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A POTENTIAL BIOSURFACTANT MEDIATED CONTROL OF FUSARIUM WILT IN TOMATO CROP AND A NOVEL METHOD FOR CLEANING PESTICIDE RESIDUES IN TOMATOES

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Abstract

The biosurfactant produced by Serratia rubidaea SNAU02 (NCBI accession number KC560769, has rhl gene KF 835609) was isolated from hydrocarbon-contaminated soils of Cuddalore district, Tamilnadu, India. The molecular characterization of the biosurfactant revealed the presence of rhamnolipid. The strain exhibited antifungal activity and demonstrated no toxicity against the seeds of Brassica oleracea and Artemia salina employed as a bio-indicator. The concentration of 250 μg/ml biosurfactant application controlled the Fusarium wilt of tomato crop. The feasibility of pesticide residues cleaning in tomatoes was studied five times using HPLC analysis. One kg of each tomato was treated with the 100ppm of Monocrotophos solution and allowed to stand for 24 h. After that, the tomatoes were collected and air dried for 1 hour. Further, the tomatoes were soaked for 30 min in the following treatments, T_1 (1000 ml distilled water), T_2 (1000 ml luke warm water), T_3 (2 per cent $NaCl_2$ in 1000 ml of distilled water),T_4 (2 per cent $NaCl_2$ in 1000 ml of luke warm) and T_5 (10 mg biosurfactant in 1000 ml distilled water) respectively. Followed that the tomatoes were carefully washed with 100ml of double distilled water and the washings were then collected and subjected to UV/Vis detector HPLC analysis. The treatment washings revealed that, there was no change in peak observation for T_1 and T_2, T_3 and T_4. Whereas, T_5 showed three broad peaks with the retention time of 2.432, 2.784 and 2.955 respectively, which denote the cleanup of pesticide into intermediate products which may be non-toxic.

Keywords: Serratia rubidaea, rhamnolipid, biocontrol, Fusarium wilt, Pesticide, Tomato

Introduction

Biosurfactants are amphiphilic compounds, produced by variety of microorganisms such as bacteria, fungi, and yeast and can reduce surface and interfacial tension of the liquids [14].The biosurfactants have several advantages over chemical surfactants including high ionic strength tolerance, high temperature tolerance, higher biodegradability, lower toxicity, lower critical micelle concentration (CMC), and higher surface activity [2].

Biosurfactant producers can only be effective when they are maintained at their optimal ambient conditions required for its growth and activity. In this regard, one of the best methodologies for optimization experiment, response surface methodology (RSM) is an empirical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivalent equations simultaneously [3]. In the past few years, numerous microorganisms with antifungal activities and their antifungal factors have been identified. In addition, the mechanisms by which microorganisms inhibit growth

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of potentially pathogenic fungi have been demonstrated [13]. Among lipopeptide and rhamnolipid biosurfactant, several species of Bacillus genus has been reported as bacterial biocontrol agents. There are limited reports on rhamnolipid biosurfactant as biocontrol agents. Rhamnolipid have found to have important antagonistic effect on economically important zoosporic plant pathogen, thus opening their use as biocontrol agents [13]. Rhamnolipids have demonstrated inhibition of zoospore forming plant pathogens that have acquired resistance to commercial chemical pesticides [10] and another investigation has shown that rhamnolipid can stimulate plant immunity which is considered as an alternative strategy to reduce the infection by plant pathogens [15].

In agriculture production, various pesticides have been used for protection against plant diseases and insect pests with the result, these chemicals persist for longer period in fruits and vegetables which causing harm to human beings. Concern over the pesticide residues in fruits and vegetables have led to the development of many clean up and analysis methods. Taking these into account and considering the need of potential biosurfactant producers, economic production processes using agro-industrial wastes, controlled the Fusarium wilt of tomato crop and cleanup of pesticide into intermediate products which may be non-toxic.

