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Mosquitocidal efficacy of lecithinase derived from entomopathogenic bacteria *Xenorhabdus* sp. strain PBU1755 against filarial vector *Culex quinquefasciatus*

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ABSTRACT

The present study describes the mosquitocidal efficacy of partially purified lecithinase enzyme against filarial vector *Culex quinquefasciatus*. Entomopathogenic bacteria *Xenorhabdus* sp. PBU1755 was isolated from entomopathogenic nematode *Steinernema* sp. and identified using 16S rRNA gene sequencing. The ability of lecithinase enzyme production was screened using egg yolk emulsion agar media. The enzyme production was carried out using shake flask method and the activity was optimized with different temperature, and pH. The produced lecithinase enzyme was partially purified using trichloroacetic acid (TCA) precipitation method followed by dialysis. The molecular weight of the partially purified lecithinase enzyme was determined to be ~ 70 kDa by SDS-PAGE analysis and further partially purified lecithinase enzyme activity was evaluated using zymogram assay. In addition, mosquitocidal activity of partially purified enzyme was determined against *Culex quinquefasciatus*. Partially purified lecithinase enzyme showed significant mosquitocidal killing efficacy and their LC_{50} value was estimated to be 6.77 mg. Collectively our finding suggests that the entomopathogenic bacterial enzymatic product might be used as a potential mosquitocidal agent in pest control industries.

1. Introduction

Arthropod borne diseases are one of the major health concerns in many countries. *Culex quinquefasciatus* (southern house mosquito) are domestic pests of many urban places, and belongs to the family of Culicidae. These mosquito larvae are found in stagnant sewage water, ditches, and drainages. They may not be a key vector for widespread mosquito-borne diseases like malaria, dengue, chikungunya and yellow fever, but they are vectors of some diseases which cause serious health problems like Eastern Equine Encephalitis, St. Louis Encephalitis, Western Equine Encephalitis, and West Nile viral fever to humans, birds and animals (Grech et al., 2013; Medeiros et al., 2017; Bhattacharya et al., 2016). *Culex quinquefasciatus* may be act as a vector of the Zika virus (Schmidt, 2016).

Lymphatic filariasis is a human disease caused by parasitic worms and it develops a symptom called elephantiasis. When the mosquito bites human, microfilaria migrate into the lymphatic vessels and develop into adult worms which cause damage in the lymphatic system, legs, arms and genitals. In order to control these mosquitoes, people depend on the use of chemical insecticide such as DDT (Dichlorodiphenyltrichloroethane), malathion and pyrethroids, but extensive and prolonged use of these chemical insecticides often resistance to such mosquitoes (Brouqui et al., 2012; Osta et al., 2012; Al-Sarar, 2010) and causes many hazardous health problems in human and other living beings (Ibarra et al., 2003; Azmi et al., 2009). Thus an alternative bio-control measure is necessary for the development of new practices.

Xenorhabdus is a genus of gram-negative bacteria from the family of *Enterobacteriaceae,* which is associated with the entomopathogenic nematode of genera *Steinernema* (Chaston et al., 2011). All strains of *Xenorhabdus* sp. produce two physiological forms on nutrient plate such as primary (phase-I) and secondary (phase-II) form (Boemare and Akhurst, 1988). Phase-I form is associated with the infective stages of nematodes are capable of producing an array of endo/exoenzymes (lipases, proteases, and lecithinase) and bioactive compounds with antimicrobial, antiparasitic, cytotoxic and insecticidal properties that stimulate macro-molecular degradation (Volgyi et al., 1998; Boemare et al., 1997). Secondary forms also produce these exo-enzymes but the biomass production was very less compared to primary form.

The main objective of the present study is principally focused on the production of lecithinase enzyme from isolated *Xenorhabdus* sp. We have demonstrated the isolation, characterization, production and

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partial purification of lecithinase enzyme from *Xenorhabdus* sp. strain PBU1755. In addition, hemolytic and mosquitocidal activity of partially purified lecithinase enzyme was performed.

