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Biological Control of Dengue Vector using *Pseudomonas Fluorescens*

Lalithambika B., Vani C.* and Arayil Nancy Tittes
Department of Biotechnology, School of Biotechnology and Health sciences, Karunya University, Coimbatore – 641114, INDIA

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Abstract

In the present study Pseudomonas fluorescens from the rhizosphere soil was isolated using Kings B medium and biochemically characterised. Nutrient broth medium, King's B medium, King's B+ yeast extract medium and glucose peptone salt medium are used to maximize the production of exotoxin and check the anti-larvicidal activity using liquid formulation of the exoproteins which will be used for the control of dengue vector and there by eradication of the disease. The result observed that the net mortality for King's B medium at protein concentration of 40µg and 80µg/ ml for 48hrs supernatant is 100% and also the 24 hrs supernatant is active against Aedes aegypti at low concentration of protein(20µg/ml), while the 24hrs supernatant from glucose peptone salt medium shows 100% mortality at 80 µg/ ml and Kings B yeast extract medium shows 100% mortality for 24hrs supernatant at protein concentration of 80µg/ml and at 40µg/ml and 80µg/ml for 48 hrs supernatant. This result shows that Kings B medium is very effective for the production of exotoxin against Aedes aegypti, since it shows their larvicidal activity at low concentration of protein.

Keywords: *Pseudomonas fluorescens, nutrient medium, King's B medium, King's B+ yeast extract and glucose peptone salt medium, anti-larvicidal activity, Aedes aegypti*.

Introduction

Vector borne diseases are a major threat to human health and are increasingly reported in the majority of tropical countries across the globe. The World Health Organization (WHO) has reported that the global prevalence of dengue fever, an arboviral disease has dramatically increased making it endemic in more than 100 countries in Africa, America and south-east Asia. WHO currently estimates that there may be 50 –100 million dengue infections worldwide every year.In India there are about 37 million dengue infections occurring every year and may be 227,500 hospitalizations," according to Dr. Scott Halstead, a tropical disease expert focused on dengue research. Dengue infection can cause a variety of illness ranging from an influenza-like diseases called dengue fever to dengue hemorrhagic fever, resulting in 50-100 million infections worldwide and 37 million particularly in India every year[1].

Dengue viruses are transmitted to humans by certain species of mosquitoes, specifically *Aedes aegypti* and *Aedes albopictus*, which is found in urban environments, while dengue fever is caused by one of 4 serotypes of dengue virus (DENV1-4), the infection confers lifelong immunity to that particular strain but not to the other three. *Aedes* (Stegomyia) *albopictus*, is currently considered of secondary importance in transmission, except in Asian countries, being present in rural or semi-urban habitats [2]. The efforts to control the disease is now centered on vector control. But the use of chemical pesticides resulted in *Aedes* mosquitoes developing resistance to all major insecticide groups such as organophosphate, pyrethroids, organochlorine and carbamate[3-8]. In order to tackle this problem natural biocontrol agent or their by products are being used as an alternative. A number of biological control agents formulated with bacteria, fungi, virus, pheromones, and plant extracts have been in use mainly for the control of mosquitoes.

Various commercial formulations of bacteria such as *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* offers high level of initial mosquito larval control but with very little residual activity, necessitating weekly application thus increasing the cost of operation[9]. Recent reports indicate development of resistance in mosquitoes against these formulations[10]. These limitations therefore necessitate the search for new control method which may replace these insecticides. This led to the identification of insecticidal activity among the metabolites of various bacteria like *Pseudomonas fluorescens, Pseudomonas pseudomallei and Pseudomonas aeruginos*[11]. According to Vector Control Research Centre, Pondicherry the liquid formulation of *Pseudomonas fluorescens* metabolite was found to be lethal to larvae as well as pupae of vector mosquitoes and safe to mammals[12]. This led to our decision to isolate new strains *of Pseudomonas fluorescens* for the eradication of dengue vector. The aim and scope of this study is to eradicate the dengue vector because other biocontrol agents have not yet reached the operational stage for the control of *Aedes* spp. Prabhakaran *et al.,* 2009 reported that there was a good effect in the control of *Aedes* spp using Pseudomonas spp[11]. The main objective of our study is to do media optimization and identify the better formulation for using it as a biocontrol agent as mosquito larvicide in conjunction with other control methods in integrated control programs will be an efficient technology.

