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

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Abstract

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

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

Introduction

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

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

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

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

Biosurfactants are biologically active compounds produced by groups of microorganisms that utilize their energy sources such as oils, hydrocarbons and simple sugars (Parthipan et al. 2017). They have the capabilities to reduce surface/interface tension with liquid and solid substances (Das and Mukherjee, 2007). Biosurfactants are extensively utilized for numerous intentions in different sectors like, oil recovery process, food processing industry, cleaning purpose, crude oil drilling lubricants, bioremediation of oil contaminated sites and pharmaceutical industry (Makkar et al. 2011; Freitas de Oliveira et al. 2013; Parthipan et al. 2017). Biosurfactants have many advantages, i.e., they are less toxic, eco-friendly, easily degradable, very stable/active in high temperature/salinity regions and can easily producible using cheap organic sources (Rienzo et al. 2016). The present research is deal with biosynthesis and characterization of biosurfactant using two bacterial strains such as *Bacillus subtilis* A1 and *Pseudomonas stutzeri* NA3 for mosquito control in laboratory conditions.

Materials and methods

Bacterial strain and culture conditions

In this study, two bacterial strains were used, *Bacillus subtilis* A1 (KP895564) and *Pseudomonas stutzeri* NA3 (KU708859), which are isolated in sample (A1 from crude oil and NA3 from injection water) collected at Indian crude oil reservoir. These bacterial strains were sub-cultured in Luria–Bertani (LB) medium (g/l 10.0 tryptone, 5.0 yeast extract, 10.0 sodium chloride with 15.0 agar (Himedia, Mumbai, India)) and incubated for 24 hrs at 40°C for *B. subtilis* A1 and 30°C for *P. stutzeri* NA3 respectively. Further inoculums was prepared by picking single colonies and inoculated in LB broth (pH: 7.0) and incubated in orbital shaker incubator (150 rpm) at 37°C for 24 hrs (Parthipan et al. 2017).

Biosurfactant production

Biosurfactant production was carried as described in Parthipan et al. (2017). In brief, sterile minimal salt medium (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, India)), supplemented with 2% sucrose as carbon source. Pre-cultured bacterial strains *B. subtilis* A1 and

Pseudomonas stutzeri NA3 were inoculated (initial load: 1.6×10^4 CFU ml^{-1}) and incubated for 120 hrs at 40°C for *B. subtilis* A1 and 30°C for *P. stutzeri* NA3 respectively in an orbital shaker (150 rpm). After incubation period, bacterial biomass was separated by centrifugation with 3400 x *g* at 4°C for 20 min (refrigerated centrifuge, Remi-India: R-248). Further biosurfactant containing solutions were acidified (pH 2.0) by help of 6 M HCl. The acidified solutions were kept at 4 °C for overnight to complete precipitation. Further precipitated biosurfactants were collected by centrifugation at 8000 g for 20 min at 4 °C and dissolved in deionized sterile water (pH 7.0), followed by extraction using 65:15 ratio of chloroform:methanol. These solvents were removed using a rotary evaporator, and the biosurfactant phase was sluiced with three volumes of hexane to eliminate free fatty acids, alcohols and alkanes. This procedure was repeated three times. The biosurfactant was further characterized using FT-IR and GC-MS spectroscopy. Both biosurfactant obtained in this method were checked for oil displacement test (Hassanshahian, 2014)

Characterization of biosurfactant

The obtained biosurfactant was characterized by Fourier transform infrared spectrum (FT-IR) and gas chromatographic mass spectrum (GC-MS). The functional groups of the biosurfactant recovered from both bacterial strains *B. subtilis* A1 and *P. stutzeri* NA3 were characterized using FT-IR (Perkin–Elmer, Nicolet Nexus - 470) (Parthipan et al. 2017). In brief, biosurfactant was mixed with KBr in the ratio of 1:100 to make pellet. Further prepared pellet was set aside in the sample holder and analysed in the IR region ranged between 400- 4000 cm^{-1}. Further biosurfactants were characterised by Gas chromatography as described by Parthipan et al (2017). Briefly 1 µl of methanol diluted samples was injected into a gas chromatograph (Shimadzu QP2010 Ultra, Rtx-5Sil MS (30 m × 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 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}, through an isothermal phase 10 min at the end of the run. The electron impact ion basis was constant at 200°C. Mass spectra were observed at 70 keV. The mass spectra were acquired with a m/z range: 40–600 ultra-high resolution approach with an acquisition speed of 6 spectra/sec. The detection of components was made in scan mode by using NIST11 and Wiley8 library.