Materials and Method

Microorganism

S. rubidaea SNAU02 (accession number KC560769) (http:// www.ncbi.nlm.nih.gov/nuccore/KC560769.1), a potent biosurfactant producer was used for the present study [6]. The strain was grown in nutrient agar (NA), sub-cultured each month and stored at 4 °C.

Substrate

Cashew apple juice was used as substrate for the present study.

Biosurfactant production

100 ml of Mineral salt medium (MSM) broth was sterilized in an autoclave at 121℃ for 15 min. The clarified cashew apple juice as such without inorganic mineral salts to prove the effect of CAJ on the biosurfactant production. For the comparison, defined medium (MSM with 2% glucose) was included in this study. The sterilized MSM broth was inoculated with 5ml of the isolate SNAU02 and incubated at room temperature over an orbital rotary shaker set at 129 rpm min^{-1} for 3 days.

Response surface methodology (RSM) for the optimization of biosurfactant production

In the experimental model, factors such as Inoculum size (gl^{-1}), Peptone(gl^{-1}),Initial pH, temperature and Shaker (rpm)were optimized by RSM. The specific codes for each independent variable and range of the variables used for this experiment are given in Table 1.The experiment was performed using central composite design (CCD) for which a total of 50 treatment combinations were generated using designer expert 7.0 software (Stat-Ease Inc. Minneapolis, USA).

From the experimental data according to this design, a second order polynomial regression model equation was derived as below:

Surface tension reduction of the media =

$$46.97482 + 0.28023A - 0.35236B - 0.29234C - 0.43173D + 0.40777E - 0.20312AB - 0.57188AC - 0.090625AD - 0.45938AE + 0.13437BC + 0.065625BD - 0.17812BE + 0.65937CD + 0.30312CE - 0.15313DE - 2.29423A^2 - 2.29423B^2 - 2.29423C^2 - 0.230307D^2 - 2.29423E^2$$

Where A: Inoculum size; B – Peptone; C - Initial pH; D – Temperature; E – Shaker; * - 72.The RSM experimental model was employed in order to study the interaction between the factors for the optimization of the production of biosurfactant. Every level was included in the run matrixs for the study on effect of various independent variables on the production of biosurfactant by *S. rubidaea* SNAU02. Here, each experiment was done in three sets.

Statistical analysis

This data was analyzed by the analysis of variance (ANOVA) technique to find out which factors had the most effective interactions for higher biosurfactant production [5].

Biosurfactant isolation

The cell free supernatant was acidified with 6 N hydrochloric acid solutions to pH 2.0. The precipitate contained biosurfactant was allowed to settle down and kept overnight at 4 °C .The precipitated biosurfactant was collected by centrifugation at 15,000 rpm for 20 min. The precipitate was neutralised and recentifuged at 12000 rpm for 10 min. The precipitate was freeze dried and stored.

Characterization of biosurfactant

Thin layer chromatography

The extracted biosurfactant was characterized by thin layer chromatography (TLC) using silica gel plate (Silica gel 60; Merck, Darmstadt, Germany) and chloroform-methanol-water (65:15:2, v/v/v) as solvent system. The spots separated were visualized by placing the silica gel plate in iodine vapour in a glass chamber.

Fourier transform infrared spectroscopy

The extracted biosurfactant was subjected to Fourier transform infrared spectroscopy (FT-IR) analysis to identify the chemical bonds or the functional groups present. One milligram (freeze dried) purified biosurfactant was ground with 100 mg KBr pellet and pressed with 7,500 kg for 30 seconds to obtain translucent pellet. For this study, AVATAR-NICOLAT FT-IR system was used with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. All the measurements consisted of 500 scans and a KBr pellet was used as background reference.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was performed on a THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II. The column used was ZB 5 - MS capillary standard non - polar column. The oven temperature program was as follows: 70 ºC raised to 260 ºC at 6 ºC /min. The electron impact ion source was maintained at 200°C. The electron energy was set at 70 eV.