Material and Methods

Isolation of Pseudomonas fluorescens: Isolation of *Pseudomonas fluorescens* was made from rhizhosphere soil of Beans plant in Nallurvayal in Coimbatore district of Tamil Nadu.The 10 cm rhizhosphere soil particles loosely adhering to the roots were gently teased out and the roots were cut into small pieces and mixed well. The soil thus obtained was crushed in a sterile mortar and pestle and then serially diluted the soil sample. 0.1ml sample were taken from the dilution 10^{-4} -10^{-7} was plated on the Kings B agar medium(KB) and incubated at 30°C for 24-48 hrs. Distinct colonies showing fluorescence under UV light at 360 nm were picked and streaked on KB fresh agar medium and the pure cultures so obtained were stored in refrigerator at 4°C for further use [13].

Mosquito culture: 4th instar larvae of *Aedes aegypti* was collected from the National centre for health and disease control, Mettupalayam.

Biochemical tests for Pseudomonas fluorescens: Isolated strains were identified based on their morphological and biochemical characteristics as *Pseudomonas fluorescens* according to Cappuccino and Sherman[14].

Culture conditions and Media: *Pseudomonas fluorescens* was cultured in different media by adding 1ml of the culture in different medium such as nutrient broth (NB), glucose peptone salt(GPS) (glucose 10 g/L, peptone 10 g/L, KH_2PO_4 1.26 g/L), King's B (KB)(peptone 20 g/L, glycerol 10 g/L, $MgSO_4$ 1.5 g/L, K_2HPO_4 1.5 g/L) and King's B + yeast extract (KBY) (peptone 20 g/L, glycerol 10 g/L, $MgSO_4$ 1.5 g/L, K_2HPO_4 1.5 g/L, yeast extract 5 g/L). The cultures were grown for 48 hrs at 28 °C. The estimation of the exoproteins in the culture supernatants of 24hrs and 48 hrs were determined by Lowry's *et.al.,* 1951[15].

Bioassay: The culture supernatants of the medium (KB, GPS, KBY and NB) of 24 and 48 hrs with protein concentrations of 20µg/ml, 40µg/ml, 80µg/ml were prepared by diluting the culture filtrate with sterile distilled water. These were added to the disposable bioassay cups containing 25ml of chlorine free tap water and 10 larvae was introduced. A bioassay cup without the culture supernatant was served as control. Larval food containing yeast and dog biscuit (1:1/w/w) was added in fine powder form to the bioassay cups containing larvae. Larval mortality was scored after 24hrs till 72hrs exposure[12].

Results and Discussion

Isolation and characterization of *Pseudomonas fluorescens*: In this Present study, *Pseudomonas fluorescens* was isolated from the rhizosphere of the beans planted fields in Nallurvayal Coimbatore was plated on KB medium and Yellowish green fluorescent colonies detected at 365nm in UV light were isolated and streaked on KB medium (figure-1,2). The *Pseudomonas fluorescens* showed positive results for Catalase, Oxidase, Citrate, TSI, Nitrate reduction, Gelatine hydrolysis, Casein hydrolysis and showed the negative result for Indole, MRVP and starch hydrolysis (table 1).

