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Di-rhamnolipid is a mosquito pupicidal metabolite from *Pseudomonas fluorescens* (VCRC B426)

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ABSTRACT

Pseudomonas fluorescens Migula (VCRC B426) produces a secondary metabolite, which was found to be active against pupae of vector mosquitoes namely *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. The mosquito pupicidal metabolite from *P. fluoescens* was mass produced and separated by ethyl acetate extraction and purified further by silica gel column chromatography, FPLC, HPLC and TLC. The purified metabolite was characterized by NMR, FT-IR, LC–MS and MALDI-TOF. The FT-IR, ^{1}H and ^{13}C NMR results showed that it is a rhamnolipid (di-rhamnolipid). The matrix assisted laser desorption and ionization-time-of-flight spectrum of the sample showed predominant pupicidal component produced by *P. fluorescens* was the molecule mass of 673.40 Da. Owing to its high toxicity to mosquito pupae, especially *Anopheles* sp., and *Aedes* sp., the di-rhamnolipd has potential in the control of the vectors of dengue, chikungunya, yellow fever and malaria. This is the first report of mosquito pupicidal di-rhamnolipid from *P. fluorescens*.

1. Introduction

Q2 Mosquito control programs have been mainly dependent mainly upon chemical insecticides for several decades. Development of resistance to insecticides and environmental concerns limit the use of these insecticides and hence the trend is being shifted gradually to an integrated approach incorporating all possible alternate tools (Brattsten et al., 1986). The biological control potential of many natural pathogens, parasites and predators of mosquitoes is being investigated for several decades. Among several biocontrol agents, extensive studies have been carried out on two potential bacterial candidates, namely *Bacillus thuringiensis* var. *israelensis* and *Lysinibacillus sphaericus*. Although these biocides are being used extensively for mosquito control, the recent reports of development of resistance in Wirth et al. (2005) prompted many workers to search for new microbial agents or their metabolites for mosquito control. It has resulted in the identification of insecticidal activity of metabolites of a wide variety of bacteria, including *Pseudomonas fluorescens* (Murty et al., 1994; Prabakaran et al., 2003), *Pseudomonas pseudomallei* (Lee and Seleena, 1990) and *Pseudomonas aeruginosa* (Chadde, 1992; De Barjac, 1989). Our search for new microbial agents has resulted in the isolation and identification of a strain of *P. fluorescens* Migula (VCRC B426), which was found to be active against different stages of mosquitoes, such as *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Prabakaran et al., 2003). The isolate is more active against pupal stage and is the first report of a di-rhamnolipid showing mosquito pupicide. The secondary metabolite or exotoxin produced by the *P. fluorescens* was found to be a biosurfactant. This paper presents the purification and characterization of the mosquito pupicidal metabolite isolated from the *P. fluorescens*.

2. Materials and methods

2.1. Organism and materials

P. fluorescens Migula (VCRC B426), obtained from the culture collection of Vector Control Research Center was used in the present study. The strain was maintained on the Nutrient Yeast Salt Medium (NYSM) (Myers and Yousten, 1980). The composition of the medium was as follows: 5.0 g L^{-1} glucose, 5.0 g L^{-1} peptone, 5.0 g L^{-1} sodium chloride, 3.0 g L^{-1} beef extract, 0.5 g L^{-1} yeast extract, 0.203 g L^{-1} $MgCl_2$, 0.102 g L^{-1} $CaCl_2$, and 0.01 g L^{-1} $MnCl_2$

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dissolved in distilled water and adjusted the pH to 7.0. All the ingredients were purchased from Hi-media Chemicals, India.

2.2. Seed culture preparation

A loopful from the slant culture of the bacterium was inoculated into 10 mL NYSM broth in tube incubated on a rotary shaker at 30 °C, 250 rpm for a period of 6 h. The first seed was prepared by transferring 1 mL of the seed culture to 50 mL of NYSM medium in a 250 mL conical flask and incubated on a rotary shaker at 30 °C, 250 rpm for a period of 6 h. Second stage seed was prepared by inoculating 2% v/v of first stage seed into 600 mL of NYSM medium in a 2 L Erlenmeyer flask and incubating the flask on a rotary shaker at 30 °C, 250 rpm for a period of 6 h.

2.3. Production of pupicidal metabolite

Actively growing second stage seed culture was used to inoculate a bioreactor (100 L capacity, Bioengineering, Wald, Switzerland) at 2% v/v. The optical density (OD) of the inocula was 0.5 measured at 600 nm. The bioreactor used in this study had a working volume of 60 L and was set to control the fermentation variables automatically. Glucose 1%, peptone 1% and potassium dihydrogen phosphate 0.01 M were prepared, pH adjusted to 7.0 and sterilized *in situ* and the fermentation was started in batch mode with the following conditions: temperature 30 °C, stirrer speed 150 rpm, pH controller was connected only with 2 N NaOH and the pH was never allowed to drop below 7.0. However, rise in pH above 7.0 was not controlled. Dissolved oxygen (DO) was maintained between 20% and 40% saturation by controlling the air flow that was set at 1 L/min. Groundnut oil was used as an antifoam agent. Fermentation was terminated after the completion of pupicidal metabolite production as assessed by bioassay, usually after 24–48 h. After the completion of fermentation the culture broth was separated by continuous centrifugation (CEPA continuous centrifuge, Germany) at 20,000 rpm. Further purification of the mosquito pupicidal compound was done by acid precipitation, solvent extraction and silica gel column chromatography method. Pupicidal metabolite yield was calculated by lyophilizing the culture supernatant and the yields are given in g L^{-1}. Pupicidal activity during different stages of purification was measured by bioassay.

