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Enhanced production of mosquitocidal cyclic lipopeptide from *Bacillus subtilis* subsp. *subtilis*

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***Background & objectives*: A cyclic lipopeptide, surfactin produced by a strain of *Bacillus subtilis* subsp. *subtilis* (VCRC B471) was found to exhibit activity against both the larval and pupal stages of mosquitoes. The present study was aimed at increasing the production of the mosquitocidal metabolite by modifying the conventional medium.**

***Methods*: Enhancement of mosquitocidal metabolite production was attempted by replacing the existing micronutrients of the conventional NYSM and supplementing the medium with additional amounts of glucose. The LC_{50} value of culture supernatant (CS) against the larval and pupal stages of *Anopheles stephensi* was determined. Crude mosquitocidal metabolite (CMM) was separated from the CS, identified by MALDI-TOF analysis and its LC_{50} dosage requirement for the pupal stage of the above mosquito species determined.**

***Results*: The medium containing a new composition of micronutrients and glucose up to 1 per cent resulted in increased metabolite production. The LC_{50} value of the CS obtained in the improved medium against larvae and pupae of *An. stephensi* was 5.57 and 0.71 µl/ml, respectively. The yield of CMM was doubled in the improved medium. MALDI-TOF analysis revealed that the CMM was surfactin.**

***Interpretation & conclusions*: The new improved medium enhanced the production of mosquitocidal metabolite as the dosage required for inciting 50 per cent mortality among the pupal stages of mosquitoes was only half of that required when the metabolite was produced in the conventional medium. The mosquitocidal metabolite was identified as surfactin, a cyclic lipopeptide and biosurfactant.**

Key words Anopheles - *Bacillus subtilis* - cyclic lipopeptide - mosquitocidal metabolite - surfactin

Among various microbial pesticides being advocated for the control of mosquitoes, those containing *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* enjoy top priority[1-3]. However, reports indicate development of resistance by certain species of mosquitoes to certain strains of *B. sphaericus*[4,5]. Though resistance development has not been a major problem encountered with *B. thuringiensis* till date, the stability, solubility and insecticidal activity of the crystal toxins of *B. thuringiensis* are known to be affected by *p*H of the habitat[6,7] and by exposure to sunlight[8]. These drawbacks call for the development of new mosquitocidal bacterial agents which can overcome the above limitations.

We have isolated a Gram-positive, aerobic, spore-forming bacterium from mangrove forests of Andaman & Nicobar islands, India. It was identified to be a strain of *Bacillus subtilis* subsp. *subtilis* (VCRC B471) based

on biochemical parameters, 16S ribosomal DNA and *gyrA* gene sequencing. The culture supernatant (CS) of this bacterium was found to kill the larval and pupal stages of three species of mosquitoes *viz*., *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*[9]. The production of the mosquitocidal metabolite from this strain was optimized using Nutrient Yeast Salt Medium (NYSM)[9,10]. In this study, this medium was modified by replacing and/or supplementing various media constituents reported for increasing the yield of this metabolite.

Material & Methods

Microorganism: The *B. subtilis* subsp. *subtilis* strain (VCRC B471) isolated from soil samples collected from the mangrove forests of Andaman & Nicobar islands was used for production of the mosquitocidal metabolite. The production strain has been deposited in Microbial Type Culture Collection (MTCC), Chandigarh, India (Accession Number No. 5368). Stock cultures were stored in 10 per cent glycerol (Sigma, USA) at -80°C. The strain was maintained on Nutrient Yeast Salt Medium (NYSM) [glucose 5 g, peptone 5 g NaCl 5 g, beef extract 3 g, yeast extract 5 g, $MgCl_2$ 203 mg , $MnCl_2$ 10 mg and $CaCl_2$ 103 mg (Hi-Media, India) per liter of water] agar slants[11] at 4ºC.

Bacterial growth conditions: For the production of mosquitocidal metabolite(s), the bacterium was grown aerobically in NYSM. Tubes containing 10 ml NYSM broth were inoculated with a loopful of bacterial cells from the slant culture. The tubes were incubated overnight on a rotary shaker (New Brunswick Scientific Co. Inc., USA) at 28 ± 2ºC and 250 rpm. After incubation, cultures were inoculated to fresh 50 ml of NYSM broth and incubated again for a further period of 7 h to synchronize the growth. From this young culture, 5 ml was added to flasks containing 100 ml of the various media (Table I) and incubated with shaking for 72 h. Bacterial cells were removed from the medium by centrifugation at 9000 x *g* for 25 min at 4°C in a Sorvall Evolution RC superspeed centrifuge (Kendro Lab. Products, Asheville, NC, USA) using SLA-1500 rotor. The culture supernatant (CS) obtained was used for bioassay.

