J Vector Borne Dis 52, March 2015, pp. 58–62

Cost-effective medium for the production of mosquito pupicidal lipopeptide from *Bacillus subtilis* subsp. *subtilis* (VCRC B471)

S. Bhuvaneswari, A.M. Manonmani & I. Geetha

Unit of Microbiology and Molecular Biology, Vector Control Research Centre (ICMR), Puducherry, India

ABSTRACT

Background & objectives: A cyclic lipopeptide (CLP), surfactin produced by a strain of *Bacillus subtilis* subsp. *subtilis* (VCRC B471) was found to exhibit mosquitocidal activity. The present study was carried out to enhance the surfactin level using low cost material in the production medium.

Methods: Two carbon sources, glucose and common sugar, and two nitrogen sources, ammonium nitrate and soya were used in the study. Different concentrations of 'C' and 'N' sources were used in the production medium to enhance the production of surfactin.

Results: A new medium (SS7) containing 2% sugar, 6% soya and 0.5% common salt with micronutrients was designed which was found to enhance the production of surfactin. The crude mosquitocidal metabolite (CMM) produced in this medium was 3 g/l which was two times higher than that obtained using synthetic medium NYSM. The LC_{50} dosage of the CMM to the pupal stages of *An. stephensi* (2.3 µg/ml) was comparable to that obtained with CMM from the conventional medium.

Interpretation & conclusion: The newly designed cost-effective medium designated as sugar soya medium (SSM) enhanced the production of surfactin and the cost of production was estimated as ₹ 6 per litre, which is six times lesser than that of the conventional medium. Replacement of sodium chloride with cooking salt further reduced the cost of the medium.

Key words *Anopheles stephensi; Bacillus subtilis;* CMM; cyclic lipopeptide; soya; surfactin

INTRODUCTION

Mosquitoes being vectors for various parasites play an important role in the transmission of several life threatening diseases like malaria, filariasis, dengue, yellow fever, encephalitis, *etc*. The two widely used microbial pesticides are *Bacillus thuringiensis* and *B. sphaericus* which serve as mosquito larvicidal agents for mosquito control[1–2]. No bacterial control agent is known till date for pupal and adult stages of mosquito. Efforts in this line at our institute resulted in a strain of *B. subtilis* subsp. *subtilis* (VCRC B471) whose metabolite(s) were found to kill the larval, pupal and adult stages of mosquitoes[3–4]. The mosquitocidal cyclic lipopeptides (CLPs) were able to withstand environmental stresses such as high temperature, pH, *etc*. and proved safe to fishes and mammals[5–7]. The CLP produced by *B. subtilis* (VCRC B471) has been characterized as surfactin which is the most powerful biosurfactant known to lower the surface tension of water from 72 to 27 mN/m[8].

Presently, a synthetic medium, nutrient yeast salt medium (NYSM) is used for the production of CLPs. Pilot scale production of metabolites for use against mosquitoes require cost-effective culture medium so that the production cost is reduced. Glucose, sugar, soya and ammonium nitrate are reported to enhance the production of CLPs[9–13]. Hence, in the present study, varying concentrations of these ingredients were tried for designing a new culture medium for enhancing the production of CLP by our indigenous isolate, VCRC B471.

MATERIAL & METHODS

Microorganism

The strain, *B. subtilis* subsp. *subtilis* (VCRC B471) obtained from the culture collection of VCRC was maintained on 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 (Himedia, India) per litre of water] agar slants[14].

Growth conditions

Tubes containing 10 ml of NYSM broth were inoculated with a loopful of bacterial cells from a slant culture. The tubes were incubated overnight on a rotary shaker (New Brunswick Scientific Co. Inc., NJ, USA) at 28 ± 2°C and 250 rpm. The overnight culture was used to inoculate 50 ml of NYSM broth at 2% level and incubated

again for a further period of 7 h to synchronize the growth. From this young culture, 5% was added to flasks containing 100 ml of different media combinations (mentioned under section, Media preparation) and incubated for 72 h as mentioned above.

Media preparation

Selection of media components: Two carbon sources, glucose and common sugar; and two nitrogen sources, ammonium nitrate and soya were used for the study. Concentration of carbon source used was 1 and 2%, whereas concentration of nitrogen source was different for ammonium nitrate and soya. For ammonium nitrate, the concentration used was 0.3 and 0.4% and for soya it was 2 and 4%. Using these concentrations of carbon and nitrogen sources, 16 different combinations were designed and production media were prepared (Table 1). All the media were supplemented with 0.5% NaCl and micronutrients ($MgSO_4$–592 mg, KH_2PO_4–1.36 g, $MnSO_4$–2 mg, $FeSO_4$–2 mg, $CaCl_2$–1 mg) per litre of water (pH 7.0 ± 0.2)[7,15].