2. Materials and methods

2.1. Materials

Lecithin, MacConkey agar and NBTA (nutrient bromothymol blue-triphenyltetrazolium chloride agar media) were purchased from Sigma Aldrich, and all other chemicals and reagents used in this study were of analytical grade. *C. quinquefasciatus* larvae were collected from National Center for Disease Control (NCDC), Mattupalayam, Tamilnadu, India

2.2. Isolation of entomopathogenic nematode

Agricultural soil was collected aseptically in thudiyalur (11°03'36.9"N 76°56'56.0"E), Coimbatore, India in radiation-sterilized polyethene zipper bag. The samples collected were from 12 cm depth of agricultural land. The entomopathogenic nematodes were recovered from the collected soil sample using insect baiting method as described by Orozco et al. (2014). *Corcyra cephalonica* was used as baiting organism, which was maintained in the laboratory conditions using artificial diet (Bernardi et al., 2000).

2.3. Isolation of entomopathogenic bacteria Xenorhabdus sp

Bacteria *Xenorhabdus* sp. was isolated from the hemolymph of *C. cephalonica* infected entomopathogenic nematodes as previously described by Fukruksa et al. (2017). Primary (phase-I) and secondary (phase-II) forms of the isolated bacteria were identified based on the utilization of bromothymol blue from NBTA indicator medium and neutral red dye from MacConkey agar medium (Boemare and Akhurst, 1988). Isolated bacteria were identified based on the morphology, biochemical and molecular characterization techniques.

2.4. Molecular identification of Xenorhabdus sp

For the molecular identification, DNA was extracted from the isolated bacteria as described by Cheng and Jiang (2006) method. The extracted DNA was amplified using PCR (Eppendrof, India) with genus-specific primer. Primers used for amplifying 16 S rRNA of *Xenorhabdus* sp. were F–5′GATGGAGGGGGATAACCACT3′ R-3′TTGTCCAGGGGGCC GCCT5′. About 50 µL of PCR reaction was carried out, which consist of 25 µL of PCR master mix (Fermentas, USA), 21 µL of nuclease free water, 1 µL each of forward, 1 µL of reverse primers, and 2 µL of template DNA. The thermal cycle programmed as follows 94 °C for 2 min for initial denaturation followed by 25 cycles of final denaturation 94 °C for 15 s, 53 °C for 30 s annealing, 72 °C for 45 s extension and 72 °C for 5 min final extension. After amplification, PCR products were examined and purified using the Gel/PCR DNA extraction kit and purified PCR product was sequenced by an automated DNA sequencer at Chromous Pvt Ltd., Bangalore, India.

2.5. Lecithinase activity of Xenorhabdus sp

The primary and secondary forms of *Xenorhabdus* sp. were screened for the production of lecithinase enzyme. A loopful of pure primary and secondary form of isolated bacterial colonies were spot inoculated on nutrient agar plate media supplemented with egg yolk emulsion (as a source of lecithin) and incubated for 48 h. After incubation, the plates were examined for the formation of opaque precipitation around the inoculated colonies (Thaler et al., 1998). Lecithinase activity was also determined by lecithovitellin or nagler's reaction by inoculating both forms of the bacterial isolates in nutrient broth supplemented with egg yolk emulsion and incubated at 28 °C for 48 h. After that, the flasks were examined for the formation of floating precipitate on the surface of broth media. The higher lecithinase producing form (primary and secondary) of bacteria was chosen for further studies.

2.6. Production and partial purification of lecithinase enzyme

The higher enzyme producing form of *Xenorhabdus* sp. were inoculated in a 500 mL conical flask containing 150 mL of egg yolk emulsion nutrient broth and incubated at 28 °C for 6 days. After incubation, cell-free supernatant was collected by centrifugation at 10,000 rpm for 30 min and precipitated using 12% (w/v) trichloroacetic acid (TCA). After precipitation process, the crude enzyme was subjected to DEAE cellulose column and the fractions were collected at constant flow rate using bio-rad fraction collector, and the amount of protein concentration were quantified using spectrophotometer (O.D. at 280 nm) (Grimsley and Pace, 2003). Each fraction was dialyzed separately against 1 mM Tris HCl buffer to remove salt from the enzyme samples.