Mosquito rearing

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

In-vitro larvicidal and pupicidal toxicity assay

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

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

Data analysis

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

Results

Biosurfactant characterization

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

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

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

Biosurfactant toxicity assay against *A. stephensi*

Table 1 indicates that the larvicidal and pupicidal activities of biosurfactant synthesized by *B. subtilis* A1 at different concentrations in laboratory conditions. Biosurfactant from A1 was found to be highly toxic to the larva and pupa of *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 the life span. Similarly, mosquitocidal activity of NA3 (Table 2) showed their toxicity with the LC 50 values of 2.61 (I), 3.68 (II), 4.48 (III), 5.55 (IV) and 6.99 (pupa) which confirms that the both biosurfactant contains

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

Discussion

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

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

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

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

Conclusion

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

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Compliance with ethical standards

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

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Captions:

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

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

Fig. 3 Mass spectrum of the biosurfactant isolated from *Pseudomonas stutzeri* NA3: (a) 1-Dodecanol; (b) oleic acid; (c) hexanoic acid, octadecyl ester.

Figure

Fig. 1

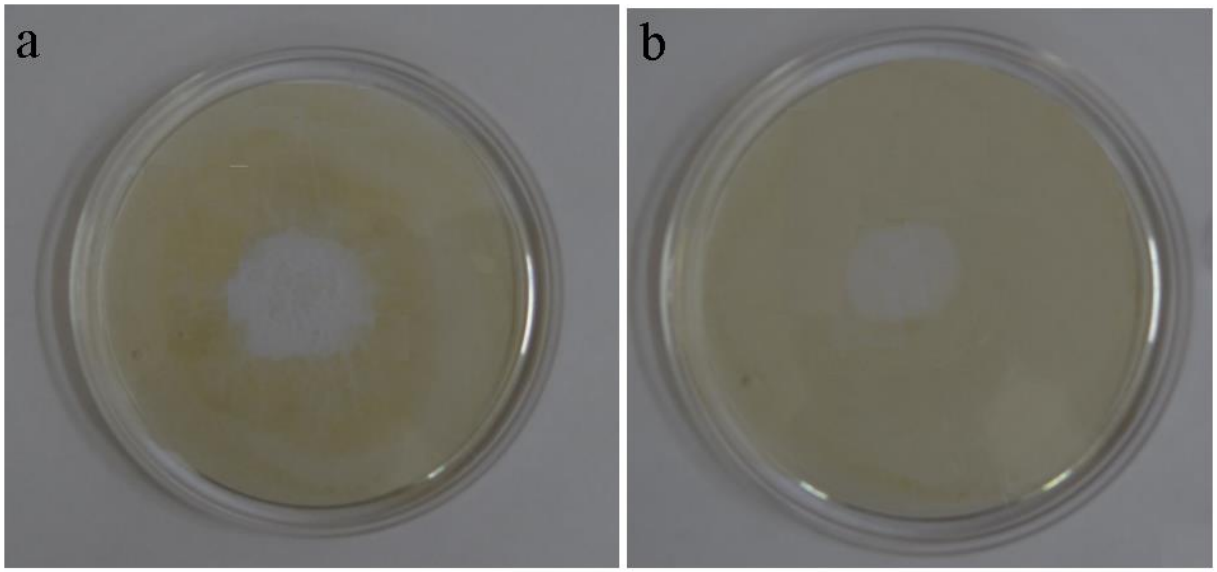
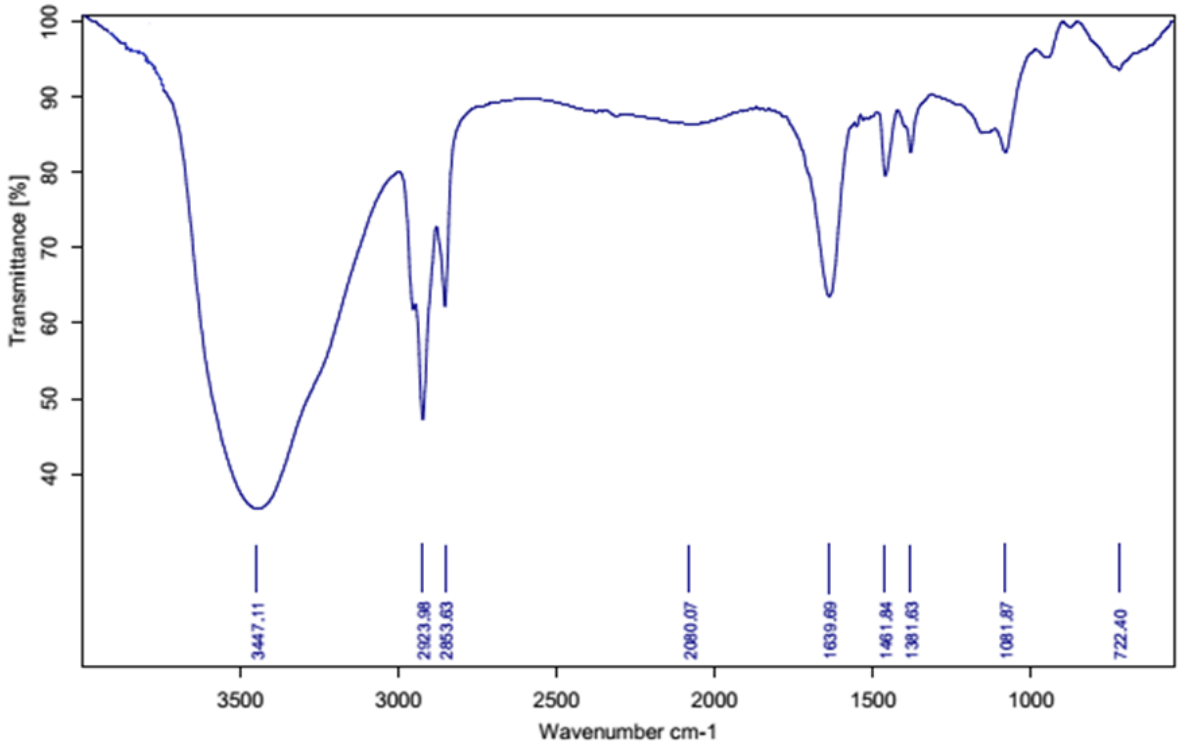


Fig. 2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90
80
70
60
50
40
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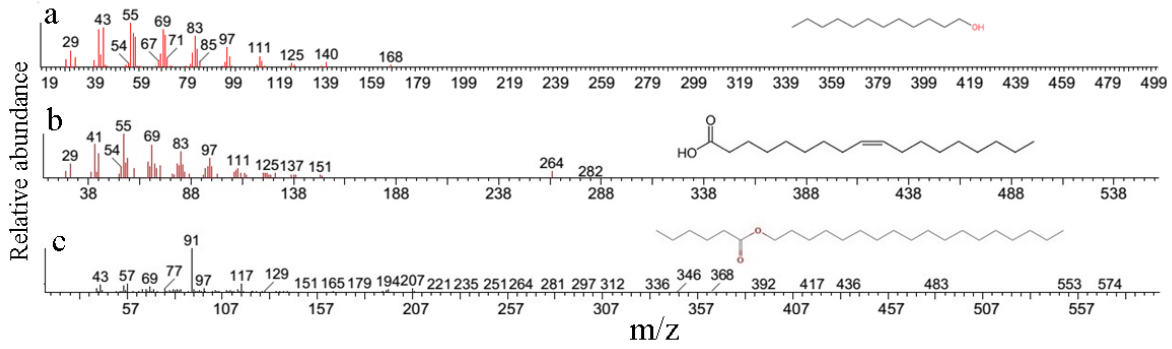
2923.98
2853.63
2080.07
1639.69
1461.84
1381.63
1081.87
722.40
3500
3000
2500
2000
1500
1000
Wavenumber cm-1

Fig. 3



Table

Table 1 Mosquitocidal effect of the biosurfactant isolated from *B. subtilis* A1 against the malarial vector *Anopheles stephensi.*

No mortality was observed in the control.

