

Biocontrol potential of extracellular proteins from *Xenorhabdus nematophilus* on dengue vectors and the enhancement by response surface methodology

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ABSTRACT

Dengue fever is a prevalent and rapidly spreading disease. It is best controlled by controlling its vectors. *Aedes aegypti* and *Aedes albopictus* are the primary and secondary worldwide vectors, respectively, which are bred in peridomestic man-made water containers. Biological control is the most effective and sustainable method as there is no resurgence effect and does not harm humans. Our study includes the use of extracellular proteins of *Xenorhabdus nematophilus*, a Gram-negative bacterium widely used as biocontrol agents belonging to the family Enterobacteriaceae. The mortality rates of fourth instar larvae *A. aegypti* when treated with 250 µg of extracellular proteins of *X. nematophilus*, recorded to be 40% after 72 hours of exposure. The rate of mortality was observed minimum even at higher concentration. The optimization of the medium through response surface methodology showed that there was an increase in the production of extracellular proteins. These proteins played a very important role in the control of *A. aegypti*. The maximum rate of mortality was recorded to be 92% when treated with 200 µg of extracellular proteins within 48 hours of treatment. Our research proved that the media optimization enhanced the production of extracellular proteins in the *X. nematophilus* and it can be used as a biocontrol agent for the control of dengue vector.

INTRODUCTION

Dengue fever is caused by any of the five serotypes⁷ (DENV 1, 2, 3, 4, and 5) of dengue virus belonging to the family Flaviviridae and transmitted mainly by the insect vector *Aedes aegypti*, of Culicidae family. It is an arboviral disease with high mortality rates in humans. An approximate of 50 million infections has been estimated, and more than 20,000 deaths occur each year. Though the disease dominates in the tropical areas of the world, it is not completely restricted to those regions, and hence any region infested with the *Aedes* spp. can lead to the disease as well. Vaccines are not available in the market, and most of them are in the clinical trial phase. If the virus cannot be targeted, then the vector has to be targeted, which calls for physical, chemical,

and biological methods of mosquito control (Huang *et al.*, 2017). A novel vaccine for dengue fever has been approved in three countries, but it is not yet commercially available (Dans *et al.*, 2018). As there is no potent drug available against these arboviral diseases, mosquito vector control is an effective measure for the control of dengue fever (Chandran and Azeez, 2015). Various types of control methods were practiced for the control of *A. aegypti* which include repellents and chemical insecticides like Organophosphates, such as diflubenzuron and methoprene, etc. (Seccacini *et al.*, 2008). The continuous usage of these insecticides increases resistance in the mosquitoes; hence, there is a need for developing alternative strategies for the control.

Xenorhabdus nematophilus, a Gram-negative bacterium, belonging to the family Enterobacteriaceae. They are symbiotically associated with the entomopathogenic nematodes (EPN) *Heterorhabditis* and *Steinernema* species. The EPNs are widely used as biocontrol agents for the control of various crops pests. The bacteria are carried as symbionts in the gut tract (often in a specialized vesicle of the entomopathogenic nematode). The

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foraging nematodes locate the prey insect pests and penetrate to the hemolymph, acting as vectors for the injection of their symbiotic bacteria. These bacteria do not kill the insects if ingested but do so quickly if they are introduced to the hemolymph, either via nematode infection or via syringe injection of laboratory cultures. Once into the hemolymph, the bacteria grow rapidly to produce a variety of extracellular proteins which plays a major role as virulent factors and kills the pests (Walia et al., 2011). This study includes the biological control of *A. aegypti* larvae using extracellular proteins from *X. nematophilus* and the enhancement of proteins by response surface methodology (RSM).

METHODOLOGY

Culture conditions of *X. nematophilus*

Xenorhabdus nematophilus (MTCC No 4691 Microbial Type Culture Collection, Gene Bank, Institute of Microbial Technology, Chandigarh, India) was procured. The culture was inoculated in Luria Bertani (LB) broth and yeast starch glucose broth. After incubation for 24 hours at 28°C, bacterial colonies which were developed was further streaked onto nutrient bromothymol agar medium (NBTA) (0.025% bromothymol blue, 0.004% triphenyl tetrazolium chloride) and was incubated for 24 hours at 28°C. Pre inoculum cultures were raised in 100-ml flask containing 15-ml media and were incubated at 24 hours at 28°C in a rotary shaker at 150 rpm. For inoculums preparation, pre inoculum cultures transferred aseptically to 250-ml shake flask containing 100-ml nutrient broth medium (Khandelwal and Bhatnagar, 2003).

Growth of *X. nematophilus* in different media composition

Xenorhabdus nematophilus was grown in different semi-synthetic media (Table 1) to compare the growth. A final pH of 7.20 ± 0.02 was adjusted and media was sterilized at 121°C for 20 minutes. Inoculum of 5% was added to 50 ml of each media in 250-ml conical flask and was incubated at 28°C for 72 hours at 150 rpm.

Estimation of biomass

About 10 ml of culture was centrifuged at 8,000 rpm for 20 minutes. The supernatant obtained after centrifugation was stored at 4°C for protein estimation, while the pellet containing cells were dried at 70°C until it reached a constant weight (Ahmad et al., 2013).