The protein profile and molecular weight of partially purified lecithinase enzyme was determined by 12% Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). It was performed as described by Borkar et al. (2009).

2.7. Zymogram assay

Detection of enzyme and their activity was done by Zymogram assay (Castelli et al., 2008). The partially purified sample was run in 7.5% Native PAGE gel without the addition of SDS and the gel was washed twice for 5 min in buffer containing 10 mM Tris-HCl (pH 8.0); 0.9% (w/v) NaCl and subsequently overlaid with 5.0 mL of 5% egg yolk suspension in 1% (w/v) agarose dissolved in buffer containing 100 mM Tris-HCl pH 8.0% and 0.9% (w/v) NaCl. The gel was incubated at 37 °C to visualize a white precipitate band for the confirmation of lecithinase activity.

2.8. Optimization of lecithinase activity

The activity of partially purified lecithinase enzyme was optimized with different pH (4 − 10) and temperature (10–60 °C). All this optimization studies were done using lecithin as a substrate. Control tubes were maintained without addition of enzyme solution. All experiments were carried out in triplicates.

2.9. Agar diffusion assay

The lecithinase enzyme producing capability of the purified enzyme fractions were tested using agar diffusion assay. About 10 µL of each fraction was loaded onto the 1% (w/v) agar gel containing 0.01% (w/v) lecithin, 0.1 M NaCl, and 0.02 M Tris-HCl and incubated for 24 h. After incubation, the plates were examined for the formation of opaque precipitation zone. The higher lecithinase activity was determined by measuring the radius (mm) of the precipitation zone in each well.

2.10. Hemolytic assay

The hemolytic activity of the lecithinase enzyme was performed using blood agar (5% blood in 2% agar). About 10 µL of purified fractions were loaded on to the 5 mm wells and incubated for 24 h. After incubation, the plates were examined for the formation of clear zone (Wang et al., 2010).

2.11. Mosquitocidal activity

Mosquitocidal activity of the partially purified lecithinase enzyme was checked against larvae *C. quinquefasciatus*. Different concentration

of lecithinase enzyme (2–10 mg/mL) was added into the sterile water containing 25 healthy third instar (stage) larvae of *C. quinquefasciatus* and incubated for 48 h at 27 °C. Lethal concentration (LC_{50}) of the lecithinase activity against *C. quinquefasciatus* was calculated.

2.12. Statistical analysis

All data in triplicates were analyzed using statistical package for the social sciences (SPSS version 14, IBM Corporation, USA) software by one way ANOVA. Linear regression analysis was performed for all dose-response experimental data, and LC_{50} was calculated using probit analysis. Results with $p \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. Isolation of entomopathogenic nematodes

The entomopathogenic nematodes were recovered by soil baiting method using *C. cephalonica*. After 48 h of the baiting action, the soil sample was examined for the presence of cadavers and it was observed that the *C. cephalonica* infected entomopathogenic nematodes were found to be brown in color. The infected larvae were secreted infective juveniles (Fig. 1). Fig. 2 shows the microscopic image of the recovered nematode from soil. Collected larvae were surface sterilized and used for the isolation of bacteria *Xenorhabdus* sp.

3.2. Isolation and morphological characterization

Bacteria *Xenorhabdus* sp. was isolated from hemolymph of entomopathogenic nematodes infected *C. cephalonica*. Microscopic and morphological observation showed that the bacterial colonies of *Xenorhabdus* sp. are circular, convex, umbonate. Initially, the appearance of dark bluish color indicates the utilization of bromophenol blue from the NBTA indicator plate (Fig. 3), which clearly showed that the isolated bacterial colonies are in primary form (phase I). After 1 week of incubation, the bacterial colonies exhibited no color in NBTA indicator medium and turned red color in MacConkey agar medium. The red color change in MacConkey agar plates showed the presence of secondary forms of bacteria (Phase II). The primary forms of the isolated bacteria were convex and smaller in diameter, whereas the secondary forms are wider and flatter.

The morphological characterization showed the isolated bacteria are rod-shaped, gram-negative, motile and non-bioluminescence. The morphological and biochemical characterization of isolated bacteria was shown in Table 1.