HPLC analysis

The derivatization and HPLC analysis were carried out using a modified method described by [9] and [4] rhamnolipid. The TLC fraction was further tested for their purity by a HPLC analysis. One mg of extract of *Serratia rubidaea* SNAU02 was dissolved in 1ml distilled water and 1ml of acetonitrile containing 2-bromoacetophenon ;Et3N)and heated for 1h at 80ºC.The mixture was filtered through 0.22 µm syringe filter to remove particulate materials. Monocrotophos was used as standard.

Detection of rhl gene (rhamnosyltransferase) and sequencing

Primers used for rhlAB were based on the gene sequences reported from *Pseudomonas aeruginosa* PA01 (Ochsner et al.1994). The amplification of rhl gene was performed using Rhlabf (5'-CAGGCCGATGAAGGGAAATA-3') and RhlAbr (5'AGGACGACGAGGTGGAAATC-3') primers. The reaction mixture was carried out in a (50µl) volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxy nucleoside triphosphate, 2 U *Taq* DNA polymerase (Invitrogen), 0.2 µM forward primer, reverse primer and 0.1 µg of template genomic DNA. The PCR condition were as follows: (i) 5min at 95 ºC (ii) 30 cycle with 1 cycle consisting of 30s at 95ºC,1 min at 50 ºC, and 2min at 72 ºC, and (iii) a final extension step of 10min at 72 ºC. The PCR product was separated and by agarose gel electrophoresis and visualized, purified and sequenced. The sequence was submitted in the GenBank database. The gene bank accession number (KF835609) for rhlAB gene was obtained from NCBI.

Antifungal activity against plant pathogens

Antifungal activity was screened by agar well diffusion method (11). The various concentration of biosurfactant (0,100, 250 and 500 µg/ml) were tested against plant pathogens, *viz. Fusarium oxysporum*. The PDA medium was poured in to the sterile Petri plates and allowed to solidify. The test fungal cultures were evenly spread over the PDA using sterile cotton swabs. Then wells (6 mm) were made in the medium using sterile cork borer. Each well was filled with various concentration of 200µl of biosurfactant solution and distilled water served as control. The plates were incubated at 27°C for 72 h and the plates were observed for formation of clear inhibition zone around the well and zone of inhibition was measured.

Biosurfactant toxicity assay

Phytotoxicity assay

The phytotoxicity assay for biosurfactant was performed according to Tiquia et al., (11)The seed germination and root elongation were carried out using cabbage *(Brassica oleracea*). Different concentration of extracted biosurfactant solutions (0,100, 250 and 500 µg/ml) were prepared with distilled water. The toxicity was assessed in sterilized Petri plate containing Whatman No.1 filter paper. The cabbage seeds were pretreated with 1% sodium hypochlorite for 15min. The Petri dish with 10 cabbage seeds was inoculated with 5.0ml of the biosurfactant solution and incubated at 27ºC for five days. Then the number seed germination, root elongation and germination index (GI) were determined as follows:

Relative seed germination = (%) x 100

Relative root length (%) = x 100

$$GI = \frac{\text{Seed germination} \times \text{Root length of treatment}}{\text{Seed germination} \times \text{Root length of control}} \times 100$$

Controls were prepared with distilled water. The analysis was performed in triplicate.

Artemia assay

The toxicity assay was performed with different concentration of the biosurfactant (0,100, 250 and 500 µg/ml) using brine shrimp (*Artemia salina)*). Brine shrimp eggs were obtained from the Center of Advance Study in Marine Biology, Annamalai University, Parangipettai, India and the larvae were used within one day of hatching. Biosurfactant solution with saline water (33.3 g/l) was taken in penicillin tube containing 10 brine shrimp larvae in 5.0 ml of saline water per tube. The brine shrimp larvae in each penicillin tube were tested using 5.0 ml per different concentration of isolated biosurfactant solution and were observed for 24 h to calculate the mortality. The toxicity threshold concentration, expressed as biosurfactant concentration per 100 ml of saline water, was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was run in triplicate and saline water was used as the control .**Cleaning pesticide residues in tomatoes.**