Table I. Media used for the production of mosquitocidal metabolite(s)

Culture medium	Media ingredients
A	NYSM + EMN (Conventional medium)
B	NYSM + EMN + 0.5% glucose
C	NYSM + EMN + 1.5% glucose
D	NYSM + NMN
E	NYSM + NMN + 0.5% glucose
F	NYSM + NMN + 1.5% glucose

NYSM, nutrient yeast salt medium; EMN, existing micro nutrient; NMN, new micro nutrient

Media used: A total of six media were prepared in 2 sets using NYSM as the basal medium, by varying the micronutrient and glucose concentration. Media set 1 was prepared with Existing Micro Nutrient (EMN) [EMN: $MgCl_2$ 203 mg, $MnCl_2$ 10 mg and $CaCl_2$ 103 mg per litre of water] and media set 2 was prepared with New Micro Nutrient (NMN) [NMN: $MgSO_4$ 592 mg, KH_2PO_4 1.36 g, $MnSO_4$ 2 mg, $FeSO_4$ 2 mg, $CaCl_2$ 1 mg per litre of water[12] *p*H 7.0±0.2]. The concentration of glucose was varied from 1 - 2 per cent in both the sets (Table I).

Source of mosquito: Third instar larvae and freshly emerged pupae of *An. stephensi* were obtained from a laboratory colony maintained at the Rearing and Colonization Unit of the Vector Control Research Centre, Puducherry, and used for bioassay of the mosquitocidal metabolite(s).

Bioassay: CS obtained from the different sets of media was used for bioassay following WHO standard protocols[13]. To 200 ml capacity, disposable wax-coated paper cups, 150 ml of chlorine-free tap water was added and 25 numbers of larvae or freshly emerged pupae of *An. stephensi* were transferred to each cup. For experimental treatment, five concentrations of CS were used. The dosages used for testing the larvicidal and pupicidal activity are given in Table II. Each experiment was performed using four replicates per dose and having an equal number of controls. The treated and control cups were held at 28 ± 2°C, 80-90 per cent relative humidity, and a photoperiod of 12 h light followed by 12 h dark. Bioassay cups used for testing the pupicidal activity were covered with mosquito net cloth to prevent the escape of emerging adults, if any. The mortality of the pupae was scored after 24 h of exposure by counting the number of live ones present in the bioassay cup. The dead pupae in the four replicates were combined and expressed as percentage of pupal mortality for each concentration. Dead pupae included those found at the bottom of the bioassay cups as straightened pupae after losing their typical coma shape as well as those which had moulted to adults but were unable to come out of the pupal exuviae. The experiments were repeated twice. In cases

Table II. Dosages of CS (µl/150 ml) of different culture media (A-F) used for determination of LC_{50} and LC_{90} against pupae and larvae of *An. stephensi*

Medium	Dose of CS for pupae	Dose of CS for larvae
A	125, 150, 175, 200, 225	1000, 1250, 1500, 1750, 2000
B	100, 125, 150, 175, 200	750, 800, 850, 900, 950
C	125, 150, 175, 200, 225	675, 750, 825, 900, 975
D	100, 125, 150, 175, 200, 225	1000, 1250, 1500, 1750
E	100, 125, 150, 175, 200	750, 800, 850, 900, 950
F	125, 150, 175, 200, 225	750, 800, 850, 900, 950

where the control mortality was between 5 and 20 per cent, the observed percentage mortality was corrected using Abbott's formula[14]. Data from all replicates were pooled for analysis.

Separation of the mosquitocidal metabolite: Three batches of the 2 sets of media (NYSM + EMN and NYSM + NMN) were prepared on different days. The crude mosquitocidal metabolite (CMM) was precipitated from the culture supernatant obtained from the six media using 6N HCl and the precipitates were collected by centrifugation (9000 × *g* rpm for 25 min at 4°C)[10]. The CMM was re-suspended in water, adjusted to *p*H 7.0, lyophilized (Freeze dryer Modulyo Edwards, B.O.C. Ltd., Crawley, England) and tested against the pupae of *An. stephensi*, which was found to be the most susceptible among the three mosquito species studied[9].

Matrix assisted laser desorption ionization - Time of flight (MALDI-TOF): MALDI-TOF mass spectra were recorded by using a Bruker Daltonik Autoflex MALDI-TOF (Technische Universitaet Berlin, Berlin, Germany) instrument equipped with a 337-nm nitrogen laser for desorption and ionization. For mass spectrometric analysis, 2 µl portions of CMM obtained from the conventional medium was mixed with an equal volume of matrix medium [a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 70% aqueous acetonitrile) containing 0.1% trifluoroacetic acid (v/v)], spotted onto the target and air dried for measurement. Positive-ion detection and reflector mode were used. The acceleration and reflector voltages were 19.0 and 20 kV in pulsed ion extraction mode. A molecular mass gate of 350 kDa improved the measurements by filtering out most of the matrix ions.