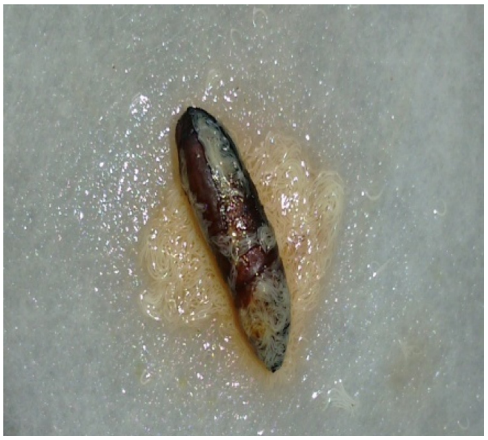

Fig. 1. Emergence of entomopathogenic nematodes from the pupal cadaver of rice moth (*Corcyra cephalonica*).

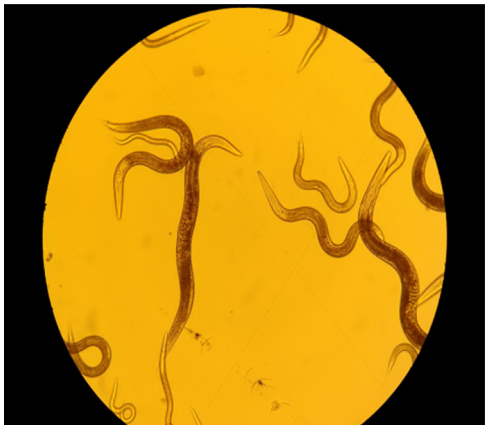

Fig. 2. Light microscopic observation of the isolated entomopathogenic nematode at 10X magnification.

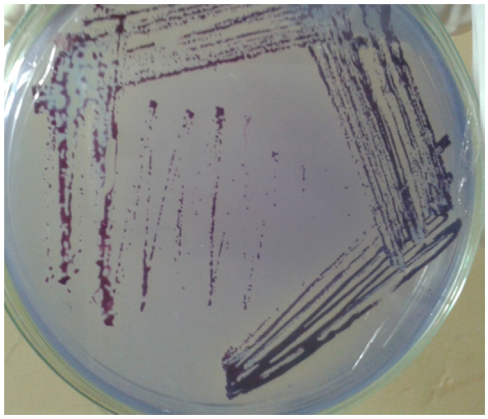

Fig. 3. Utilization of bromophenol blue from NBTA agar plate by primary forms of the isolated bacteria *Xenorhabdus* sp. PBU1755.

Table 1
Morphological and biochemical characterization of the isolated entomopathogenic bacteria *Xenorhabdus* sp. PBU1755.

S.no.	Characterization Test (s)	Observation
1	Gram's staining	–
2	Motility	+
3	Utilization of Bromothymol blue from NBTA	+
4	Utilization of Methyl red from MacConkey	+
5	Bioluminescence	–
6	Methyl red	+
7	Vogus Proskauers	–
8	Catalase	–
9	Oxidase	–
10	Indole	–
11	Urease	–
12	Lactose fermentation	–
13	Maltose fermentation	+
14	Glycerol fermentation	–
15	Nitrogen reduction	+
16	Gelatin liquefaction	+

Note: + denotes positive and – denotes negative.

3.3. Molecular characterization

PCR amplification of DNA extracted from entomopathogenic bacteria resulted in the product size of 720 bp using genus-specific primer (Fig. 4). The bacterial sequence obtained in this study was compared with National Center for Biotechnology Information (NCBI) database of *Xenorhabdus* sp. using BLASTn search tool (www.blast.ncbi.nlm.nih.