One kg of each tomato was treated with the 100 ppm of Monocrotophos solution and allowed to stand for 24 h. After that, the tomatoes were collected and air dried for 1 hour. Further, the tomatoes were soaked for 30 min in the following treatments, T_1 (1000 ml distilled water), T_2 (1000 ml luke warm water), T_3 (2 per cent $NaCl_2$ in 1000 ml of distilled water), T_4 (2 per cent $NaCl_2$ in 1000 ml of luke warm) and T_5 (10 mg biosurfactant in 1000 ml distilled water) respectively. Followed that the tomatoes were carefully washed with 100ml of double distilled water and the washings were then collected and subjected to UV/Vis detector HPLC analysis.

Result and Discussion

The isolate *Serratia rubidaea* SNAU02 was identified as potential biosurfactant producer. The study resulted in a novel bacterial isolate, characterized as *Serratia rubidaea* SNAU02 and found possessing rhl gene. The detection of rhl gene confirmed the ability of *S. rubidaea* SNAU02 for the production of rhamnolipid type of biosurfactant. The expected size 777bp (Fig.1) for rhamnosyltransferase gene, which eventually showed biosynthetic pathway of rhamnolipid in *S. rubidaea* SNAU02.The strain exhibited antifungal activity (Fig.2) and demonstrated no toxicity against the seeds of *Brassica oleracea* and *Artemia salina* employed as a bio-indicator [6,7].

RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously [8]. RSM is not only used for optimization of culture parameters in the fermentation process, but also for studying the combined effects of medium components [1]. The model was designed with the result of 50 treatments (runs) presented, actual and predicted value of ST reduction in Table 1. .The ANOVA analysis results showed that Inoculum size (gl^{-1}), Peptone (gl^{-1}),Initial pH, temperature and Shaker (rpm) had a significant effect on biosurfactant production (Table 2). The fit of the model was expressed with the coefficient of determination R^2 that was 0.9471, indicating that 94.71% of variability in the response could be explained by this model. The adjusted R^2 value of the model was 0.9106 and predicted R^2 value was 0.8112. These results showed that application of RSM enhanced the biosurfactant production with the combination of inputs and proved that cashew apple juice could be effectively used as substrate for the production of biosurfactants.

The results of the HPLC chromatograms of all the five treatments (Table 3) on pesticide washing in the tomato were presented in Fig. 4.a-d. There was no change in peak observation for the treatments of T_1 and T_2. Whereas a slight bend was observed in the retention time of 2.98 in T_3. In treatment T_4 a single peak was observed in the retention time 3.0 which indicated the presence of pesticide residues in a little quantity.

In treatment T_5 which was treated with 10 ppm concentration of biosurfactant solution showed three broad peaks with the retention time of 2.432, 2.784 and 2.955 which denote the cleanup of pesticide as well as the breakdown of parent compound i.e. Monocrotophos, into intermediate products which may be non-toxic. The peak observed with the retention time of 3.147 for the standard was used in the comparative study for cleaning up pesticide residues. Thus, it was found that, biosurfactants were able to disintegrate the Monocrotophos compound sediment in tomato vegetables and it was evidenced by the different peak values for the treatment T_5.

Conclusion

The biosurfactant produced by *Serratia rubidaea* SNAU02 isolated from hydrocarbon-contaminated soils of Cuddalore district, Tamilnadu, India. The molecular characterization of the biosurfactant revealed the presence of rhamnolipid. The strain exhibited antifungal activity and demonstrated no toxicity against the seeds of *Brassica oleracea* and Artemia salina employed as a bio-indicator. The concentration of 250 µg/ml biosurfactant application controlled the *Fusarium* wilt of tomato crop.

