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Isolation and Characterization of Organophosphate Pesticides Degrading Bacteria from Contaminated Agricultural Soil

Soni Yadav, Sitansu Kumar Verma and Hotam Singh Chaudhary

Department of Biotechnology, Madhav Institute of Technology and Science, Gwalior, M.P.-474005, India

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Corresponding Author:
Hotam Singh Chaudhary
Department of Biotechnology,
Madhav Institute of
Technology and Science,
Gwalior, M.P.-474005, India
Tel: +91-9981203686
Email: hotamsingh@gmail.com

Abstract: The soil sample was collected from Agriculture University Gwalior, Madhya Pradesh, India which is having a history of repeated pesticide application. Bacterium capable of degrading Malathion and Dichlorvos were isolated and identify as *Staphylococcus sp. Micrococcus sp. Entrobacte sp. Bordetella sp. Pseudomonas sp. and Klebsella sp*. The growth of all six pesticide degrading isolates was assessed in Mineral Salt Medium (MSM) canting 100 mg/lit of each pesticide. The maximum growth rate by the isolates *Pseudomonas* sp. AUG12 were 1.564 and 1.435 for Malathion and Dichlorvos respectively after 140 h Plate assay revealed that *Pseudomonas* sp. AUG12 could grow with high concentration of Malathion (1900 mg/lit) and Dichlorvos (1500 mg/lit). The total protein concentration was higher in the supernatant of *Pseudomonas* sp. AUG12 extracellular fraction which is 97 µg/mL for Malathion and 99 µg/mL for Dichlorvos. The beast *Pseudomonas* sp. AUG12 was therefore used in further experiment. The maximum growth ret of *Pseudomonas* was recorded at 30°C and pH 7.0 the growth of *Pseudomonas* was maximum in the presence of sucrose fallowed by starch maltose the least growth was recorded in lactose the growth of *Pseudomonas* sp. AUG12 was maximum in the presence of peptone fallowed by yeast extract and beef extract. The least growth was recorded in urea as nitrogen source. The result of this study suggested a relation among microbial growth pH and temperature in MSM medium with different carbon and nitrogen source. The Organophasphorus Phosphatase (OPP) production and that OPP concentration were higher in the supernatant of *Pseudomonas* sp. AUG12 extracellular fraction.

Keywords: Malathion, Dichlorvos, *Pseudomonas* sp. Pesticides, OPP

Introduction

Although some persistent pesticide have been banned from agricultural and public health use during the past few years. High concentration of pesticides has been contaminating in soil, water and sediment samples. Bioremediation is an environmental cleanup process that currently being investigated for use on a wide verity of chemical (Aruna *et al*., 2014). It is the use of natural occurring microorganism to enhance biodegradation to detoxify the contaminant. Pesticides are organic compounds manufactured and used control destructive pests such as insect, plant disease organism and weeds in agricultural field. The application of pesticides may cause adverse effect. Among the different from of life and among the ecosystem the quality of ground water soil in land and terrestrial water and air are all affected by pesticide contamination (Eddleston *et al*., 2008; Ahn *et al*., 2011).

The use of microbes in the bioremediation and detoxification of many toxic xenobiotics, especially toxic pesticide is an efficient tool for the remediation of contaminated sites in the environment (Jamaluddin *et al*., 2012). Many microorganisms that are able to degrade organophosphate pesticide have been isolated from soil around the world. Previous research have shown that pesticide Malathion, Chlorochlorpyrifose, Dichlorvos degrading bacteria applied as single strains or as consortia to increase the rate of degradation of pesticide Malathion, Chlorochlorpyrifose, Dichlorvos in soil (McCoy *et al*., 2012; Qi *et al*., 2012; Yonar *et al*., 2014). Organophosphate pesticide constitutes a group of widely used very heterogeneous compound that

share a phosphoric acid derivative chemical structure (Basarslan *et al*., 2014; Hamer, 2010).