Optimization of sugar soya medium (SSM): Based on the results of the above experiment, sugar and soya were selected for further optimization. Table 2 shows different concentrations of sugar and soya tried in the medium. Micronutrients and salt concentrations were same as used earlier. All the media were inoculated at 5% level and the culture supernatant (CS) obtained from 72 h old culture was tested for mosquito pupicidal and larvicidal activity and the crude mosquitocidal metabolite (CMM) also quantified.

Replacement of sodium chloride with common salt: The medium which yielded the maximum CMM and pupicidal activity was selected and NaCl was replaced with common salt. CMM quantification and its pupicidal activity were determined.

Table 1. Media combinations used for production of mosquitocidal lipopeptide

S. No. of culture medium	Media combinations
1.	1% Glucose + 0.3% Ammonium nitrate
2.	2% Glucose + 0.3% Ammonium nitrate
3.	1% Glucose + 0.4% Ammonium nitrate
4.	2% Glucose + 0.4% Ammonium nitrate
5.	1% Sugar + 0.3% Ammonium nitrate
6.	2% Sugar + 0.3% Ammonium nitrate
7.	1% Sugar + 0.4% Ammonium nitrate
8.	2% Sugar + 0.4% Ammonium nitrate
9.	1% Glucose + 2% Soya
10.	2% Glucose + 2% Soya
11.	1% Glucose + 4% Soya
12.	2% Glucose + 4% Soya
13.	1% Sugar + 2% Soya
14.	2% Sugar + 2% Soya
15.	1% Sugar + 4% Soya
16.	2% Sugar + 4% Soya

Table 2. Optimization of sugar and soya concentrations for sugar soya medium (SSM)

S. No. of culture medium	Sugar (%)	Soya (%)
SS1	1	4
SS2	2	4
SS3	4	4
SS4	6	4
SS5	8	4
SS6	1	6
SS7	2	6
SS8	4	6
SS9	6	6
SS10	8	6
SS11	1	8
SS12	2	8
SS13	4	8
SS14	6	8
SS15	8	8

Bioassay

Bacterial cells were removed from the production medium by centrifugation at 8000× g for 20 min at 4°C in a Sorvall Evolution RC superspeed centrifuge (Kendro Lab. Products, Asheville, NC, USA) using SLA-1500 rotor. The CS obtained from different sets of media was used for bioassay following WHO standard protocol[16]. To a 200 ml capacity, disposable wax-coated paper cups, 100 ml of chlorine-free tap water was added and 25 larvae or freshly emerged pupae of *An. stephensi* were introduced. Each experiment was performed using four replicates per dose while having an equal number of controls. All experiments were conducted at 28 ± 2°C, 80–90% 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 larvae and pupae was scored after 24 h and corrected for control mortality by applying Abbott's formula[17]. The experiment was done thrice on different days. Data from all the replicates were pooled for analysis.

Separation of the crude mosquitocidal metabolite and bioassay

The CMM was precipitated from the culture supernatant obtained from various media using 6N HCl and the precipitates were collected by centrifugation (8000 rpm for 25 min at 4°C)[18]. The CMM was re-suspended in water, adjusted to pH 7.0, lyophilized (Freeze dryer

Modulyo Edwards, B.O.C. Ltd., Crawley, England) and used for bioassay against the pupal stages of *An. stephensi* as mentioned in the above paragraph.

Statistical analysis

Data obtained from the bioassay experiments were pooled and the observed percentage mortality was corrected by applying Abbott's formula. LC_{50} and LC_{90} values were calculated from a log dosage-probit mortality regression line using SPSS 19.0 for windows (IBM Corp; USA) yielding a level of effectiveness at 50 and 90% mortality and 95% confidence intervals. The LC_{50} values obtained with different media were compared using 95% confidence interval. LC_{50} values with non-overlapping confidence interval were considered to be significant at $p < 0.05$.