Screening of medium components by Plackett Burman design

Plackett Burman design can be used for screening various medium components and it also examines ($n-1$) variables in at least “ n ” number of experiments. (Stowe and Mayer, 1966). In each of the experimental run, the frequency of every level of a variable is constant and the number of high and low variables is equal. While determining the effect of a particular variable, the effects of changing the other variables cancel out. The main effect is calculated from the difference of the average observations at high (H) level and average observations at the low level (L) of each factor. In the case of *X. nematophilus*, 11 factors including two dummy variables were screened in 12 combinations (Table 2) and all trials were performed in duplicate and average of observation was used as a response of design (Fattah et al., 2002).

Table 1. Composition of semi-synthetic media (Fang et al., 2010; Wang et al., 2008).

Media	Composition (g/l)	
TSB	Tryptone—17.00	
	Soy peptone—3.00	
	Glucose—2.50	
	NaCl—5.00	
	K ₂ HPO ₄ —2.50	
	WYH	Glucose—6.13
		Peptone—21.29
		MgSO ₄ ·7H ₂ O—1.50
		(NH ₄) ₂ SO ₄ —2.46
		KH ₂ PO ₄ —0.86
Nutrient Broth	K ₂ HPO ₄ —1.11	
	Na ₂ SO ₄ —1.72	
	Beef extract—1	
	Yeast extract—2	
	Peptone—5	
	NaCl—5	
	Modified nutrient broth	NB + NaCl—5.00
LB	Tryptone—10.00	
	Yeast extract—5.00	
	NaCl—10.00	
YSG	Glycerol—5.00	
	Yeast extract—5.00	
	1 M—MgSO ₄ —5 ml	
	(NH ₄) ₂ SO ₄ —2.00	
	1M—KH ₂ OP ₄ —5 ml	
YS	1 M—K ₂ HOP ₄ —5 ml	
	1M—Na ₂ SO ₄ —10 ml	
	Yeast extract—5.00	
	(NH ₄) ₂ SO ₄ —5.00	
	MgSO ₄ ·7H ₂ O—0.20	
	KH ₂ PO ₄ —0.50	

Table 2. Plackett–Burman experimental design.

Exp Run	A	B	C	D	E	F	G	H	J	K	L	Protein(g/l)
1	H	L	L	L	H	H	H	L	H	H	L	933.00
2	L	H	H	L	H	L	L	L	H	H	H	1,066.47
3	L	L	L	L	L	L	L	L	L	L	L	904.17
4	H	H	H	L	H	H	L	H	L	L	L	932.00
5	L	H	H	H	L	H	H	L	H	L	L	1,043.55
6	L	L	H	H	H	L	H	H	L	H	L	1,466.67
7	L	L	L	H	H	H	L	H	H	L	H	985.42
8	H	L	H	L	L	L	H	H	H	L	H	972.72
9	H	L	H	H	L	H	L	L	L	H	H	1,475.75
10	H	H	L	H	L	L	L	H	H	H	L	968.33
11	H	H	L	H	H	L	H	L	L	L	H	800.55
12	L	H	L	L	L	H	H	H	L	H	H	915.00

Where uncoded values in g/l (H, L); A—glucose (5, 1), B—peptone (12.5, 5), C—tryptone (12.5, 5), D—NaCl (4, 0.5), E—MgS.70 (1, 0.05), F—KPHP (4, 0.05), H—yeast extract (1.5, 0.25), I—(NS, J—agitation (150 rpm), and K—temperature (28°C).

Table 3. RSM experiment design.

Experiment	Glucose	Peptone	NaCl	Predicted response protein (g/l)	Experimental response protein (g/l)
1	-1	0	-1	1,220.1	1,291.667
2	-1	-1	0	1,076.8	1,041.667
3	-1	0	1	1,238.3	1,260.417
4	1	0	-1	1,688.9	1,666.667
5	0	-1	1	882.8	895.8333
6	1	1	0	1,183.6	1,218.75
7	0	-1	-1	1,099	1,062.5
8	0	0	0	1,429.4	1,645.833
9	1	0	1	1,279.9	1,208.333
10	0	1	-1	929.8	916.6667
11	0	1	1	755.2	791.6667
12	0	0	0	1,429.4	1,437.5
13	0	0	0	1,429.4	1,395.833
14	1	-1	0	1,462.2	1,520.833
15	-1	1	0	1,058.6	1,000

Where uncoded values (-1, +1) in g/l: glucose (5, 20), peptone (5, 20), and NaCl (1, 10).

Response surface methodology

The significant variables obtained from screening experiments were optimized using RSM. A three-factor and a three-level (-1, 0, and +1) Box Behnken design (BBD) consisting of 15 experimental runs was used (Table 3). The experiments were carried out in duplicates and average values of the response are taken. The analysis of the responses was done with Minitab v18 (Minitab Inc., State College, PA). A quadratic model is obtained to study the interaction between the variables. The BBD is a spherical design and also have fewer runs as compared with other three-level factorial designs (Box and Behnken, 1960; Ferreira *et al.*, 2007; Khuri and Mukhopadhyay, 2010; Myers *et al.*, 2009).