Degradation of pesticides is usually a combination of a number of processes, including microbial degradation and chemical hydrolysis and is also influenced by some physicochemical properties such as temperature, pH and carbon and nitrogen source (Gunther and Gunther, 1973). However, biodegradation is the primary mechanism of pesticide degradation and detoxification in soils. Thus bacteria and other microbes may have a major effect on the persistence of most organophosphate pesticides in soil (Surekha *et al*., 2008). Biodegradation is a common method for the removal (degradation and detoxification) of organophosphate pesticides because of its low cost and low collateral destruction of indigenous animal and plant organisms (Liu *et al*., 2007). Bacterial degradation is considered to be a major factor determining the fate of Malathion, Dichlorvos and other organophosphorus pesticide in the environment.

Malathion and Dichlorvos are belongs to organophosphate class of pesticide are most commonly used by farmers in India. Most synthetic organophosphate pesticides are highly toxic and are powerful inhibitors of acetylcholinesterase, an important enzyme involved in neurotransmission, in the form of acetylcholine substitutes (Goda *et al*., 2010; Singh *et al*., 2011). Environmental hazards and health risks caused by pesticides could therefore potentially affect human health and environment. So in situ degradation and detoxification of pesticides contaminated water and soils are very important (Chen *et al*., 2012).

The aim of the present study was to isolate and characterize new bacteria capable of degrading Malathion and Dichlorvos from agricultural soil. Many physicochemical parameters have been optimizing for the best growth of bacteria. Protein concentrations and OPP activity were also determined by various methods.

Materials and Methods

Soil

The soil sample used for the isolation of pesticide degrading bacteria were collected from a soya been field form agriculture University Gwalior. Triplicate soil sample were collected from the 5cm to 10cm layer. Soil sample were partially air dried and characterized. The properties of soil sample are listed in Table1.

Pesticide

Commercial grade Malathion (50%), Dichlorvos (76%) were procured from local pesticide shop from Gwalior, Madhya Pradesh. These pesticides belong to organophosphate class of pesticides and structures of pesticide are shown in Fig. 1.

Chemicals

The Mineral Salt Media (MSM) consist of (g L^{-1}) $Na_2HPO_4.7H_2O$ 3.6; $(NH_4)_2SO_4$ 1.0; KH_2PO_4 1.0; $MgSO_4$ 1.0; Fe $(NH)_4$ citrate 0.01; $CaCl_2.2H_2O$ 0.1 and 10 mL of trace element solution contained (mg L^{-1}) $ZnSO_4$. $7H_2O$ 10; Mncl2. $4H_2O$ 3.CoCl. $6H_2O$ 1; $NiCl_2$ $6H_2O$ 2; Na_2MoO_4. $2H_2O$ 3; H_3BO 3; H_3BO_3 30; $Cucl_2$. $2H_2O$ 1. The pH of the medium was adjusted to 7.0 (Cycon *et al*., 2009; Abo-Amer and Aly, 2011).

Isolation of Pesticide Degrading Bacteria

All the samples were used for isolation of pesticide degrading bacteria by enrichment culture technique using (MSM) supplemented with Sucrose 2.0, Yeast extract 3.0 containing each pesticide (Malathion, Dichlrovose) with the final pesticide concentration 100 mg/lit. The procedure to isolated bacteria consisted of the addition of 1.0 gm of soil sample to a flask containing 100 mL of (MSM) medium with 100 mg/lit of each pesticide as a fungal inhibitor (Atit *et al*., 2013). The culture flasks were incubated on orbital shaker with 120 rpm at 30°C. Two ml cultures were then transfer to a fresh medium containing 100 mg/pesticide and incubated. From fifth transfer 200 µL were plated on nutrient agar medium and incubator for 24 h at 30°C. The pure culture colonies bacteria stain were maintain by striking on nutrient agar slant and stored at 4°C (Lederberg and Lederberg, 1952).