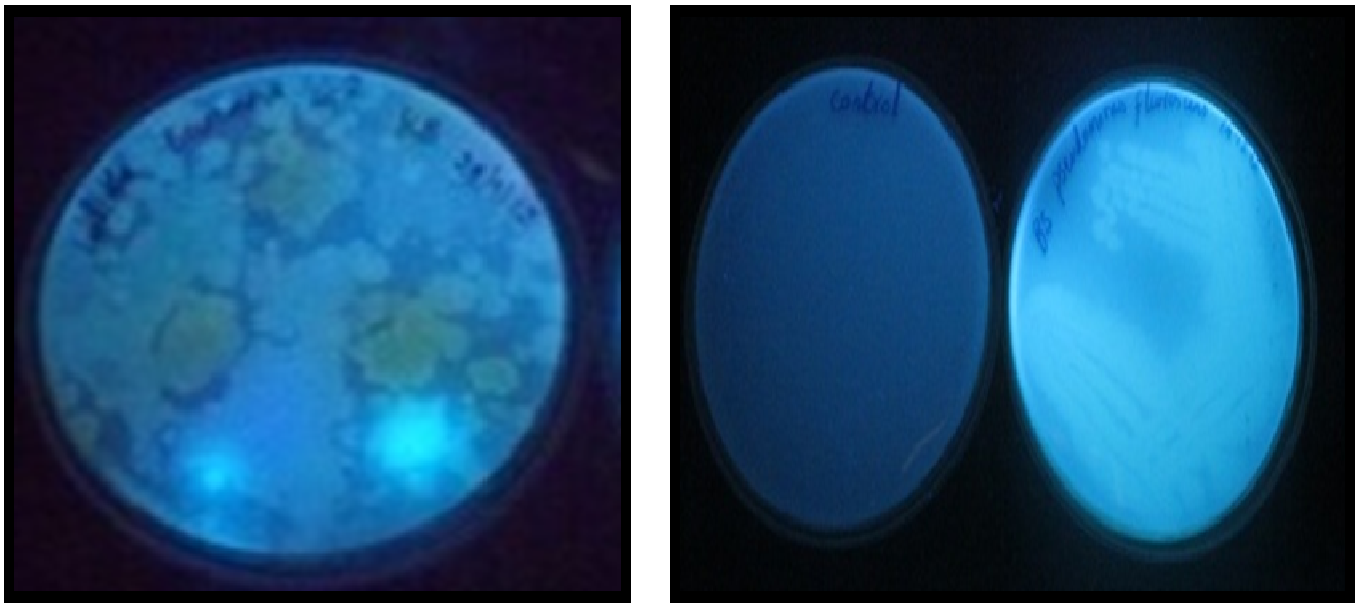

Figure-1 and 2
The emission of fluorescent pigment by *Pseudomonas fluorescens* in UV light at 365nm.

Table-1
Represents the Biochemical characteristics of the *Pseudomonas fluorescens*

S.no	Name of the biochemical test	Characteristics
1.	Catalase	Positive
2.	Oxidase	Positive
3.	Citrate	Positive
4.	TSI	Positive
5.	Nitrate reduction	Positive
6.	Gelatin hydrolysis	Positive
7.	Casein hydrolysis	Positive
8.	Indole	Negative
9.	MR	Negative
10.	VP	Negative
11.	Starch hydrolysis	Negative

Media optimization for the production of exoprotein: The protein concentration in the supernatant of the *Pseudomonas fluorescens* grown in NB, KB, KBY, GPS medium for every 24 hrs and 48 hrs was recorded. At 24hrs culture supernatant of *Pseudomonas fluorescens* showed 160µg/ml of protein in KBY, 178µg/ml of protein in KB, 99µg/ml of protein in GPS and 140µg/ml of protein in Nutrient medium, while in 48hrs culture supernatant, the protein concentration in KBY medium was noted that 117µg/ml, 120µg/ml of protein in KB, 157µg/ml of protein in GPS and 142µg/ml of protein in Nutrient medium. The concentration of protein was found to be highest in the 24 hrs culture supernatant of KB medium (178µg/ml) and in 48 hrs culture supernatant of GPS (157 µg/ml) when compared to the other mediums (figure-3).

Bioassay on the dengue vector *Aedes aegypti:*Mortality rate of larvae in supernatant of King's B + Yeast extract mediu: The rate of Mortality of *Aedes aegypti* was recorded using the *Pseudomonas fluorescens* when cultured with KBY medium. At 24hrs treatment, the rate of mortality of 24hrs supernatant was recorded as 4.5% in 20µg/ml, 85% 40µg/ml and 98% in 80µg/ml concentration of protein and the 48hrs culture supernatant showed 7% in 20µg/ml, 40% in 40µg/ml and 98% in 80µg/ml. At 48hrs treatment, the rate of mortality of 24hrs supernatant was noted that 38% in 20µg/ml, 98% 40µg/ml and 100% in 80µg/ml concentration of protein. While in 48hrs supernatant,80% in 20µg/ml, 96% in 40µg/ml and 100% in 80µg/ml. At 72hrs exposure the 24hrs culture supernatant showed 80% in 20µg/ml, 97% in 40µg/ml, 100% in 80µg/ml. while in 48hrs culture, 90.5% in 20µg/ml, 100% in 40µg/ml, 100% in 80µg/ml.The maximum mortality was observed in 48hrs and 72hrs exposure at 80µg/ml of protein (figure-4).

Mortality rate of larvae in supernatant of King's B medium: Culture supernatant of KB medium showed the mortality rate against the larvae of *Aedes aegypti* are as follows. At 24hrs treatment, the rate of mortality of 24hrs supernatant was recorded as 32.5% in 20µg/ml, 46.5% 40µg/ml and 85% in 80µg/ml concentration of protein and the 48hrs culture supernatant showed 7.5 % in 20µg/ml, 90.5% in 40µg/ml and 95% in 80ug/ml. At 48hrs treatment, the rate of mortality of 24hrs supernatant was noted that 37% in 20µg/ml, 97.5% 40µg/ml and 100% in 80µg/ml concentration of protein. While in 48hrs supernatant, 80% in 20µg/ml,95.5 % in 40µg/ml and 100% in 80µg/ml. At 72hrs exposure the 24hrs culture supernatant showed 80 % in 20µg/ml, 95% in 40µg/ml, 96 % in 80µg/ml. while in 48hrs culture, 93.5 % in 20µg/ml, 94.5 % in 40µg/ml, 97.5 % in 80µg/ml.The maximum mortality was observed in 48hrs exposure at 80µg/ml of protein (figure-5).