Statistical analysis: Data obtained from the bioassay experiments were pooled and LC_{50} and LC_{90} values were calculated from a log dosage-probit mortality regression line using SPSS 10.0 (SPSS, India) yielding a level of effectiveness at 50 and 90 per cent mortality and 95 per cent confidence intervals. The LC_{50} values obtained with different media were compared using 95 per cent confidence interval. LC_{50} values with non overlapping confidence interval were considered to be significant at $P<0.05$.

Results

Effect of CS of different production media on An. stephensi: The CS of VCRC B471 grown in six different culture media were bioassayed against larvae and pupae of *An. stephensi* and results are presented in Tables III and IV. Among the combinations NYSM + EMN and NYSM + NMN, the metabolite produced in the latter medium was more effective. Pupicidal and larvicidal activity of the CS obtained from NYSM + EMN was 1.31 and 10.6 µl/ml whereas with NYSM + NMN it was 0.97 and 9.27 µl/ml. The replacement of EMN with NMN in NYSM showed significant difference in mosquitocidal activity ($P<0.05$).

With the further addition of 0.5 and 1.5 per cent glucose to both the above media (NYSM + EMN and

Table III. Efficacy of CS of *B. subtilis* grown in different media against the pupae of *An. stephensi*

Medium	LC_{50}	LCL	UCL	SE	LC_{90}	LCL	UCL	SE
A	1.31	1.253	1.36	0.022	1.97	1.77	2.2	0.06
B	0.95	0.91	1	0.024	1.59	1.4	1.77	0.056
C	1.01	0.961	1.1	0.03	1.67	1.52	1.88	0.054
D	0.97	0.926	1.02	0.04	1.5	1.4	1.61	0.05
E	0.71	0.67	0.77	0.04	1.23	1.13	1.35	0.05
F	0.75	0.7	0.8	0.034	1.27	1.2	1.38	0.043

Values are given as µl/ml; LCL, lower confidential level; UCL, upper confidential level; SE, Standard error

Table IV. Efficacy of CS of *B. subtilis* grown in different media against the larvae of *An. stephensi*

Medium	LC_{50}	LCL	UCL	SE	LC_{90}	LCL	UCL	SE
A	10.6	10.21	11	0.02	15	13.83	16.35	0.043
B	5.68	5.54	5.83	0.013	7.47	7.1	7.83	0.025
C	5.9	5.8	6	0.01	7.33	6.89	7.41	0.02
D	9.27	8.93	9.53	0.02	12.67	11.7	14	0.05
E	5.57	5.47	5.68	0.01	6.8	6.53	7.05	0.01
F	5.63	5.53	5.74	0.01	6.87	6.6	7.14	0.02

Values are given as µl/ml; LCL, lower confidential level; UCL, upper confidential level; SE, Standard error

NYSM + NMN), there was significant difference in the production of mosquitocidal metabolites ($P<0.05$), as evident from the LC_{50} dosage. However, there was no significant difference in the 95 per cent confidence interval between the media containing 1 and 2 per cent glucose indicating that higher levels of glucose did not have a significant impact on the production of the mosquitocidal metabolite.

Among the six media used, the combination NYSM + NMN + 1 per cent glucose was found to be optimal for the production of the metabolite. In this medium, the LC_{50} dose required for larva (5.57 µl/ml) and pupa (0.71 µl/ml) of *An. stephensi* was almost half of that required when the metabolite was produced in the original medium *i.e.*, NYSM + EMN (LC_{50} values 10.6 µl/ml and 1.3 µl/ml). However, the LC_{50} dose requirement of NYSM + NMN + 1 per cent glucose, for inciting 50 per cent mortality in the larvae (5.57 µl/ml) of *An. stephensi* was found to be 7.84 times higher than that required for the pupae of the same species (0.71 µl/ml).

Production of CMM in different culture media: It was observed that upto a concentration of 1 per cent glucose in the medium the production of mosquitocidal metabolites kept increasing. This trend was same in NYSM + EMN and NYSM + NMN. The yield of the metabolite in the media containing NYSM + EMN was 1.62 g/l compared to 2.05 g/l in the combination, NYSM + NMN. Among the different concentrations of glucose used, 1 per cent glucose gave the maximum yield of CMM, 2 g/l with the combination, NYSM + EMN and 2.48 g/l with the combination, NYSM + NMN. The LC_{50} dose of the CMM produced in the latter medium was found to be 2.2 g/l as against 2.6 g/l required for that produced in the same combination containing EMN (Fig. 1).