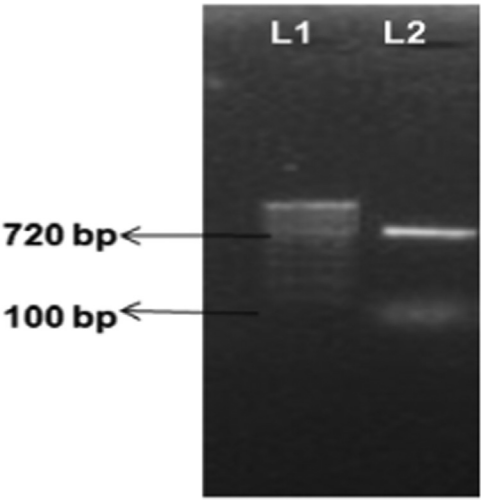

Fig. 4. PCR- amplification: L1 - Molecular marker (1 kb-size), and L2- PCR-amplified DNA of *Xenorhabdus* sp.

gov). The result showed 98% similarity with *Xenorhabdus* sp. PBU1755, and further which was deposited in the Genbank database under accession number KY825131.

3.4. Lecithinase producing activity of Xenorhabdus sp

Primarily, lecithinase producing ability of the both forms of isolated bacteria was identified by the formation opalescent zone on egg yolk emulsion nutrient agar media (plate assay). In this study, primary forms of *Xenorhabdus* sp. bacteria exhibited a clear opalescent zone around the inoculated colonies and secondary forms showed a moderated opalescent zone (Fig. 5a). Form this plate assay, it was observed that the primary forms of *Xenorhabdus* sp. showed higher lecithinase enzyme producing ability compared to secondary forms. Further, obtained results were supported by lecithovitellin or nagler's reaction broth assay. In this assay, it was observed that the primary forms of bacteria produced opalescent floating suspension on egg yolk emulsion nutrient broth medium (liquid) and the secondary forms did not show any floating or opalescent formation (Fig. 5b).

From the above analysis, primary forms of the bacteria *Xenorhabdus* sp. was chosen for the further studies because of their high enzyme producing activity. Shake flask method used for the large scale production of lecithinase enzyme from chosen primary forms (Phase I) of *Xenorhabdus* sp. bacteria. After 6 days of incubation period, produced biomass was collected from the flask. The collected cell free supernatant was used as a crude enzyme for the precipitation and dialysis process. The crude enzyme was precipitated using 12% trichloroacetic acid. After that, obtained precipitate was subjected to DEAE cellulose column and then collected fractions from cellulose column were partially purified by dialysis method with the help of dialyzing membrane.

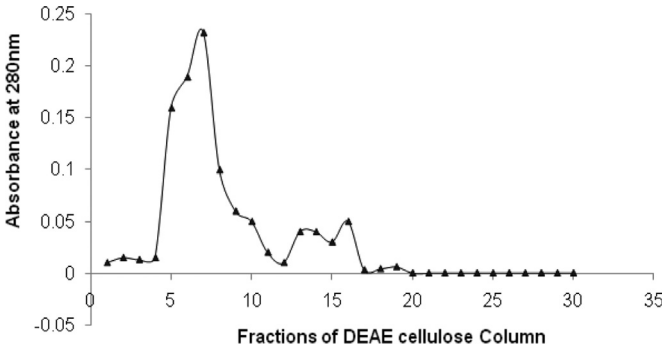

Fig. 6. Spectrophotometric determination of lecithinase enzyme concentration. All the fractions are collected from DEAE- cellulose column.

The protein concentrations of the partially purified fractions form DEAE cellulose column was quantified using spectrophotometer and the values are shown in Fig. 6. From this quantification analysis, higher concentration was observed in seventh fraction of DEAE cellulose column and also this fraction exhibited clear opalescent zone on egg yolk emulsion nutrient agar plate (Fig. 7), other fraction showed comparably less zone (data not shown).

The bacterial activity was optimized with different pH and temperature. Fig. 8 shows the results of different optimization conditions. The bacteria *Xenorhabdus* sp. showed higher activity at pH 7.0 and also it was observed that the increasing pH level showed the decrease in enzyme activity. Higher activity of lecithinase was observed at 24 °C.

Molecular weight of the lecithinase enzyme was determined using SDS-PAGE and are depicted in Fig. 9. From SDS-PAGE analysis, molecular weight of the lecithinase enzyme produced from *Xenorhabdus* sp.