The treatment washings revealed that, there was no change in peak observation for T_1 and T_2, T_3 and T_4. Whereas, T_5 showed three broad peaks with the retention time of 2.432, 2.784 and 2.955 respectively, which denote the cleanup of pesticide into intermediate products which may be non-toxic. Based on the present findings, the use of agro-industrial wastes as substrates would be an ideal process for the production of rhamnolipid biosurfactant. Considering the growing concern over the waste management challenges, the using of agro-industrial waste can be used as a cleaner bioprocess for the utilization of industrial waste as alternate substrate and the use of rhamnolipid in washing of pesticide indicates that it will be an efficient agent for cleaning of pesticide residues in tomato.

Table-1 Experimental conditions of 2^5 factorial central composition design showing experimental and predicted surface tension reduction

Run No.	Run order	Inoculum size (gl^{-1})	Peptone (gl^{-1})	Initial pH	Temperature (°C)	Shaker (rpm)	Experimental ST reduction (mN/m)	Predicted ST reduction(mN/m)
1	16	4	7	8	45	120	34.200	34.777
2	39	3	5	7	30	150	34.100	34.962
3	13	2	3	8	45	120	34.200	34.571
4	50	3	5	7	40	150	47.00	46.973
5	9	2	3	6	45	120	34.200	33.568
6	18	4	3	6	35	170	38.100	38.557
7	25	2	3	6	45	170	34.200	34.746
8	11	2	7	6	45	120	34.200	33.489
9	46	3	5	7	40	150	47.000	46.973
10	40	3	5	7	50	150	34.100	32.907
11	23	2	7	8	35	170	38.200	36.245
12	31	2	7	8	45	170	37.300	36.707
13	44	3	5	7	40	150	47.000	46.973
14	22	4	3	8	35	170	35.200	35.847
15	17	2	3	6	35	170	39.100	35.847
16	21	2	3	8	35	170	37.500	37.184
17	26	4	3	6	45	170	37.500	36.762
18	30	4	3	8	45	170	37.500	35.756
19	14	4	3	8	45	120	36.200	35.684
20	20	4	7	6	35	170	37.500	35.131
21	42	3	5	7	40	200	34.100	36.690
22	2	4	3	6	35	120	39.200	34.955
23	1	2	3	6	35	120	35.200	38.604
24	4	4	7	6	35	120	39.400	35.394
25	7	2	7	8	35	120	32.100	37.450
26	38	3	5	9	40	150	34.200	33.955
27	33	1	5	7	40	150	34.100	33.289
28	49	3	5	7	40	150	32.100	33.318
29	35	3	0	7	40	150	47.000	46.973
30	8	4	7	8	35	120	34.100	34.823
31	43	3	5	7	40	150	34.200	34.065
32	5	2	3	8	35	120	47.000	46.973
33	24	4	7	8	35	170	34.200	33.759
34	34	5	5	7	40	150	34.200	34.518
35	32	4	7	8	45	170	34.200	34.657
36	41	3	5	7	40	100	33.100	34.617
37	29	2	3	8	45	170	34.200	33.014
38	3	2	7	6	35	120	34.100	35.052
39	45	3	5	7	40	150	47.000	46.973
40	12	4	7	6	45	120	34.200	35.524
41	36	3	9	7	40	150	34.200	35.524
42	10	4	3	6	45	120	34.200	33.146
43	47	3	5	7	40	150	47.000	46.973
44	15	2	7	8	45	120	34.200	35.029
45	19	2	7	6	35	170	34.100	36.129
46	28	4	7	6	45	170	34.200	34.152
47	6	4	3	8	35	120	34.100	34.682
48	27	2	4	6	45	170	34.100	33.954
49	48	3	5	7	40	150	47.000	46.973
50	37	3	5	5	40	150	34.100	34.680