Gram Reaction and Cell Morphology

Grams staining of pure culture of all isolates were performing to study gram reaction and cell morphology. The isolates colonies color, transparency, shape and texture were observed directly.

Biochemical Characterization

The isolates were subjected to biochemical test included indole production, methyl red, citrate utilization, triple sugar iron, arginine hydrolysis, Voges Proskauer, casein utilization test and sugar fermentation test. Bergey's Manual of Determinative Bacteriology and 'PIBWIN' online software for bacteria identification were used as a reference to identify the isolates (Buchanan and Gibbons, 1984; Cowan, 1974).

Plate Assay for Pesticides

The maximum concentration of Malathion and Dichlorvos tolerated by the bacterial strains was determined by streaking the isolated strains on MSM agar plates containing various concentrations of each pesticide 100-2,000 mg L^{-1}. All the plates were incubated for 30°C until visible growth was observed (Shafiani and Malik, 2003).

Table 1. Characteristics of soil sample collected from Agriculture University, Gwalior

Sample No.	Depth	pH	Moisture contain (%)	Clay (%)	Silt (%)	Sand (%)
1	10	7.3	4.71	65.0	8.16	26.8
2	15	6.8	4.71	70.2	8.30	21.4
3	10	6.9	7.54	69.1	6.80	24.0
4	15	7.3	11.32	59.0	8.80	32.0
5	10	6.3	7.54	54.0	10.80	35.1
6	15	6.7	7.54	59.1	9.00	26.1
7	10	6.5	5.66	63.3	7.60	29.0
8	15	6.7	7.54	51.8	10.30	37.6
9	10	6.8	12.26	60.0	15.00	25.0
10	15	7.2	20.75	56.8	14.60	142.1
11	10	7.1	9.43	62.0	9.16	28.8
12	15	7.3	15.09	55.5	10.30	34.1

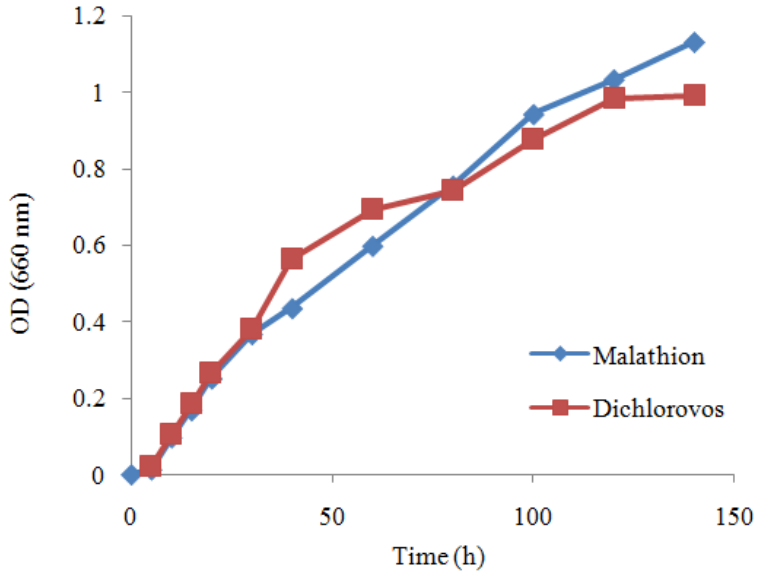

Fig. 1. Bacterial growth by Staphylococcus sp. AUG6, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

Degradation Studies of Pesticide

The all isolated bacterial colonies were pre cultured in 10mL LB medium and incubated 37°C. Bacterial cultures with 1 OD at 660 nm were used as inoculum. Degradation analyses were performed in 250 mL conical flask containing 100 mL of MSM supplemented with sucrose (10.0 g^{-lit}) and peptone (2.0 g^{-lit}). Sterilized Malathion and Dichlorvos (100 mg^{-lit}) were added after autoclaving the MSM medium (Yang *et al*., 2006).