Mortality rate of larvae in supernatant of Glucose Peptone Salt medium: The Exoproteins in the culture filterate of GPS medium showed the mortality rate against the larvae of *Aedes aegypti* was recorded at 24hrs treatment, the rate of mortality of 24hrs supernatant was recorded as 3.5 % in 20µg/ml, 84.5 % 40µg/ml and 97.5 % in 80µg/ml concentration of protein and the 48hrs culture supernatant showed 5 % in 20µg/ml, 40% in 40µg/ml and 97.5% in 80µg/ml. At 48hrs treatment, the rate of mortality of 24hrs supernatant was noted that 37% in 20µg/ml, 97.5% 40µg/ml and 100% in 80µg/ml concentration of protein. while in 48hrs supernatant,80% in 20µg/ml,96% in 40µg/ml and 100% in 80µg/ml. At 72hrs exposure the 24hrs culture supernatant showed 80% in 20µg/ml, 97% in 40µg/ml, 100% in 80µg/ml.while in 48hrs culture, 95.5% in 20µg/ml, 100% in 40µg/ml,100% in 80µg/ml. The maximum mortality was observed in 48hrs and 72hrs exposure at 40µg/ml and 80µg/ml of protein (figure-6).

Mortality rate of larvae in supernatant of Nutrient Broth: The culture filtrate of Nutrient broth which shows the mortality rate against the larvae (Aedes aegypti) are as follows. At 24hrs treatment, the rate of mortality of 24hrs supernatant was recorded as 4 % in 20µg/ml,18.5 % 40µg/ml and 57.5 % in 80µg/ml concentration of protein and the 48hrs culture supernatant showed 4% in 20µg/ml, 37% in 40µg/ml and 76.5% in 80µg/ml. At 48hrs treatment, the rate of mortality of 24hrs supernatant was noted that 41% in 20µg/ml, 80% in 40µg/ml and 95.5% in 80µg/ml concentration of protein. while in 48hrs supernatant,41.5% in 20µg/ml,80.5% in 40µg/ml and 96% in 80µg/ml. At 72hrs exposure the 24hrs culture supernatant showed 76.5% in 20µg/ml, 96% in 40µg/ml, 97.5% in 80µg/ml. while in 48hrs culture, 80% in 20µg/ml, 97% in 40µg/ml, 98% in 80µg/ml. Hence there is no 100% mortality rate was observed in the nutrient medium (figure-7).