*Table-2 ANOVA: Effect of five variables on biosurfactant production**

Source	Sum of squares	Df	Mean square	F value	p-value Prob > F
Model significant	1025.13	20	51.26	25.94	< 0.0001
A-A3.40	1	3.40	1.72	0.1998	
B-B5.38	1	5.38	2.72	0.1098	
C-C	3.70	1	3.70	1.87	0.1816
D-D	8.07	1	8.07	1.09	0.0526
E-E7.20	1	7.20	3.65	0.0662	
AB1.32	1	1.32	0.67	0.4203	
AC10.47	1	10.47	5.30	0.0287	
AD0.26	1	0.26	0.13	0.7180	
AE6.75	1	6.75	3.42	0.0747	
BC0.58	1	0.58	0.29	0.5928	
BD0.14	1	0.14	0.070	0.7936	
BE1.02	1	1.02	0.51	0.4792	
CD13.91	1	13.91	7.04	0.0128	
CE2.94	1	2.94	1.49	0.2323	
DE0.75	1	0.75	0.38	0.5425	
$A^2$292.49	1	292.49	148.03	< 0.0001	
$B^2$292.49	1	292.49	148.03	< 0.0001	
$C^2$292.49	1	292.49	148.03	< 0.0001	
$D^2$294.74	1	292.74	148.17	< 0.0001	
E^2 292.49	1	292.49	148.03	< 0.0001	
Residual	57.30	29	1.98		
Lack of fit	57.30	22	2.60		
Pure error	0.0000	7	0.0000		
Cor Total	1082.42	49			

Table-3 Cleaning of pesticide residues in Tomato using different washing solution

Treatments	Washing solutions
T1	1000ml distilled water
T2	1000ml lukewarm water
T3	2 percent sodium chloride in 1000ml of distilled water
T4	2 percent sodium chloride in 1000ml of lukewarm water
T5	10ppm of biosurfactant solution(10mg in 1000ml distillled water)

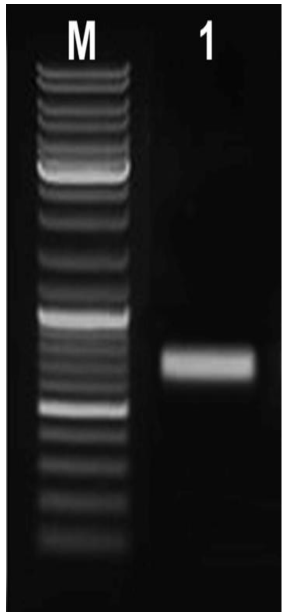

Figure 1: Agarose gel electrophoresis for PCR product of rhamnosyltransferase gene in S. rubidaea SNAU02 (Lane M(Marker)-1kb DNA ladder, Lane 1- rhamnosyltransferase gene - 777bp).

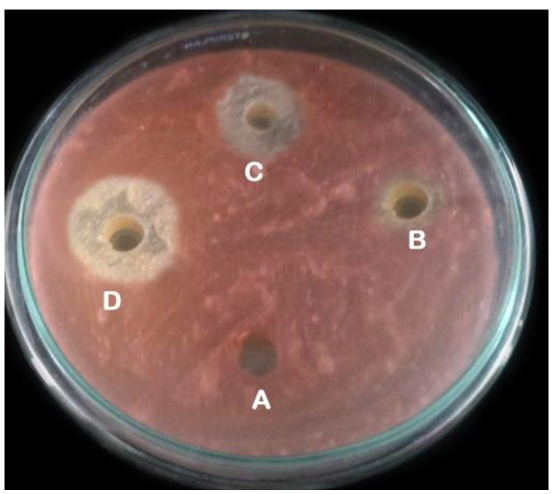

Figure 2: Antifungal activity of biosurfactant at various concentrations (A-control, B-100µg/ml, C-250µg/ml, D- 500µg/ml) against Fusarium oxysporum