RESULTS

Screening of media components

The CS of VCRC B471 grown in four sets of four different culture media (listed in Table 1) containing glucose + ammonium nitrate, sugar + ammonium nitrate, glucose + soya and sugar + soya were bioassayed against pupae of *An. stephensi* and the results are presented in Table 3. Among the carbon sources used, sugar was found to enhance the metabolite production as seen by the LC_{50} values. The CS from medium containing soya exhibited higher pupicidal activity than the medium containing ammonium nitrate. Increase in the carbon source from 1 to 2% in all sets of media enhanced the production of pupicidal metabolite as evident from the LC_{50} dosage and there was a significant difference ($p < 0.05$) in the activity in media containing 1 and 2% carbon source. Increasing the soya concentration from 2 to 4% level and sugar from 1 to 2% level further increased the pupicidal activity with $p < 0.05$. Maximum pupicidal activity was exhibited by the metabolites produced in the media containing 2% sugar + 4% soya. The LC_{50} dose was 1.4 µl/ml which is nearly equal to that obtained with the synthetic NYSM (1.3 µl/ml) medium. Hence, sugar was selected as the carbon source and soya as the nitrogen source and used for further optimization experiments.

Optimization of sugar soya medium (SSM)

A total of 15 different media were used for optimizing the concentration of soya and sugar in the production medium (Table 2). When the pupicidal activity of the CS obtained from different media were compared, highest activity was observed with SS13 (LC_{50} dose 0.6 µl/ml) followed by SS8 (LC_{50} dose 0.75 µl/ml) and SS7 (LC_{50} dose 0.85 µl/ml) (Fig. 1). When the larvicidal activity of the metabolite from different media were compared, highest activity was observed with SS14 (LC_{50} dose 1.3 µl/ml) followed by SS13 (LC_{50} dose 1.6 µl/ml) and SS9 (LC_{50} dose 1.8 µl/ml) (Fig. 1).

Table 3. Efficacy of CS of *B. subtilis* grown in different media combinations against pupal stages of *An. stephensi*

S.No. of culture medium	LC_{50} dose	LCL	UCL	SE
1.	4.45	4.3	4.6	0.14
2.	4	3.89	4.21	0.14
3.	4.22	4.08	4.37	0.11
4.	3.59	3.44	3.74	0.14
5.	4.12	3.79	4.45	0.09
6.	3.52	3.37	3.67	0.11
7.	3.8	3.5	4.1	0.09
8.	3	2.9	3.24	0.1
9.	3.77	3.69	3.86	0.18
10.	3.46	3.37	3.55	0.16
11.	2.27	2.19	2.36	0.12
12.	1.7	1.6	1.8	0.09
13.	3.28	3.1	3.37	0.16
14.	2.93	2.6	3.25	0.15
15.	1.96	1.65	2.27	0.1
16.	1.38	1.08	1.52	0.09

Values are given as µl/ml; LCL — Lower confidential level; UCL — Upper confidential level; SE — Standard error.

VCRC B471 was found to exhibit higher activity on the pupal stages of mosquito rather than larval stages. As our focus was designing a cost-effective medium for enhancing the pupicidal activity, the medium SS7 (sugar 2% and soya 6%) was selected. In this medium, the LC_{50} dose requirement for larval and pupal stages were 3.4 and 0.85 µl/ml as compared to 10.6 and 1.3 µl/ml required with the metabolites produced in NYSM. And, the LC_{50}

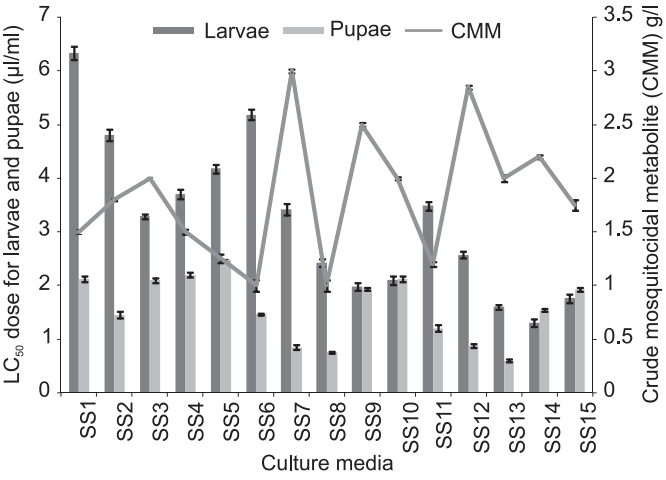

Fig. 1: LC_{50} dosages and quantity of CMM produced in different media combinations.

dose required for inciting 50% mortality in the larval stages of *An. stephensi* was found to be four times higher than that required for the pupal stages of the same species.

CMM production and bioassay

The CMM production in the 15 media combinations are given in Fig. 1. The highest production of the CMM, 3 g/l was achieved with medium SS7. This was a two fold increase when compared to the quantity of CMM obtained in the synthetic NYSM medium. The LC_{50} dosage of the CMM obtained from the medium SS7 to the pupae of *An. stephensi* (2.4 µg/ml) was comparable to that obtained in NYSM.