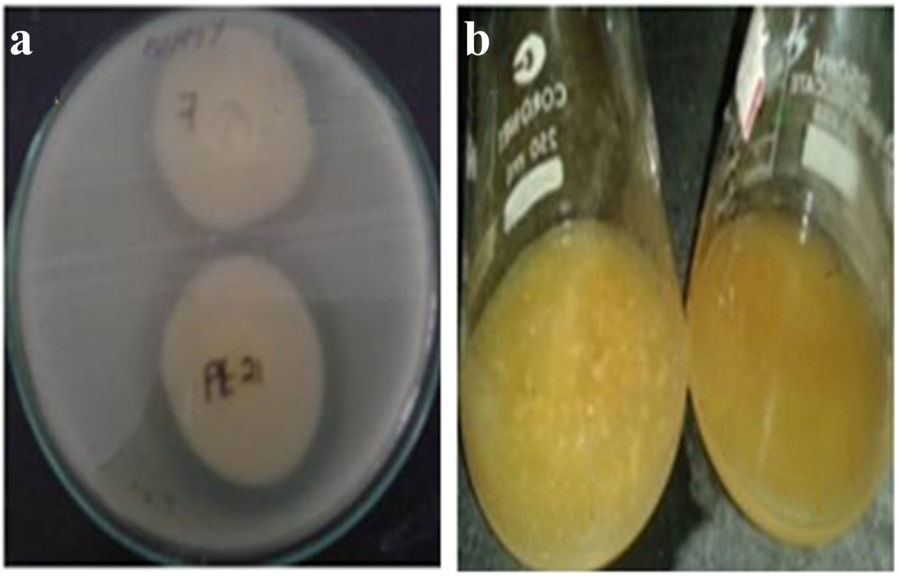

Fig. 5. (a) Screening of lecithinase enzyme production on egg yolk emulsion agar plate method, and (b) screening of lecithinase enzyme production on liquid broth method.

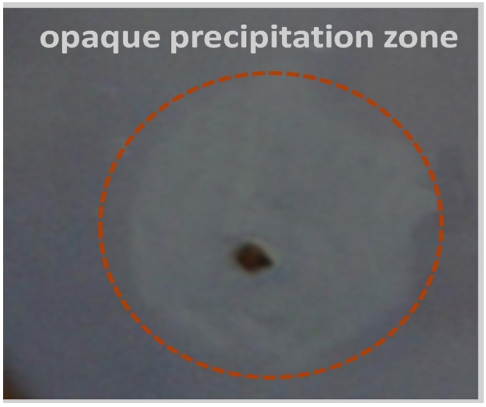

Fig. 7. Detection of lecithinase enzyme production, as determined by agar well diffusion assay at seventh fractions of DEAE- cellulose column.

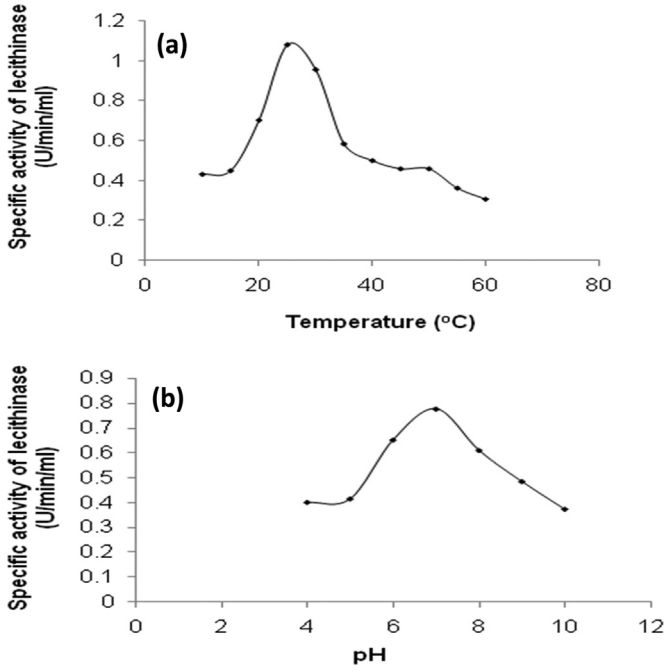

Fig. 8. Optimization studies of lecithinase enzyme production: (a) temperature optimization and (b) pH optimization.