Estimation of extracellular protein from *X. nematophilus*

The supernatant of culture was precipitated in 70% ammonium sulfate using a magnetic stirrer in ice-cold condition and kept overnight at 4°C. After 24 hours, the precipitated supernatant was centrifuged at 10,000 rpm for 30 minutes at 4°C. The pellet was suspended in 0.1 M Tris buffer (pH 7.5) and transferred into a dialysis bag. The buffer was changed for two–three times. The protein thus obtained was transferred into Eppendorf tubes and stored at -20°C (Ramasamy *et al.*, 2008). Protein content was estimated by Lowry's *et al.* (1961) method.

Mosquito culture

The mosquito larvae of *A. aegypti* were collected and identified from the National Center for Disease and Control, Mettupalayam. The eggs of *A. aegypti* were collected and kept in the tray in the laboratory at room condition and allowed to hatch within 2 days after hatching the fourth instar larvae were used for the present study.

Larvicidal activity on *A. aegypti* using extracellular proteins of *X. nematophilus*

Different concentrations of 50, 100, 200, 800, and 1,000 µg of extracellular proteins of 24 and 48 hours culture supernatant were treated against fourth instar larvae of *A. aegypti*. The concentrations

of protein in µg/ml were dissolved in 25 ml of chlorine-free tap water and five larvae were introduced per treatment. Each treatment was repeated three times for replication (Brammacharry and Paily, 2012). The rate of mortality was recorded for every 24, 48, and 72 hours by using Abbott's formula (1925).

$$\text{Percentage of mortality} = \left(\frac{\text{Number of larva dead}}{\text{number of larvae}} \right) \times 100$$

Statistical analysis

All the values are expressed as the mean ± standard deviation (SD). All the experiments were performed in triplicates. Statistical analyses were performed with graph pad Prism 4 software; using two-way analysis of variance (ANOVA) followed by Turkey Kramer multiple comparison tests. The significance was set at $p < 0.05$.

RESULT AND DISCUSSION

Biomass of *X. nematophilus* cultured in different media

The effects of different medium (Table 1) on the growth of *X. nematophilus* are represented in Figure 1. TSB medium gave maximum growth when compared with the other media and it was employed for further optimizations. TSB has been reported for high biomass and antibiotic productivity in *Xenorhabdus* by Fang *et al.* (2010).

Larvicidal activity of the extracellular proteins of *X. nematophilus* cultured in TSB medium against *A. aegypti* fourth instar larvae

Figure 2 represents the rate of mortality of *A. aegypti* larvae treated with extracellular proteins from 24 hours culture supernatant of *X. nematophilus*. The rate of mortality was recorded for every 24, 48, and 72 hours of treatment. It was observed that the mortality rate was recorded 0% in the treatments with 50, 100, and 150 µg/ml of extracellular proteins. Maximum rate of mortality was observed to be 40.0% at 72 hours of treatment.