MALDI-TOF mass spectrometric analysis: MALDI-TOF spectrum of CMM showed well-resolved groups of peaks at *m/z* values between 1000 and 1080 (Fig. 2).

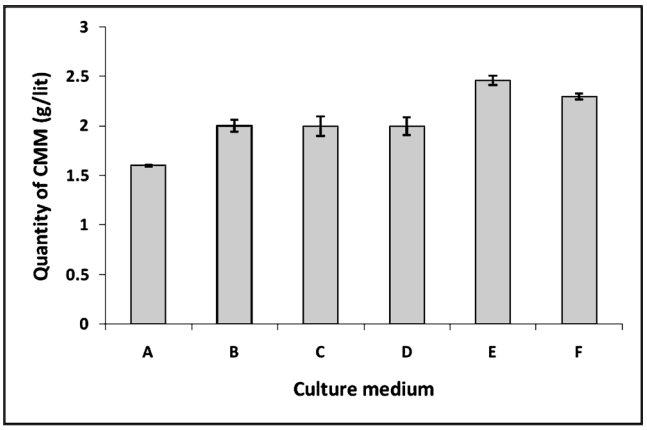

Fig. 1. Production of crude mosquitocidal metabolite (CMM) in different culture media by *B. subtilis* subsp. *subtilis.*

The mass peaks obtained were at *m/z* 1030.6, 1046.7, 1044.6, 1052.6, 1058.7, 1060.7, 1066.7 and 1074.8.

Discussion

Very few reports exist on the anti-mosquitocidal activity exhibited by strains of *B. subtilis*[15,16]. Unlike in the case of *B. thuringiensis* var. *israelensis* and *B. sphaericus,* where the spore crystal complex is known to be larvicidal, the cyclic lipopeptides (CLPs) present in the culture supernatant of *B. subtilis* have been reported to be responsible for mosquito larvicidal property[16]. The CLPs of our strain however, were found to be effective both on the larval as well as pupal stages of various species of mosquitoes[9].

Trace elements and carbon level in the culture medium have been reported to increase the lipopeptide production in strains of *B. subtilis*[12,17]. Using a statistical experimental design (Taguchi method), Wei *et al*[12] have optimized the trace element composition for surfactin production by *B. subtilis*. These trace elements (micronutrients) were incorporated into the production media (NYSM) used in this study. As observed by them, replacement of the new trace elements (NMN) was found to increase