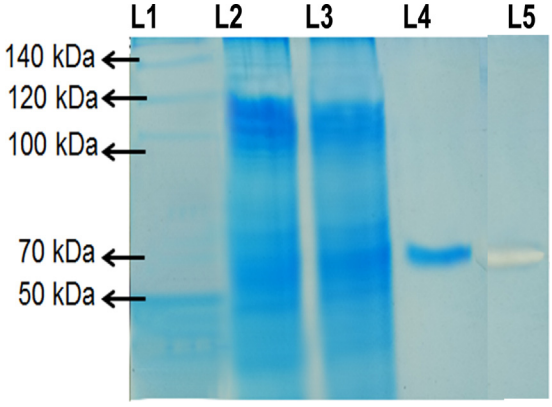

Fig. 9. Molecular weight determination by SDS-PAGE, L1 - Molecular marker (50–140 kDa), L2- Crude enzyme, L3- Dialyzed enzyme, L4- Partially purified enzyme, and L5 – Opalescent band of partially purified lecithinase enzyme. kDa denotes Kilo Daltons.

was found to be ~70 kDa and a single very faint and thin band was observed in Zymogram assay (Lane 5).

3.5. Toxicity assay

The hemolytic activity of purified lecithinase enzyme was performed using blood agar plate. The purified lecithinase enzyme exhibited no hemolytic activity which was confirmed by the absence of clear zone in the blood agar plate.

The toxicity of purified lecithinase against the mosquito larvae *C. quinquefasciatus* was performed. The mortality rate of the larvae was increasing with the increase in the concentration of lecithinase (Fig. S1). The lethal concentration (LC_{50}) of lecithinase was found to be 6.77 mg which was found to be significant at $p < 0.01$.

4. Discussion

Mosquito control is an important strategy for the prevention of widespread outbreak of infectious diseases (Gubler, 1998; Beier et al., 2008). Mosquito infection leads to loss of socio-economic wealth in many countries due to the death of millions of people every year (Benelli and Duggan, 2018). Past few decades, many chemical insecticides have been introduced into the environment in order to kill the diseases causing mosquitos, but the usage of chemical insecticides leads to the potential health risks and environmental problems (Aktar et al., 2009). In recent times, the production of bio-insecticides from microorganisms and plant extract are used to control the mosquito borne diseases due to their biodegradability and eco-friendly (Hemingway and Ranson, 2000; Sharma, 2001; Ramkumar et al., 2016). Thus, the present study deals with the mosquitocidal activity of lecithinase enzyme produced from entomopathogenic bacteria *Xenorhabdus* sp., as a bio-insecticide.

In the present study, the entomopathogenic bacteria *Xenorhabdus* sp. was isolated from the hemolymph of *C. cephalonica* infected by entomopathogenic nematodes. Similar to our study, Fukruksa et al. (2017) and Yooyangket et al. (2018) isolated *Xenorhabdus* sp. from the hemolymph of *Galleria mellonella* infected entomopathogenic nematodes. The isolated bacterium was identified using morphological, biochemical and molecular characterization techniques.

The molecular based 16S rRNA gene sequencing was used for the accurate identification of isolated bacteria. The BLASTn analysis of 16S rRNA sequence showed maximum similarity (98%) with the genus of *Xenorhabdus* sp. PBU1755. In agreement with our identification, Liu et al. (2001) and Godjo et al. (2018) identified the bacteria *Xenorhabdus* sp. based on 16S rRNA gene sequencing. Primary (Phase I) and secondary (Phase II) form stages of the isolated *Xenorhabdus* bacteria were screened using NBTA and MacConkey agar utilization indicator assay. Similar screening studies were reported by El-Sadawy et al. (2016).