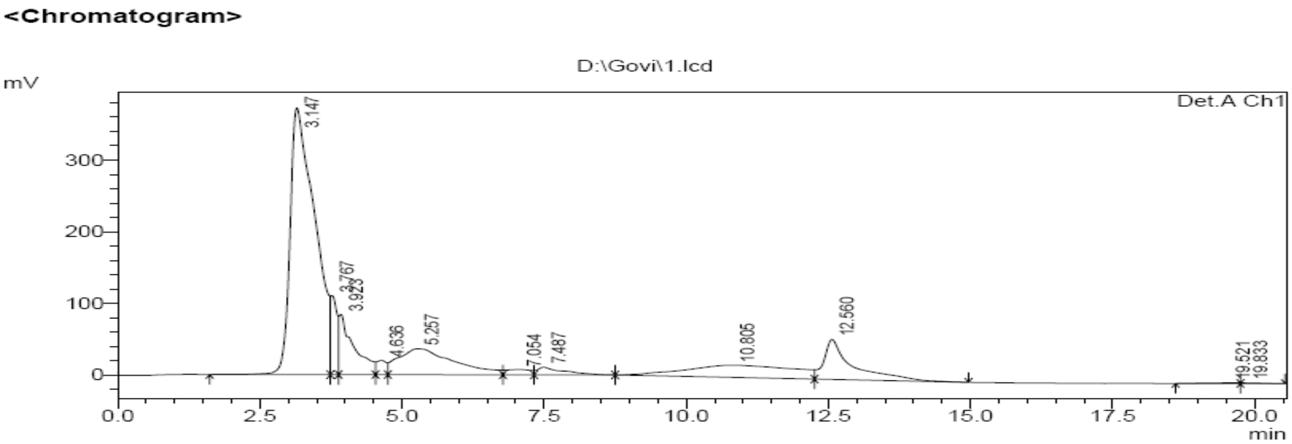

Figure 4: a. Monocrotophos Standard

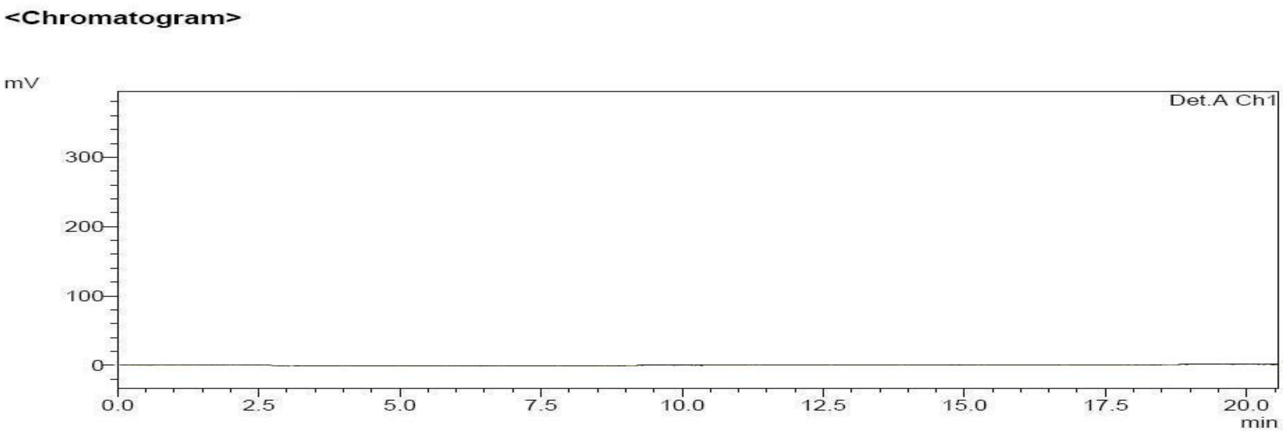

Figure 4: b. T1- 1000 ml distilled water

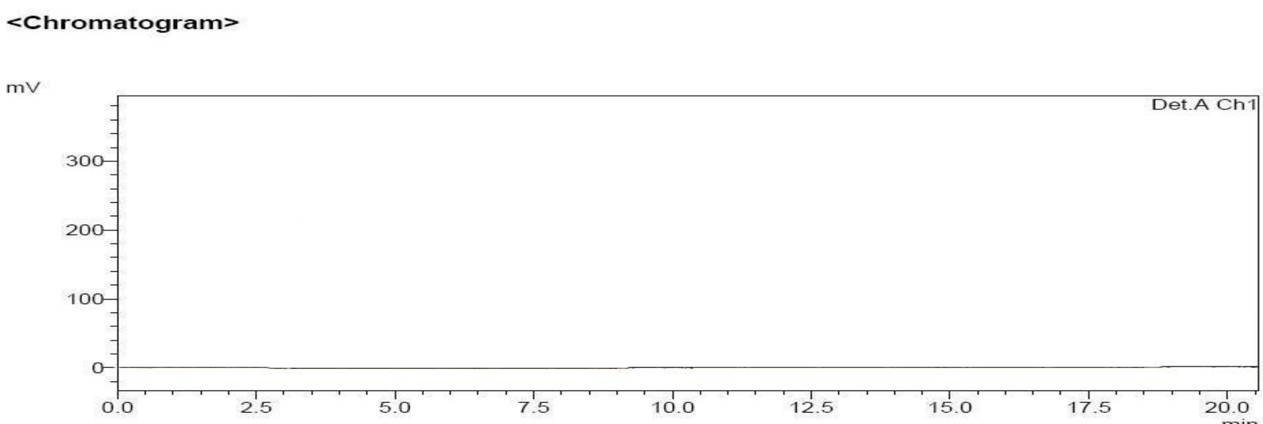