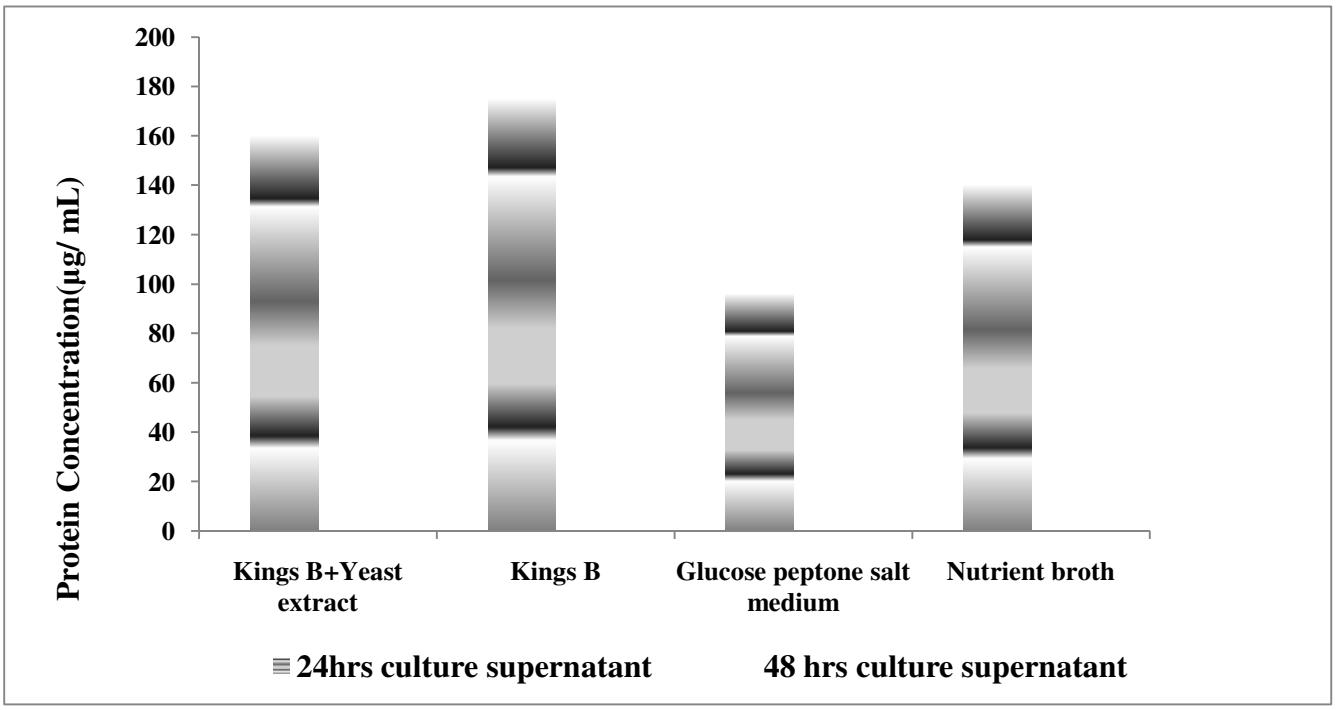

Figure-3
The concentration of protein in four different medium for 24 hrs and 48 hrs

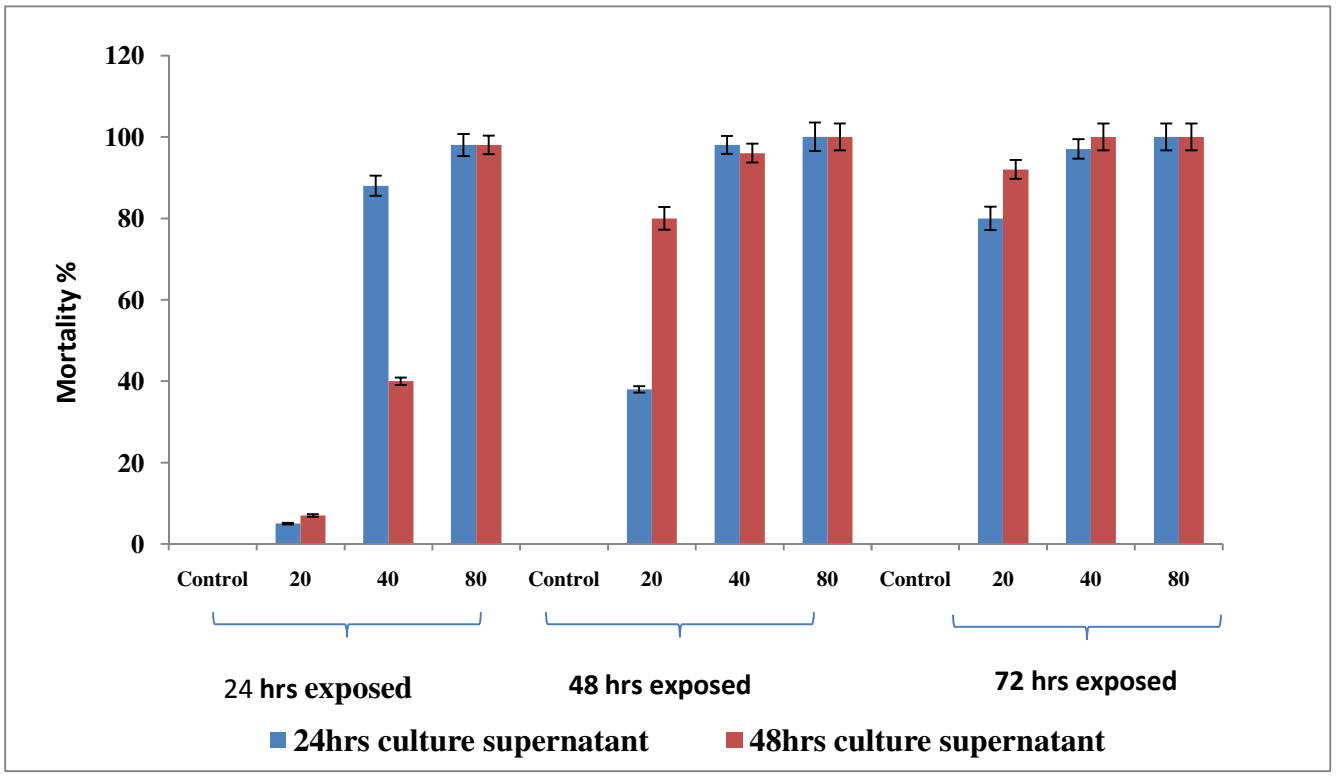

Figure-4
Represents the mortality percentage of *Aedes aegypti* treated with culture supernatant of KBY medium when exposed to 24hrs, 48hrs, 72hrs