Replacement of sodium chloride with common salt

The medium SS7 was taken up for this study wherein NaCl was replaced with common salt. In this medium, the CMM quantity was 3 g/l and its LC_{50} dosage for pupae of *An. stephensi* was 2.3 µg/ml, which was comparable to that obtained from the control medium (with NaCl). The results indicated that the replacement of sodium chloride with common salt did not affect the pupicidal activity and CMM quantity.

Cost analysis

The mosquitocidal CLP from the strain VCRC B471 is presently produced in a synthetic NYSM medium which cost ₹ 36 per litre. The cost of the medium SS7 used in the present study worked out to ₹ 6 per litre. Hence, the cost of production of the mosquitocidal metabolite was brought down six times when compared to NYSM.

DISCUSSION

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 present in the culture supernatant of *B. subtilis* have been reported to be responsible for mosquito larvicidal property[5]. The CLPs of our strain *B. subtilis* subsp. *subtilis* (VCRC B471) was found to be effective both on the larval as well as pupal stages of various species of mosquitoes[3]. However, large scale production of the lipopeptide was hindered due to low yields and high production costs[19–20]. Hence, enhancing the lipopeptide production was attempted in this study using previously reported low cost raw materials. Initially, selection of carbon and nitrogen source was done which made us to arrive at sugar and soya respectively. As per USDA (United States Department of Agriculture) nutrient data base, soya contains 36.49% of proteins (essential amino acids) and 30.16% of carbohydrates (starch and sugar). Though, soya contained carbohydrates, it was not found to be sufficient for lipopeptide production (VCRC unpublished data). Also high level of carbon source is known to play a vital role in increasing the production of surfactin[21]. Therefore, addition of carbon source to the soya was found to be essential. Among the various carbon and nitrogen sources reported for enhancing the production of surfactin in *B. subtilis*, common sugar and soya occupy top priority[10–13]. Hence, these ingredients were tried in the present study. Increasing the sugar concentration to 4% level was found to enhance the mosquitocidal activity. This is in agreement with the findings of Cooper *et al*[9] and Wei *et al*[15] who found 4% glucose in mineral salts medium to result in a good yield of this compound. Trace elements and carbon levels are known to play a crucial role in the production of lipopeptides[9, 22]. Hence, trace elements known to increase surfactin production were used to enhance the mosquitocidal lipopeptide production.

The medium SS7 containing 2% sugar and 6% soya yielded highest metabolite production (3 g/l) with an LC_{50} value of 0.85 µl/ml for the pupal stages. Though, the medium SS13 (4% sugar and 8% soya) and SS8 (4% sugar and 6% soya) showed higher pupicidal activity than the medium SS7, the LC_{50} dosage was not found to be significant. Moreover, CMM production in these two media was also found to be low. Additionally, medium SS7 is cost-effective when compared to media SS13 and SS8 respectively. Hence, medium SS7 was selected as the optimum medium for production of lipopeptides from *B. subtilis* (VCRC B471). Our results corroborate with Gu *et al*[23] who have reported 2.2% sugar to be optimal for lipopeptide production. Increasing the concentration of sugar further did not enhance the metabolite production. This may be due to the production of acidic compounds in the medium with excess of carbon source which leads to lowering of the pH followed by decrease in growth and metabolite production[24].

Further, studies are underway for designing appropriate formulations for use against the immature stages of mosquitoes in operational programmes.

CONCLUSION

Optimization of media ingredients, sugar and soya for the production of mosquitocidal lipopeptides by *Bacillus subtilis* subsp. s*ubtilis* (VCRC B471) resulted in a newly designed sugar soya medium (SSM). Replacement of sodium chloride with cooking salt reduced the cost of the medium. The medium SS7 containing sugar 2%, soya

6%, cooking salt 0.5% and micronutrients not only enhanced the production of CLP but also lowered the cost of the medium (₹ 6 per litre) by six times.

ACKNOWLEDGEMENTS

We are grateful to Dr P. Jambulingam, Director, Vector Control Research Centre for the support and the facilities provided. This study was supported by a research grant from the Department of Science and Technology (DST), New Delhi. The Senior Research Fellowship provided by DST to the first author (SB) is gratefully acknowledged.