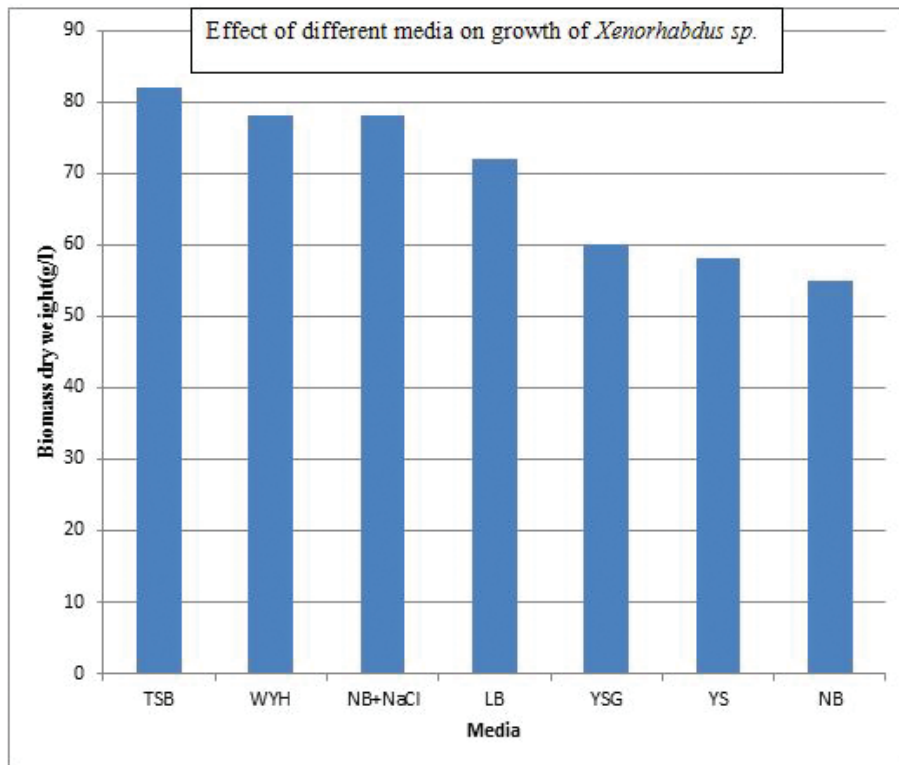


Figure 1. Effect of different media on the growth of *Xenorhabdus sp.*

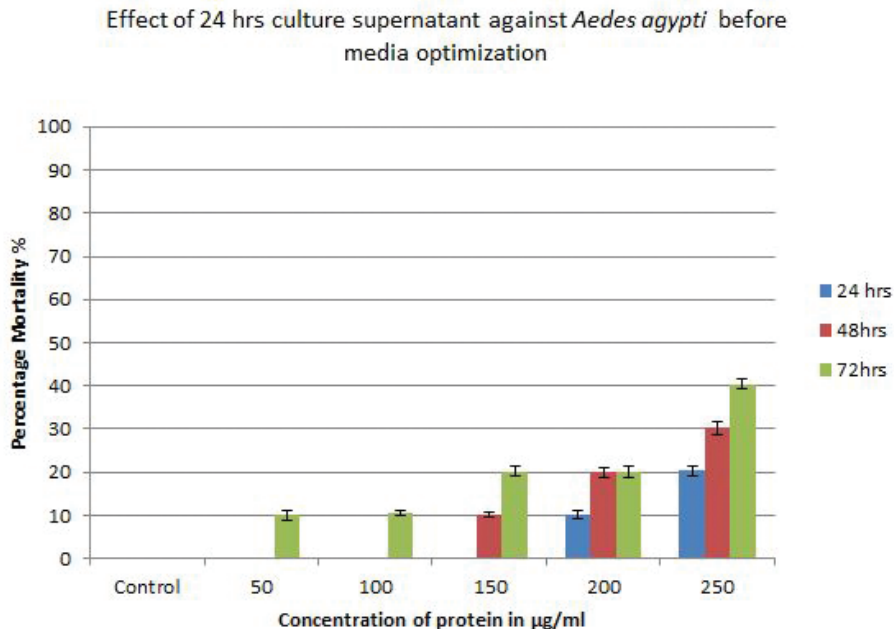


Figure 2. Effect of 24 hours supernatant against *A. aegypti* before media optimization.

Figure 3 represents the mortality rate of fourth instar larvae of *A. aegypti* treated with extracellular proteins from 48-hour culture supernatant. Maximum mortality rate was recorded to be 20% when treated with 200 µg/ml of extracellular proteins after 24 hours of exposure. Minimum mortality of 13.2% was recorded after 24 and 48 hours of exposure when treated with 50, 150,

and 250 µg/ml of extracellular proteins. However, there was no substantial mortality observed after 72 hours of exposure. Considerable mortality rate was not observed in *A. aegypti* fourth instar larvae treated with extracellular proteins of *X. nematophilus*. This result was not on a par with the result of Forst *et al.* (1997) who reported that the *Xenorhabdus* spp. killed 90% of the larvae

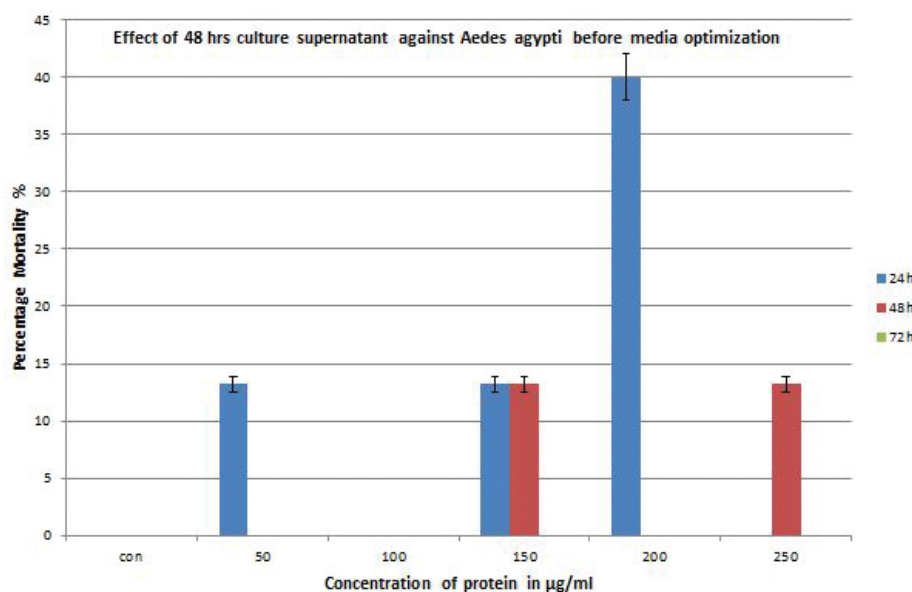


Figure 3. Effect of 48 hours supernatant against *A. aegypti* before media optimization.

of *Manduca sexta* which is a pest of tobacco. The lower mortality rate may be due to the composition of the media which has led to the lesser production and virulence of extracellular proteins. From the results it was observed that the biomass production of *X. nematophilus* in TSB medium was recorded as the highest (82 g/l); however, the larvicidal activity of extracellular proteins was minimal (40.0%) in TSB medium. The larvicidal activity of the extracellular proteins against *A. aegypti* was recorded minimum of 40.0%. Hence, the observations recommend the optimization of TSB medium to enhance the protein production thereby the efficiency of larvicidal activity of the *X. nematophilus*.