Lecithinase enzyme is a type of phospholipase and used as a toxic determinant. Lecithinase enzyme has been produced by many bacteria such as *Bacillus cereus*, *pseudomonas aeruginosa* (Sharaf et al., 2014; Younis et al., 2015). In this study, isolated *Xenorhabdus* bacteria used for the production lecithinase enzyme and the production ability was screened based on the formation of opalescent zone on egg yolk emulsion nutrient agar and broth media. The primary forms (phase-I) of entomopathogenic *Xenorhabdus* sp. exhibited higher lecithinase production compared to secondary forms (phase-II). In evidence to the above finding, the phase-I variants of *Xenorhabdus nematophilus* and *Xenorhabdus bovienii* strains formed higher opalescent zone on a solid lecithin medium (Thaler et al., 1998). Similarly, studies on extracellular enzymes including lecithinase from the bacteria *Stenotrophomonas maltophilia* showed precipitation zone around the inoculum spot in egg yolk agar plate (Thomas et al., 2014)

Higher lecithinase enzyme production was carried out by submerged fermentation process. Recently, research reports proved that the submerged fermentation is highly effective in large scale production

of industrially important enzymes such as lecithinase, lipases, and pectinases (Bharathi et al., 2018; Ahmed et al., 2016). Shake flask method was employed as a submerged system for the production of lecithinase enzyme from *Xenorhabdus* bacteria.

In present investigation, the optimization of lecithinase enzyme production was carried out with different pH and temperature conditions. Higher activity of lecithinase was observed at 24 °C. Researchers reported that the slight decrease in temperature up to 25 °C enhance the enzyme activity (Snellman and Colwell, 2004). *Xenorhabdus* bacteria showed high yield of lecithinase activity at pH 7. It has been reported that maximum lecithinase activity was achieved at pH at 6–7 (Al-Juamily and Al-Zaidy, 2012).

In the present study, the molecular weight of the produced lecithinase enzyme was determined using SDS-PAGE. The obtained molecular weight of partially purified lecithinase (~ 70 kDa) was quite similar to Singh et al. (1999) and the zymogram assay showed the stable lecithinase activity. The detection of lecithinase enzyme using zymogram assay showed opalescent band. Similar studies on lecithinase enzyme isolated from *Pseudomonas fluorescens* resulted in opalescent band in zymogram assay (Rossignol et al., 2008)

The investigation on the pathogenicity of extracellular product lecithinase against *C. quinquefasciatus* exhibited increased mortality rate. Increasing concentration of lecithinase enzyme showed increased mortality rate. In this study, the third instar larvae stage of *C. quinquefasciatus* was used for toxicity assay. Similar to our study, same stage of *C. tritaeniorhynchus* was used for the determination of larvicidal activity (Mishra et al., 2016). Researchers have been investigated that the extracellular products of symbiotic bacteria associated with entomopathogenic nematode against various mosquitoes. In agreement with our studies, the bioactive compounds produced by *Xenorhabdus indica* such as taxlllaids A-G showed potential toxic activity on *Plasmodium falciparum* (Kronenwerth et al., 2014). Similarly, Vani and Lalithambika (2014) reported that the extracellular protein from *Xenorhabdus* sp. ranging from 20 to 97 kDa can be kill the third larval instar of *Anopheles gambiae* with 93.32% of mortality. The toxic complexes isolated from the nematode symbiotic bacteria *Photorhabdus* sp. has also been used against *Culex pipiens* (Ahmed et al., 2017). Similar to our studies, soil borne entomopathogenic fungus mediated synthesized silver nanoparticles exhibited significant larvicidal activity against *C. quinquefasciatus* and other two larvae (Vivekanandhan et al., 2018). Our finding suggests that the bacterial derived lecithinase enzyme could be used as a potential alternative to chemical mosquito control agents.

5. Conclusion

In this study, we demonstrated the larvicidal potential of lecithinase enzyme against filarial vector *C. quinquefasciatus*. A potent lecithinase enzyme producing bacteria *Xenorhabdus* sp.was isolated from entomopathogenic nematode. The production of lecithinase enzyme was carried out using submerged fermentation system and the activity was optimized with different pH and temperature. Partial purification of the produced lecithinase was done by TCA precipitation, DEAE, and dialysis method. Furthermore, partially purified lecithinase enzyme showed significant larvicidal activity against *C. quinquefasciatus* in concentration dependent manner. Overall, our finding suggests that the nematode associated bacterial product could be used to control various insect pests in pest control industries.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2019.01.003.

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