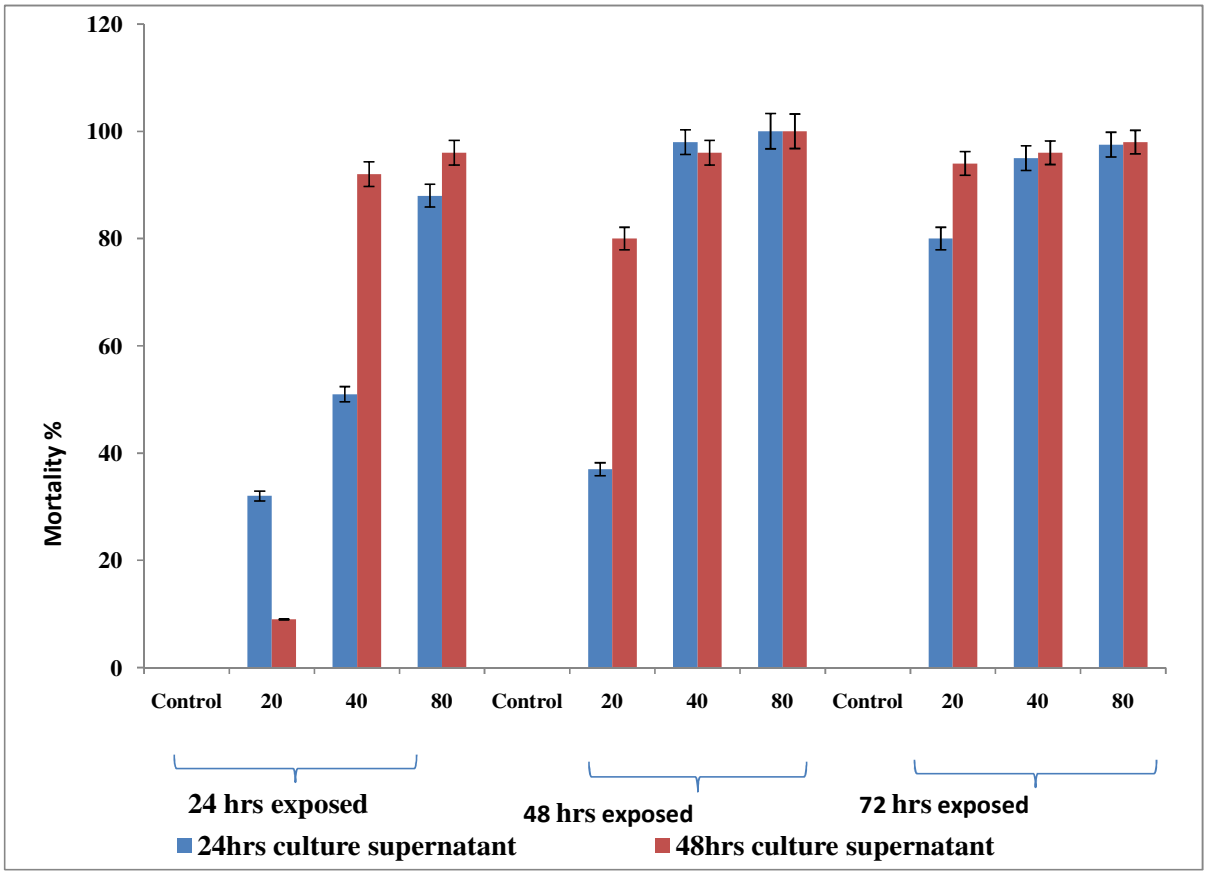

Figure-5
Represents the mortality percentage of *Aedes aegypti* treated with culture supernatant of KB medium when exposed to 24hrs, 48hrs, 72hrs

