanoic acid, octadecadienoic acid and octadecenoic acid (Parthipan et al. 2017). Similarly NA3
183 also predicted with numerous fatty acid peaks at different retention time (RT) as below, 1-Dodecanol (Fig. 3a) (RT:
184 16.16, molecular weight (MW): 186, chemical formula (CF): $C_{12}H_{26}O$)), oleic acid (Fig. 3b) (RT: 20.55, MW: 282,
185 CF: $C_{18}H_{34}O_2$), hexanoic acid, octadecyl ester (Fig. 3c) (RT: 22.82, MW: 368, CF: $C_{24}H_{48}O_2$).

186

187 **Biosurfactant toxicity assay against *A. stephensi***

188

189 Table 1 indicates that the larvicidal and pupicidal activities of biosurfactant synthesized by *B. subtilis* A1 at different
190 concentrations in laboratory conditions. Biosurfactant from A1 was found to be highly toxic to the larva and pupa of
191 *A. stephensi* whose LC50 values were 3.58 (I), 4.92 (II), 5.73 (III), 7.10 (IV) and 7.99 (V) for the different stages of
192 the life span. Similarly, mosquitocidal activity of NA3 (Table 2) showed their toxicity with the LC 50 values of
193 2.61 (I), 3.68 (II), 4.48 (III), 5.55 (IV) and 6.99 (pupa) which confirms that the both biosurfactant contains

194 mosquitocidal components and its efficiency of the extermination was increased with increasing of concentrations
195 (ppm).
196

197 **Discussion**

198

199 In recent times, plentiful reports were available to support that *Bacillus* sp. and *Pseudomonas* sp. were effective
200 biosurfactant producers and also widely used for extensive range of application in many field such as: oil recovery,
201 bioremediation, industrial application and biodegradation (Pereira et al. 2013; Pacwa-Plociniczak et al. 2014;
202 Cubitto et al. 2004 Greenwell et al. 2016; Ismail et al. 2013; Parthipan et al. 2017). Unfortunately very few reports
203 were available on the mosquitocidal application of the biosurfactant produced by Bacillus sp. and *Pseudomonas* sp.
204 (Das and Mukherjee, 2006; Geetha and Manonmani, 2010; Geetha et al. 2010). Biosurfactants are habitually a
205 mixture of molecules such as fatty acids, peptides and polysaccharide; it could be any one form as like lipopeptides,
206 lipoproteins, glycolipids, phospholipids and lipopolysaccharides based on the biosurfactant producers. Interestingly
207 some of these compounds were highly toxic to the many of organisms such as insects and mosquitos, which are very
208 dangerous to the human health (Das and Mukherjee, 2006; Geetha and Manonmani, 2010; Geetha et al. 2010). The
209 FTIR analysis reveals that occurrence of ester carbonyl groups, phosphines in phosphoserine, carboxylic acids and
210 Carbon–Iodine. Based on this observation biosurfactant obtained from bacterium *B. subtilis* A1 and *P. stutzeri* NA3
211 was categorized as lipopeptide in nature (Rodrigues et al. 2006; Parthipan et al. 2017).

212 The LC50 values of the present study revealed that the significant difference in the production of
213 mosquitocidal metabolites of both biosurfactant produced by the bacterial strains *B. subtilis A1* and *P. stutzeri* NA3.
214 Pupae of mosquitoes needed the accessible atmospheric oxygen for their respiration and other functions. Due to
215 reduction in surface tension of water by the auctions of lipopetides, the pupae were incapable to come up to the
216 surface of water for their oxygen needs and had to remain submerged in water, these unusual circumstances lead
217 them to fatality (Piper and Maxwell, 1971). Hence, the mosquito pupal mortality observed in our study could be
218 primarily by reduction in surface tension of the water caused by the bacterial biosurfactant. However, the possibility
219 of its action on the cuticle of the pupae cannot be ruled out as there are reports on the action of surfactin on
220 biological membranes (Vollenbroich et al. 1997a&b; Buchoux et al. 2008). Trace elements and carbon level in the
221 culture medium have been reported to enhance the lipopeptide production by the strains *B. subtilis* (Wei et al. 2007).

222 These lipopeptides will get bind with the sulphur group of DNA, leading to the rapid denaturation of organelles and
223 enzymes in mosquito. Subsequently, decreases in the membrane permeability and disturbance in proton motive force
224 may cause loss of cellular function and also cell death. Further research on this issue is required (Benelli, 2016a,b).

225 Present research outcomes were supported by some of the previous research findings as the biosurfactant
226 used as mosquito controlling agent. Deepali et al. (2014) isolated biosurfactant from *Stenotrophomonas maltophilia,*
227 also identified that biosurfactant was rhamnolipid in nature and it has no activity for 4mg/l concentration but
228 increasing the concentration to 10 mg/l gives larvicidal properties. Recently Geetha and Manonmani, (2010) isolated
229 surfactin a biosurfactant compound from the strain *Bacillus subtilis* ssp. *subtilis* and reported that surfactin showed
230 mosquito pupicidal activity (Geetha et al. 2010). Similarly Das and Mukherjee, (2006) used lipopeptides extracted
231 from *B. subtilis* strain for mosquito larvicidal uses. Recently Silva et al. (2015) reported that the rhamnolipid as an
232 eco-friendly surfactants and it has many activities like larvicidal, insecticidal, and repellent activities against *A.*
233 *aegypti*, but this achievements were obtained using very high level of biosurfactant 1 g/l as reported. As compared to
234 these studies, present study achieved higher mortality rate in very low concentration (2-10ppm). These observations
235 signify that the biosurfactant produced by strains A1 and NA3 were highly active at low concentrations and may be
236 used as potential mosquitocidal agent.

237

238 **Conclusion**

239

240 In conclusion, these observations showed that the biosurfactant produced by both bacterial strains *B. subtilis A1* and
241 *P. stutzeri NA3* were lipopeptide in nature and which are accountable of the *A. stephensi* mosquito larvicidal and
242 pupicidal activities. This mosquitocidal action of the both biosurfactant on the larvae and pupae may be due to
243 reduction in the surface tension of water, its direct to the oxygen deficiency at underwater where larvae and pupae
244 exit and this abnormal condition lead them to dead. There are limited reports only available on the mosquitocidal
245 effects of the biosurfactant produced by bacterial strains. As there are limited bio-control methods only available
246 against mosquito to eradicate, it could be a hopeful method for control of the mosquito spread. Further studies
247 needed to extend these observations to external uses in the form of effective controlling agent.

248

249

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251

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257

258 **Compliance with ethical standards**

259

260 All applicable international and national guidelines for the care and use of animals were followed. All procedures
261 performed in studies involving animals were in accordance with the ethical standards of the institution or practice at
262 which the studies were conducted

263

264 **Conflict of interest**

265

266 The authors declare that they have no conflict of interest.

267

268 **References**

269

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390 **Figure Captions:**

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392 **Fig. 1** Oil displacement activity of the biosurfactant: (a) *B. subtilis* A1; (b) *Pseudomonas stutzeri* NA3.

393 **Fig. 2** FT-IR spectrum of biosurfactant produced by strain *Pseudomonas stutzeri* NA3.

394 **Fig. 3** Mass spectrum of the biosurfactant isolated from *Pseudomonas stutzeri* NA3: (a) 1-Dodecanol; (b)

395 oleic acid; (c) hexanoic acid, octadecyl ester.

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