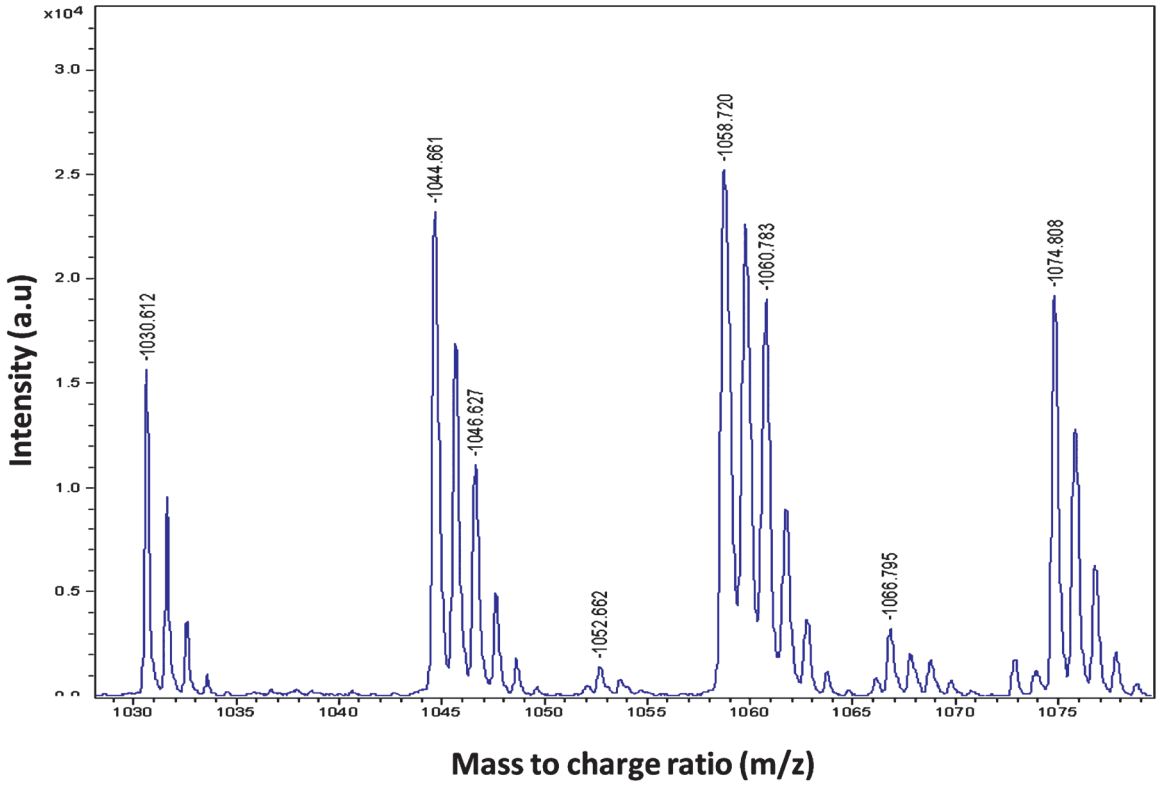

Fig. 2. MALDI-TOF spectrum of crude mosquitocidal metabolite (CMM) produced by *B. subtilis* subsp. *subtilis*.

the yield of the metabolite with subsequent increase in the mosquitocidal activity. Among various carbon substrates, glucose is reported to increase the yield of lipopeptide[17]. Hence, the conventional medium NYSM was supplemented with glucose at 1 and 2 per cent level. It was observed that metabolites produced in NYSM + NMN + 1 per cent glucose, showed highest mosquitocidal activity and highest metabolite production. Since the metabolites produced in the six culture media were not quantified and tested for mosquitocidal activity, the LC_{50} values are given in terms of µl/ml. In the next step, this needs to be done. The effect of glucose on surfactin production is rather indirect. Phosphotransacetylase (*pta*) is an enzyme required for the conversion of the end product acetyl coA to acetyl phosphate in the glycolysis pathway. The latter compound is a phospho donor responsible for activating the ComA protein required for the transcription of the *srf* operon which is essential for the production of surfactin by *B. subtilis* strains[18]. The expression of this *pta* enzyme was found to require high levels of glucose in the growth medium which might be the reason for the enhancement in the production of mosquitocidal metabolite in media containing high levels of glucose.

The purified mosquito pupicidal metabolite from *B. subtilis* subsp. *subtilis* was earlier identified as a cyclic lipopeptide, surfactin by IR, NMR and MALDI-TOF analysis[19]. This study also showed that the CMM when subjected to MALDI-TOF analysis exhibited peaks indicative of surfactin. The group of peaks could be attributed to the isoform ensembles of surfactins produced by the mosquitocidal strain. It is clear that the cyclic lipopeptide surfactin is the main component produced by our strain. Surfactin is one of the most powerful biosurfactants known for reducing the surface tension of water from 72 to 27 mN/m and reduction to levels 41-31 mN/m have been found to result in total pupal mortality[17,20,21]. Pupae of mosquitoes depend on atmospheric oxygen for respiration. Due to reduction in surface tension of water by surfactin, the pupae were unable to come up to the surface of water for oxygen and had to remain submerged in water, followed by death[22]. Hence, the mosquito pupal mortality observed in our study could be primarily by reduction in the surface tension of the water caused by the biosurfactant, surfactin. 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[23-25].

The larvicidal cyclic lipopeptides produced by *B. subtilis* is reported to be non-toxic to fishes and aquatic organisms[16]. Toxicological tests *viz.* acute oral toxicity/pathogenicity tests conducted in Wistar rats and primary skin irritation test conducted in rabbits using CMM showed that it is non-toxic and safe to mammals as there was no adverse effect on body weight, food consumption, rectal temperature, haematological and biochemical parameters. This part of the study was done by outsourcing at International Institute of Biotechnology and Toxicology (IIBAT), Padappai, Tamil Nadu, India (VCRC, unpublished data). This bacterium assumes great importance as the first ever reported spore forming bacterium possessing activity against the pupal stage, a non feeding stage in the life cycle of a mosquito. Further, unlike the toxins of the commonly used biolarvicides, *B. thuringiensis* subsp. *israelensis* and *B. sphaericus*, this mosquitocidal metabolite was found to withstand wide range of water temperatures, *p*H, exposure to long hours of sunlight, UV radiation as well as moist heat, upto 121^0C[26]. However, for use in operational control programmes it has to be made into a suitable formulation and currently studies are underway for designing an appropriate formulation as well as for developing a cheap production medium based on inexpensive and locally available raw materials.

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