2.4. Extraction of pupicidal metabolite

2.4.1. Acid precipitation

1 L of cell free supernatant obtained after centrifugation was acidified with 6 N HCl to pH 2.0 and held overnight at 4 °C to enhance the precipitation of biosurfactant. The resulting precipitate was separated by centrifugation at 15,000 × *g*, 30 min, 4 °C. The pellet obtained was dried by lyophilization to calculate the metabolite yield and the activity was done by bioassay method.

2.4.2. Solvent extraction

The dried powder obtained by acid precipitation was used for the solvent extraction. The dried powder was extracted three times with equal volume of ethyl acetate. The mixture was continuously shaken at 250 rpm and 30 °C for 2 h on a rotary shaker. Then the aqueous layer was discarded and the solvent layer was transferred to a glass beaker and allowed to evaporate on a water bath. After evaporation the left over viscous brown colored residue is the partially purified crude metabolite.

2.5. Surface tension measurement

Surface tension of the metabolite was determined with a Kruss Easy Dyne Tensiometer K-20 model, Kruss, Germany, by Wilhelmy plate method. 10 μg of crude metabolite was dissolved in 100 mL of water and the surface activity was measured. All the assays were performed in triplicate along with distilled water as a control.

2.6. Fast performance liquid chromatography (FPLC) using silica gel column

Purification of the crude biosurfactant was carried out by column chromatography using Silica gel 60 (200–425 mesh, 0.035–0.075 mm) prepared in chloroform. Crude biosurfactant (1 g) was dissolved in 2 mL chloroform and injected into the column. The purification was carried out using chloroform to elute neutral lipids, followed by chloroform:methanol 50:5 (to elute monorhamnolipid fraction) and finally chloroform:methanol 50:50 (to elute di-rhamnolipid fraction). In each elution step 10 mL fractions were collected and the pupicidal activity was tested against pupae of *A. stephensi*. The fraction showing 100% pupicidal activity was pooled and evaporated to dryness for further analysis by thin layer chromatography (TLC), FT-IR, NMR, HPLC, MALDI-TOF and LC–MS.

2.7. Structural characterization

Structure of the most abundant compound in the mixture was preliminarily evaluated by TLC in comparison with the known glycolipids. This was followed by Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectral analysis which confirmed detailed structure of di-rhamnolipid 1. Further structural confirmation of di-rhamnolipid 1 was derived by mass spectroscopy.

2.7.1. Thin layer chromatography (TLC)

The chromatograms were developed with chloroform/methanol/acetic acid (5:1:0.16, v/v/v) and visualized by TLC reagents, *i.e.* iodine vapors for lipid staining and α-naphthol–H_2SO_4 (5 drops of H_2SO_4 were added using a Pasture pipette to a solution of 10% α-naphthol in 95% ethanol, Molish reagent) for sugar detection. By using TLC we compared purified rhamnolipids with the standard rhamnolipids which is a mixture of mono- and di-rhamnolipids. Mono-rhamnolipids and di-rhamnolipids separate into two bands on TLC. The spot nearer to the point of origin corresponds to the di-rhamnolipids, while the spot further from the point of origin are due to the mono-rhamnolipids. Mono-rhamnolipids display higher retention factor (R_f) value compared to di-ramhnolipids.

2.7.2. Fourier transform infrared spectroscopy (FT-IR)

One milligram of the sample was taken in a mortar and to this 10 mg of KBr was added. Then the mixture was ground in a mortar with a pestle. The mixture was loaded in a dye and pressed in a hydraulic press. This resulted in a KBr solid solution pellet. The pellet was subjected to IR spectrum analysis in a Nicolet-6700 spectrometer. The spectrum was generated at a resolution of 0.1 cm^{-1}, 32 scans in the range of 4000–700 cm^{-1}.

2.7.3. Nuclear magnetic resonance spectroscopy (NMR)

For NMR the sample was prepared by dissolving 5 mg of purified rhamnolipid in 0.4 mL of $CDCl_3$. ^1H (400 MHz), ^{13}C and DEPT-135 NMR (100 MHz) spectra were recorded for $CDCl_3$ solution on an Avance Bruker – 400 MHz spectrometer with tetramethyl silane (TMS) as the internal standard.

2.7.4. High performance liquid chromatography (HPLC)

The purified fraction obtained after column chromatography showing pupicidal activity was analyzed by using a high performance liquid chromatography (HPLC). Column used Reverse Phase Column, Phenomenex Luna® 5 μm C18, 100 Å, LC Column

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