Effect of different medium components on the extracellular protein production

Standard methods proposed by Plackett–Burman were employed for experimental data analysis (Plackett and Burman, 1946). The effect of each variable was calculated with the following equation:

$$Ex_i = 2 \left(\frac{\sum H_{xi} - \sum L_{xi}}{N} \right)$$

Where Ex_i is the effect (concentration) of the tested variable, H_{xi} and L_{xi} are the protein yield at the high and low level, respectively, of the same variable, and N is the number of trials. The positive effect implies that the impact of the variable on protein is at high concentration while negative effect implies that the impact on protein is greater at low concentration.

Mean squares of each variable (the variance of effect):

$$V_{xi} = \frac{(\sum H_{xi} - \sum L_{xi})^2}{N}$$

The experimental error: $R = \sum V_{xd}/n$ is calculated by taking an average of mean squares of dummy variables

F test is used to identify the factors with larger: $F = V_{xi}/R$, where R is the experimental error. V_{xd} is the mean

Table 4. Influence of variables on protein production.

Factors	Effect	F test
Peptone	203.4983	19.87
NaCl	203.4017	19.85
Glucose	168.305	13.59
Tryptone	-150.04	10.80
KH_2PO_4	-139.90	9.391
Yeast extract	76.87	2.835
Agitation	51.58	1.276
Temperature	38.81	0.723
$(NH_4)_2SO_4$	-37.54	0.676
K_2HPO_4	35.04	0.589
$MgSO_4$	26.13	0.327

square of dummy variable; V_{xi} is the mean square of variable and n is the number of dummy variables. Table 4 summarizes the effect and F test value for all the variables.

Peptone, NaCl, and glucose have shown high F value and hence very significant for the protein production. The Pareto chart (Fig. 4) generated also displays the significant factor for protein yield. Variables like peptone, NaCl, glucose, yeast extract, K_2HPO_4 , and $MgSO_4 \cdot 7H_2O$ have shown positive effect while other variables like tryptone, KH_2PO_4 , and $(NH_4)_2SO_4$ have a negative effect on the protein production.

Optimization of medium by RSM

Three variables which included glucose, peptone, and NaCl were examined for their optimum combination using BBD and the actual responses and the responses predicted by the model are compared (Table 2). ANOVA was carried out for responses (Table 5). A second-order polynomial equation was developed for the experimental data by applying multiple regression analysis.

Protein = 1,429.4 + 127.6 Glucose - 74.2 Peptone - 97.7 NaCl + 103.0 Glucose * Glucose - 337.1 Peptone * Peptone - 175.6

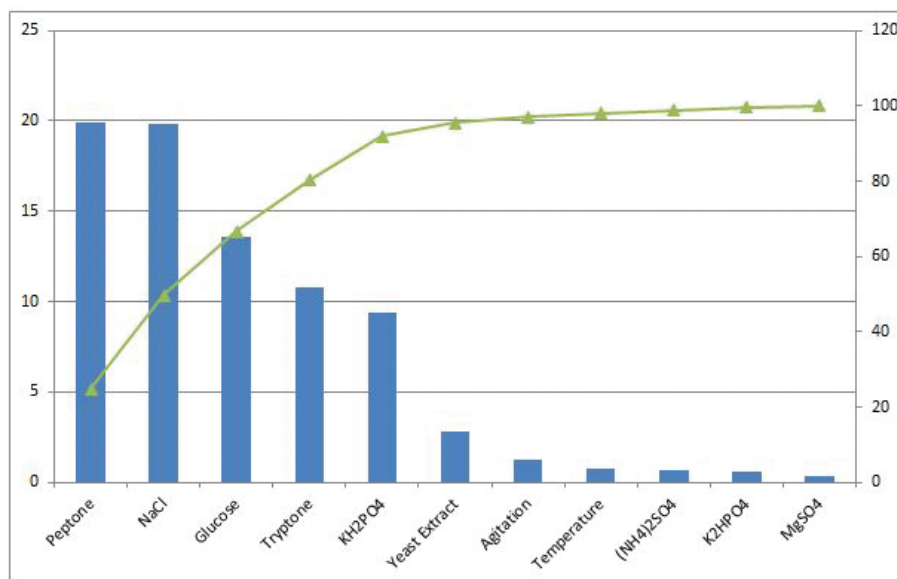


Figure 4. Pareto chart of effects on extracellular protein production.