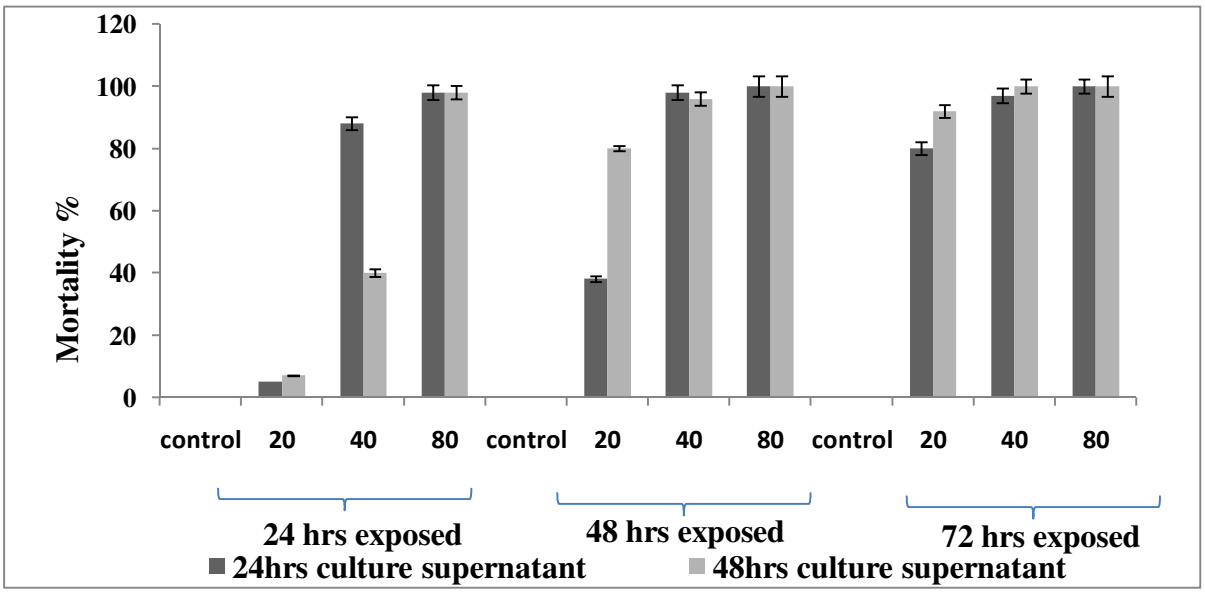

Figure-6
Represents the mortality percentage of *Aedes aegypti* treated with culture supernatant of GPS medium when exposed to 24hrs, 48hrs, 72hrs

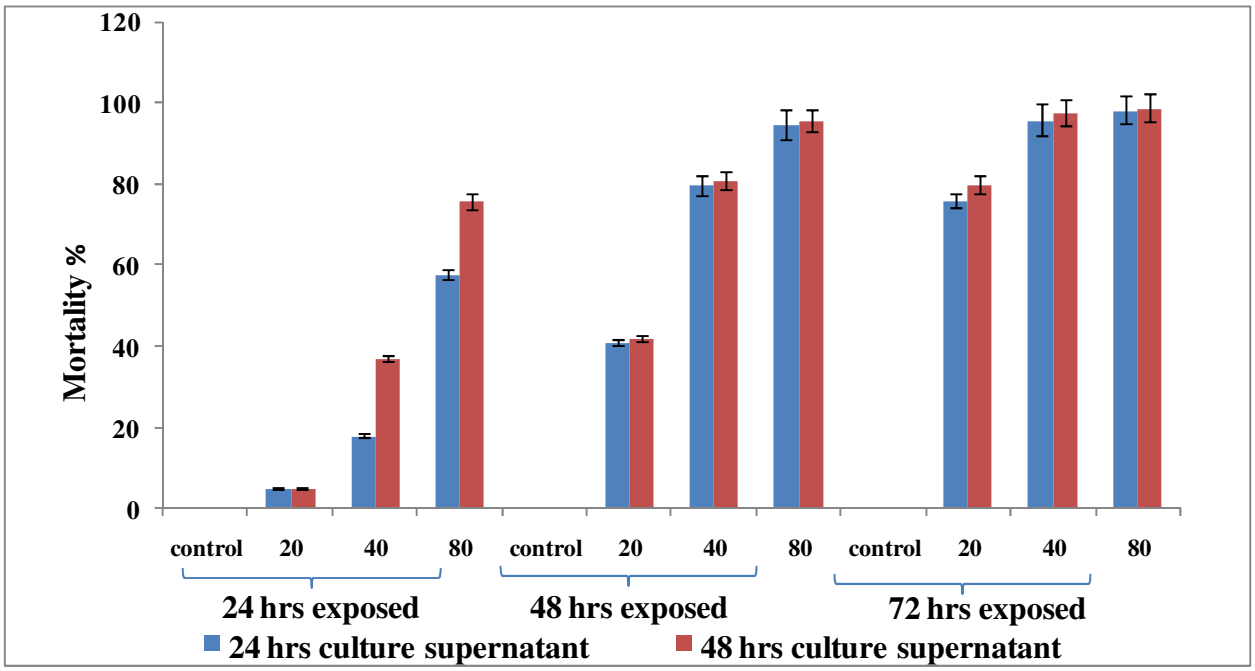

Figure-7
Represents the mortality percentage of *Aedes aegypti* treated with culture supernatant of NB medium when exposed to 24hrs, 48hrs, 72hrs