REFERENCES

1. Balaraman K. Mosquito control potential of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. *ICMR Bull* 1995; *25:* 45–51.
2. Armengol G, Hernandez J, Velez JG, Orduz S. Long-lasting effects of a *Bacillus thuringiensis* serovar *israelensis* experimental tablet formulation for *Aedes aegypti* (Diptera*:* Culicidae) control. *J Econ Entomol* 2006; *99:* 1590–5.
3. Geetha I, Prabakaran G, Paily KP, Manonmani AM, Balaraman K. Characterisation of three mosquitocidal *Bacillus* strains isolated from mangrove forest. *Biol Control* 2007; *42:* 34–40.
4. Geetha I, Paily KP, Manonmani AM. Mosquito adulticidal activity of a biosurfactant produced by *Bacillus subtilis* subsp. *subtilis*. *Pest Manag Sci* 2012; *68:* 1447–50.
5. Das K, Mukherjee AK. Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. *Acta Trop* 2006; *97:* 168–73.
6. Geetha I, Manonmani AM. Surfactin: a novel mosquitocidal biosurfactant produced by *Bacillus subtilis* sub sp *subtilis* (VCRC B471) and influence of abiotic factors on its pupicidal efficacy. *Lett Appl Microbiol* 2010; *51:* 406–12.
7. Manonmani AM, Geetha I, Bhuvaneswari S. Enhanced production of mosquitocidal cyclic lipopeptide from *Bacillus subtilis* subsp. *subtilis*. *Indian J Med Res* 2011; *134:* 476–82.
8. Geetha I, Manonmani AM, Paily KP. Identification and characterization of a mosquito pupicidal metabolite of a *Bacillus subtilis* subsp. *subtilis* strain. *Appl Microbiol Biotechnol* 2010; *86:* 1737–44.
9. Cooper DG, Macdonald CR, Duff SJ, Kosaric N. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl Environ Microbiol* 1981; *42:* 408–12.
10. Reis FASL, Servulo EFC, De Franca FP. Lipopeptide surfactant production by *Bacillus subtilis* grown on low-cost raw materials. *Appl Biochem Biotechnol* 2004; *115:* 899–912.
11. Fonseca RR, Silva AJR, De Franca FP, Cardoso VL, Servulo EFC. Optimizing carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain. *Appl Biochem Biotechnol* 2007; *137–140:* 471–86.
12. Ohno A, Ano T, Shoda M. Production of a lipopeptide antibiotic, surfactin, by recombinant *Bacillus subtilis* in solid-state fermentation. *Biotechnol Bioeng* 1995; *47:* 209–14.
13. Yoneda T, Miyota Y, Furuya K, Tsuzuki T. Production process of surfactin, Tokyo, Japan. US patent 2006; No. 7,011,969. Available from: http://www.patentbuddy.com/potent/7011969
14. Myers PS, Yousten AA. Localization of a mosquito-larval toxin of *Bacillus sphaericus* 1593. *Appl Environ Microbiol* 1980; *39:* 1205–11.
15. Wei YH, Lai CC, Chang JS. Using taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. *Process Biochem* 2007; *42:* 40–5.
16. *Guidelines for laboratory and field testing of mosquito larvicides.* Geneva: World Health Organization 2005. WHO/CDS/WHOPES/GCDPP/2005.13.
17. Abbott WS. A method for computing the effectiveness of an insecticide. *J Econ Entomol* 1925; *27:* 265–7.
18. Geetha I, Manonmani AM. Mosquito pupicidal toxin production by *Bacillus subtilis* subsp. *subtilis*. *Biol Control* 2008; *44:* 242–7.
19. Davis DA, Lynch HC, Varley J. The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. *Enzy Microbiol Technol* 1999; *25:* 322–9.
20. Mukherjee S, Das P, Sen R. Towards commercial production of microbial surfactants. *Trends Biotechnol* 2006; *24:* 509–15.
21. Kim S, Shin BS, Choi SK, Kim CK, Park SH. Involvement of acetyl phosphate in the *in vivo* activation of the response regulator ComA in *Bacillus subtilis*. *FEMS Microbiol Lett* 2001; *195:* 179–83.
22. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev* 1997; *61:* 47–64.
23. Gu XB, Zeng ZM, Yu HQ, Wang J, Liang FL, Liu RL. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. *Process Biochem* 2005; *40:* 3196–201.
24. *Guidelines for production of Bacillus thuringiensis H-14*. UNDP/WORLD BANK/WHO special programme for research and training in tropical diseases. Geneva: World Health Organization 1982; p. 98–102.

Correspondence to: Dr A.M. Manonmani, Unit of Microbiology and Molecular Biology, Vector Control Research Centre (ICMR), Indira Nagar, Puducherry–605 006, India.
E-mail: ammanonmani@yahoo.com

Received: 29 October 2014 *Accepted in revised form:* 16 January 2015