Table 5. ANOVA for experimental results.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	9	889,216	98,802	19.79	0.002
Linear	3	250,625	83,542	16.74	0.005
Glucose	1	130,262	130,262	26.10	0.004
Peptone	1	44,068	44,068	8.83	0.031
NaCl	1	76,294	76,294	15.29	0.011
Square	3	575,602	191,867	38.44	0.001
Glucose*Glucose	1	39,178	39,178	7.85	0.038
Peptone*Peptone	1	419,569	419,569	84.06	0.000
NaCl*NaCl	1	113,901	113,901	22.82	0.005
2-way interaction	3	62,990	20,997	4.21	0.078
Glucose*Peptone	1	16,954	16,954	3.40	0.125
Glucose*NaCl	1	45,601	45,601	9.14	0.029
Peptone*NaCl	1	434	434	0.09	0.780
Error	5	24,957	4,991		
Lack-of-Fit	3	23,574	7,858	11.36	0.082
Pure error	2	1,384	692		
Total	14	914,173			

NaCl * NaCl – 65.1 Glucose * Peptone – 106.8 Glucose * NaCl + 10.4 Peptone * NaCl

From the ANOVA, the model was highly significant with a very low p -value of 0.002. Lack of fit of the model is insignificant as it was >0.05 (Table 5). The value of the coefficient of determination ($R^2 = 97.27$) suggested that the model is a good fit. The adjusted R^2 is 92.36% which implies that the insignificant terms are negligible as it is close to R^2 . Among the parameters, all the three variables have a significant impact on protein production as its $p < 0.05$. The maximum interaction occurred between glucose and NaCl with the p -value 0.02. The 3-D plots (Fig. 5a–c) represent a visual interpretation of the interaction between two factors. From Figure 5a, it is obvious that increasing glucose

and peptone concentration increased protein and further increase reduced the protein production. Figure 5b and c showed a similar effect on protein and the factors.

The optimal values for the factors were calculated using the response optimizer with the goal of maximizing protein. The optimum protein production conditions were predicted to be with a concentration of glucose—10 g/l, peptone—16 g/l, and NaCl—2.4 g/l using response optimizer and response surface plots.

The experimental validation of the model was attempted with the optimized media constituents as glucose—10 g/l, peptone—16 g/l, and NaCl—2.4 g/l and observed that 1,700 g/l extracellular protein was produced and is very close to the expected observation.

Kumar *et al.* (2014) reported significant role of soya peptone 4.9 g/l and NaCl 1.45 for the production of fibrinolytic alkaline metalloproteases (66.31% increase) from *Xenorhabdus indica* KB-3; however, other major components in media include carbon sources (tryptone—5.71, dextrose—1.45,) and metal ion dipotassium phosphate—0.47 g/l. Cations in the media induce the secretion of enzyme and improve the thermostability of enzymes. There are reports on glucose as the optimal carbon source for the production of secondary metabolites with antimicrobial activity in *X. nematophila* (Wang *et al.*, 2008; Yang *et al.*, 2001)

Larvicidal activity of extracellular proteins of *X. nematophilus* cultured in optimized media against *A. aegypti* fourth instar larvae

In 24-hour culture supernatant (Fig. 6), the rate of mortality of *A. aegypti* fourth instar larvae was recorded maximum of 92% and 93.2% at 72 hours of treatment with 200 and 250 μ g/ml extracellular proteins, respectively. The minimum mortality rate of 46.6% was observed in the larvae when treated with 50 μ g/ml of extracellular protein after 48 hours of treatment. Whereas in 48-hour culture supernatant (Fig. 7), maximum mortality rate of 53.2% was recorded after 72 hours of exposure when treated with 150 μ g/ml of extracellular protein and a minimum mortality