Discussion: Naturally, *Pseudomonas fluorescens* is more abundantly present in the rhizosphere region of the soil influences the growth of various crops in the agricultural field by its plant growth promoting factor and it also act against various virulent plant pathogens and it leads to the idea to use *Pseudomonas fluorescens* as effective biocontrol agent to control various Plant pathogen such as rice fungal pathogen [16] and also against the larvae and pupae of mosquitoes [17]. With this result in the present study, *Pseudomonas fluorescens* was isolated from the rhizosphere soil and it was identified using morphological, cultural and biochemical characteristics as *Pseudomonas fluoresecens*. The strain shows gram negative and showed positive result for oxidase, catalase, citrate and gelatin and casein hydrolysis, carbohydrate fermentation test [14]. This result was in accordance with the study of Rekha *et al*., 2010[18]. They have isolated *Pseudomonas fluorescens* from the rhizosphere soil and characterised morphologically, culturally and biochemically as *Pseudomonas fluorescens* and they showed the antimicrobial activity of *Pseudomonas fluorescens* against bacterial pathogen.

Different media were used for comparative studies to know the optimal nutrient medium for the recovery of maximal amount of exotoxin. Nutrient media used in the present study are as follows, NB, KB medium, KBY and GPS medium. Among the four medium KB medium gives the high concentration of protein in 24 hrs culture supernatant (178µg/ml) and GPS medium showed the high concentration of protein in 48 hrs culture supernatant (157µg/ml). This may be due to the carbon and nitrogen sources of the above media which play an important role for increase in the production of metabolites and exoproteins by the *Pseudomonas* species. This result was similar to the findings of Wang *et al*., 2011[19].

After determining the suitable medium for exoprotein production from different sources of media, the exoproteins were tested for their insecticidal efficacy against *Aedes aegypti* Mosquito. It was reported that, the control of these species using chemical agents cost resurgence effect in humans and animals and with time they become resistant to these insecticides. Our present study reported that the control of *Aedes* species was much effective when treated with the cell- free supernatant of *Pseudomonas fluorescens* which contain exoproteins.

Net mortality for the day 1 treatment showed that 24 hrs culture of the KB medium is active against *Aedes aegypti* at low concentration when compared to other three media supernatants (Figure-4) and at 40µg and 80µg concentration mortality is high in KBY medium and GPS as compared to KB media(Figure- 3 and 5). Whereas in 48 hrs culture at both GPS and KB showed high mortality at 40µg/ml and 80µg/ml (Figure-4 and 5). On second day treatment, both the 24 and 48 hrs supernatant showed highest mortality percentage of 100% at 80µg/ml in KBY, GPS and KB medium and also showed high mortality rate in 40µg/ml. (figure-3,4,5).

On the 3rd day treatment, 24hrs supernatant shows high mortality rate in 40µg/ml and 80µg/ml in all the four medium, whereas the 48 hrs supernatant shows 100% mortality at 40µg/ml and 80µg/ml in KBY and GPS medium. It was

interesting to record that 100% mortality of the larvae of *Aedes* species was obtained when treated with the cell- free supernatant obtained from the KB medium, KBY and GPS medium. However, KB and GPS media shows the high concentration and mortality rate, we have concluded the KB medium is the optimum medium KB medium is the optimum medium for the maximal recovery of protein because it have the high virulency against mosquito larvae (*Aedes aegypti*) at low concentration of exotoxin (20µg/ml). This result was in accordance with the study against rice fungal pathogens by *Pseudomonas fluorescens*[16]. They showed that KB medium is very good for the production of secondary metabolite for the *Pseudomonas fluorescens* and it was very effective against the rice fungal pathogen. our result was also in accordance with the study against *Musca domestica* which was reported by Padmanabhan *et al.,* 2005[12]. They assayed with the lyophilised form of *Pseudomonas fluorescens* culture filterate at different concentration as 1,5,10, 15, 20 and 25 % which were equivalent to 1.13, 5.63, 11.25, 16.88, 22.50 and 28.13 µg of the toxic protein/ g of rearing medium against *Musca domestica* and found out that the net mortality of larvae was higher at the concentration of 20%. The net mortality of pupae was higher than that of larvae at all the concentrations except at 20 per cent.

Conclusion

Our study thus reveals that exotoxins of *Pseudomonas fluorescens* are toxic to the larvae of *Aedes aegypti*. Though the protein concentration was less in the 24hrs supernatant of glucose peptone salt medium, it showed highest mortality rate when compared with other media. Also, the 48hrs supernatant of King's B medium shows highest mortality rate even when protein concentration is low and also it was very active against *Aedes aegypti* even at very low concentration compared to other media (20µg/ml concentration). This leads us to conclude that this occurrence is probably due to different exoproteins being responsible for the insecticidal effect. Thus, this shows that the medium has a very important role to play with respect to release of various exoproteins.

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