Figure 4: c. T2. 1000 ml lukewarm distilled water

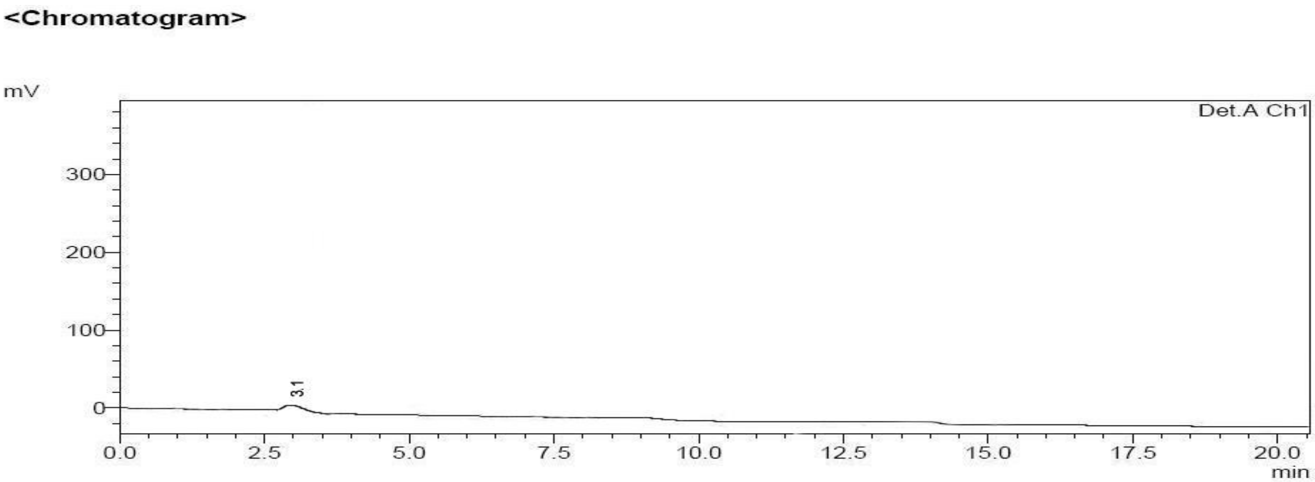

Figure 4: d. T3. 2 per cent NaCl in 1000 ml distilled water

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