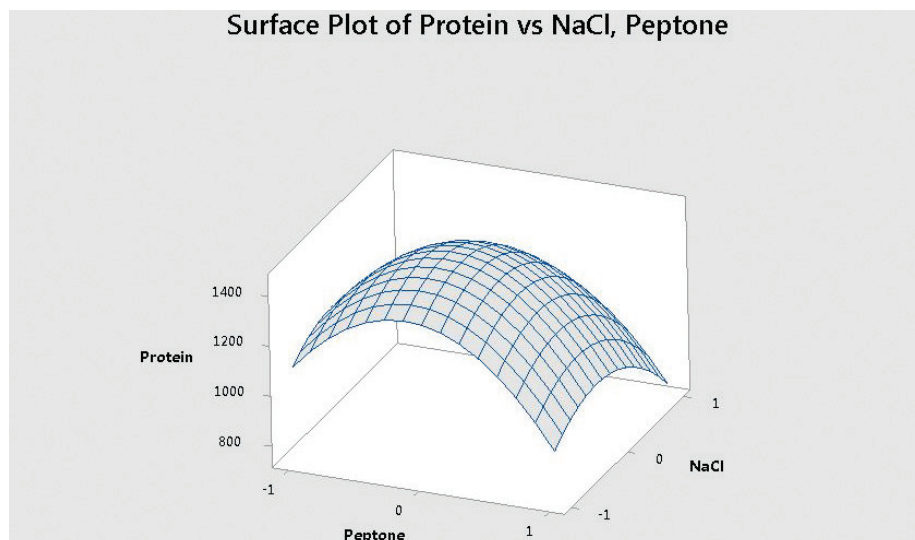
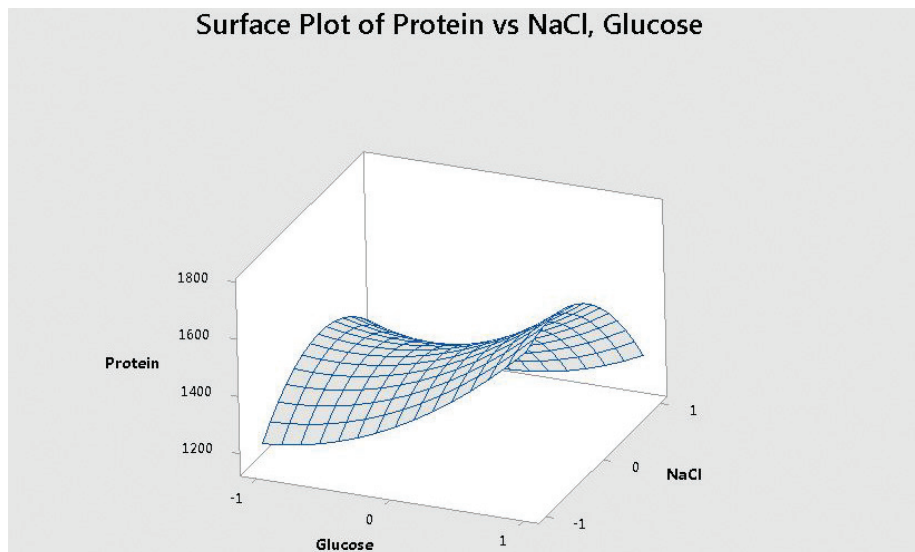
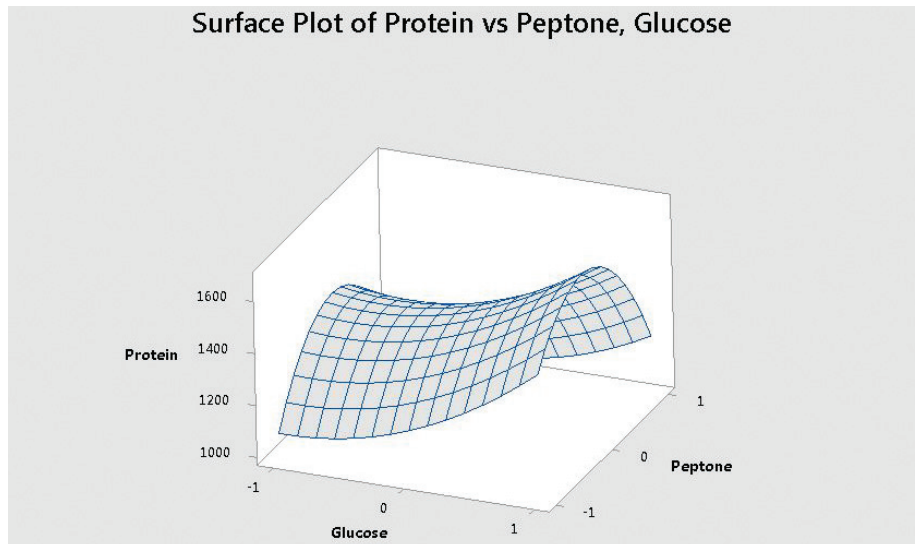


Figure 5. (a) Surface plot of protein (g/l) versus glucose (g/l) versus peptone (g/l). (b) Surface plot of protein (g/l) versus glucose (g/l) versus NaCl (g/l). (c) Surface plot of protein (g/l) versus NaCl (g/l) versus peptone (g/l).

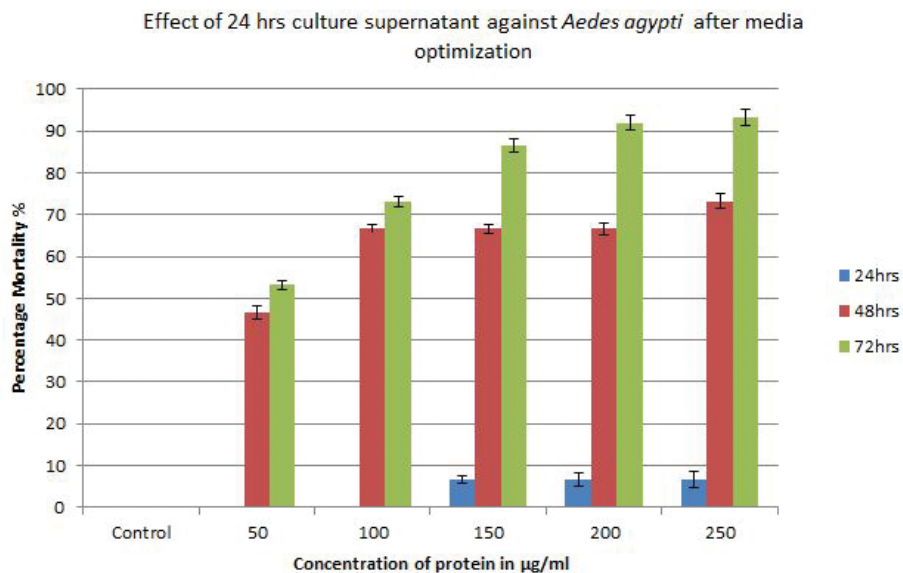


Figure 6. Effect of 24 hours culture supernatant of *X. nematophilus* against *A. aegypti* fourth instar larvae after media optimization.