Mosquito life stages	Percentage of larval and pupal mortality					LC_{50} and (LC_{90})	95% confidence Limit		Regression equation	x^2 (*d.f.*=4)
	Concentration (ppm)						LC_{50} (LC_{90})			
	2	4	6	8	10		Lower	Upper		
I	37.2 ±1.30	51 ± 2.12	70.2±1.92	87.6 ±2.07	98.6 ±1.14	3.589 (8.211)	2.987 (7.548)	4.085 (9.108)	y = -0.995 +0.277 x	3.352 *n.s*
II	29.6 ±1.67	35.2±1.64	61.8±1.30	73.6 ±1.67	89.8±0.83	4.925 (10.596)	4.335 (9.652)	5.453 (11.939)	y= -1.113+0.226 x	3.494 *n.s*
III	22.2± 1.30	34.4±1.51	56.2 ±1.48	67.8±2.86	78.8±1.64	5.733 (12.128)	5.126 (10.918)	6.318 (13.930)	y= -1.149 +0.200 x	1.015 *n.s*
IV	19.2 ±1.09	31 ±1.22	43.4±2.30	59.4±1.14	65±1.58	7.109 (15.036)	6.397 (13.112)	7.953 (18.224)	y= - 1.149+0.162 x	1.066 *n.s*
Pupa	15 ± 1.87	26.6± 0.89	37 ± 2.23	51.4± 1.67	61±0.70	7.993 (15.778)	7.247 (13.737)	8.979 (19.170)	y= - 1.316+0.165 x	0.548 *n.s*

LC50= lethal concentration (ppm) that kills 50% of the exposed organisms.

LC90= lethal concentration (ppm) that kills 90% of the exposed organisms.

χ^2=chi-square.

d.f.= degrees of freedom.

n.s.= not significant (α=0.05).

Table 2 Mosquitocidal effect of the biosurfactant isolated from *P. stutzeri* NA3 against the malarial vector *Anopheles stephensi.*

Mosquito life stages	Percentage of larval and pupal mortality					LC_{50} and (LC_{90})	95% confidence Limit		Regression equation	x^2 (*d.f.*=4)
	Concentration (ppm)						LC_{50} (LC_{90})			
	2	4	6	8	10		Lower	Upper		
I	45.4± 1.34	63.6± 0.89	80.8± 1.30	94.4± 1.94	99.8± 0.44	2.618 (7.151)	1.874 (6.534)	3.186 (7.986)	y = -0.740 +0.283 x	0.846 *n.s*
II	39 ± 0.70	51.6± 1.51	64.2± 1.92	83± 1.87	92.6±1.51	3.680 (9.709)	2.893 (8.784)	4.299 (11.054)	y= -0.782 +0.213 x	1.815 *n.s*
III	32.8± 1.09	47.6± 1.14	58 ± 2.91	75.6± 2.07	84± 1.58	4.481 (11.541)	3.679 (10.287)	5.137 (13.480)	y= -0.813 +0.182 x	0.553 *n.s*
IV	27.4± 1.14	39.2± 1.92	54.6± 2.07	68.4± 2.50	73.6±1.81	5.553 (13.490)	4.786 (11.825)	6.264 (16.216)	y= -0.897 +0.161x	1.024 *n.s*
Pupa	22.6± 1.81	32.8± 1.48	44± 1.58	57.6± 2.30	66± 2.54	6.999 (15.641)	6.231 (13.464)	7.912 (19.408)	y= - 1.038 +0.148 x	0.187 *n.s*

No mortality was observed in the control.

LC50= lethal concentration (ppm) that kills 50% of the exposed organisms.

LC90= lethal concentration (ppm) that kills 90% of the exposed organisms.

χ^2=chi-square.

d.f.= degrees of freedom.

n.s.= not significant (α=0.05).