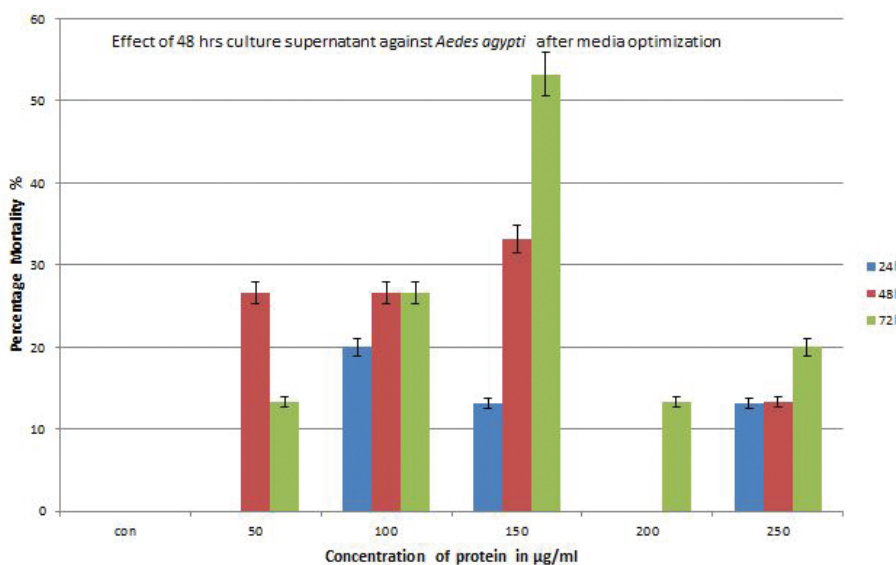


Figure 7. Effect of 48 hours culture supernatant of *X. nematophilus* against *A. aegypti* fourth instar larvae after media optimization.

(13.2%) was recorded after 24 hours of exposure with 150 and 250 µg/ml of extracellular proteins treated.

After the media optimization, it was interesting to note that the rate of mortality was observed to be 53.2% even at a lower 50 µg/ml concentration of extracellular proteins of *X. nematophilus* (24-hour culture supernatant) treated against *A. aegypti* at 72 hours of treatment. Our results proved that the mortality rate was higher when compared with the results of Fukruksa *et al.* (2017). They reported that *Xenorhabdus stockiae* recorded 60% mortality after 96 hours of treatment in *A. aegypti* larvae. Cannibalism was observed when the larvae were exposed to suspensions of *X. nematophila* and lead to a maximum of 72% mortality in 96 hours of incubation (Silva *et al.*, 2013). Owuama (2001) reported that proteinases resulted in histological lesions leading to the death of the insect. This higher mortality rate obtained in *A. aegypti* larvae was due

to the improved protein production after media optimization. The optimization parameters glucose, peptone, and NaCl involved in the production of extracellular proteins in the medium.

This may be due to the increased production of lipase, protease, phospholipases, and chitinases in the medium because of the optimization of carbon and nitrogen sources. Our results are in accordance with the previous work of Caldas *et al.* (2002). They observed that the increased production of extracellular enzymes in the medium involved in the insecticidal properties and it is not harmful to humans. Herbert and Goodrich-Blair (2007) had a similar report that *X. nematophila* controls the insects by inhibiting both at its cellular and humoral immunity and can inhibit the synthesis of antimicrobial peptides. The virulence factors comprising proteases, lipases are responsible in this larvicidal activity.

Although the 48-hour culture supernatant improved the larvicidal activity, it is not as significant as 24-hour culture supernatant protein. The extracellular proteins produced by media optimization in *Xenorhabdus* spp. will be an effective biopesticide for the eradication of dengue vectors. Further, this research can be extended for the identification and characterization of the extracellular proteins.

CONCLUSION

From the above study, it was interesting to note that media optimization played an important role in the production of extracellular proteins of *X. nematophilus* in the medium which caused 93.2% mortality in *A. aegypti* fourth instar larvae after 72 hours of treatment. Before optimization, the rate of mortality was 40% after 72 hours of treatment. This research would pave way for the eradication of dengue vectors for the control of dengue fever. The screening of different variables, responsible for the production of extracellular protein, was carried out with the help of Plackett–Burman design and obtained that glucose, peptone, and NaCl are the major factors which significantly improve the production of extracellular proteins. RSM was followed in the optimization of selected factors and observed that glucose—10 g/l, peptone—16 g/l, and NaCl—2.4 g/l can maximize the extracellular protein yield. A two-fold increase in the protein was attained after the media optimization. Further research has to be carried out in the purification of the specific protein with the insecticidal activity and this process can be scaled up to effectively control the dengue vector.

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