Media were inoculated with 5 mL^{-lit} biomass and incubated in the dark on an orbital shaker (100 rpm) at 30°C for 7 days. Two experimental sets for cell growth were prepared in duplicate. The biomass concentration was estimated by optical density measurement in 1 cm cuvettes at wavelength of 660 nm using UV-Visible spectrophotometer.

Production of Protein

All the isolates were used for production of protein by using (MSM) supplemented with Sucrose 2.0, Yeast extract 3.0 containing each pesticide (Malathion, Dichlrovose) with concentration 100 mg/lit for 7 days. Cultures were centrifuged for 15 min at 7000 rpm and 4°C. The supernatants were used for the amount of total protein described by the quantitation method by Lowry *et al.* (1951). The Lowry Assay quantitates protein depending on measuring changes in absorbance at a wavelength of 660 nm when Folin reagent interacts with protein.

Optimization of Physicochemical Condition

The growth efficacy of AUG in pesticides was studied of different temperature (25-50°C) and pH (5 to 10). The optimized pH 7.0 and temperature 30°C were selected to studying the various physicochemical

parameters such as initial pesticide concentration (100-800 mg/L), effect of NaCl and effect of Yeast extract on the growth pattern of AUG12.

Software for Data Analysis and Bacteria Identification

MS Excel 7 also used for the preparation of various graph. An offline software 'PIBWIN' was used for identification of the isolated bacteria http://www.tgw1916.net/bacteria_logare.html.

Extraction of Enzyme and Organophosphorous Phosphatase Assay

The cells grown in MSM broth containing 100 mg/L pesticides were harvested and pelleted by centrifugation at 12,000 rpm for 120 min. The supernatant was used to determine extracellular organophosphorous phosphatase activity (Wang *et al*., 2008). All the experiments were repeated two times. Organophosphorous phosphatase activity was measured by adding 100 µL of crude enzyme to 900 µL of Tris HCl containing 10 mg/mL p-nitrophenol phosphate and the mixture was incubated for 15 min at 37°C. After that reaction was terminated by addition of 1 mL of 10% trichloroacetic acid and 1 mL of 10% sodium carbonate and the liberated yellow colored end product p-nitrophenol was measured in a spectrophotometer at 410 nm. One unit (U) of OPP activity is defined as the amount of enzyme liberating 1 lmol of p-nitrophenol per minute at 37°C (Alvarez-Macarie *et al*., 1999).

Results

Isolation and Identification of Pesticide Degrading Bacteria

Six different microorganisms were isolated from the pesticide contamination Soil samples by enrichment culture method. All isolates were found to shown the ability to grow in the presence of pesticide, the strains named as AUG 6, 8, 9, 11, 12, 13 and stored in the glycerol stock for further studies.

Identification of Pesticide Degrading Bacteria

Morphological, Gram staining and biochemical test were perform with Bergey's Manual of Determinative Bacteriology and using 'PIBWIN' online software for tentative identification of the isolates. The results of gram staining and biochemical test are shown in Table 2. It was found that, the isolates AUG6, AUG8, AUG9, AUG11, AUG12 and AUG13 were *Staphylococcus sp. Micrococcus sp. Entrobacte sp. Bordetella sp. Pseudomonas sp. and Klebsella sp.* respectively (Table 2 and 3).

Growth by the Isolates in Mineral Salt Medium (MSM)

The degradation of Malathion and Dichlorvos perform under the aerobic condition by each strain for a period of 140 h in Mineral Salt Medium. Cell growth was measured by measuring OD 660 nm by following periodic interval. The bacterial growth increased rapidly during 10 h of incubation. The results for the isolates *Pseudomonas* sp. AUG12 showed best growth patterns for Malathion and Dichlorvos within 140 h (Fig. 1-6).

Plate Assay for Malathion and Dichlorvos

Plate assays revealed that *Pseudomonas sp. Staphylococcus sp. Entrobacte sp. and Klebsella sp.* had a higher tolerance to Malathion (1700-1900 mg/L) and the least tolerance was noted for *Micrococcus* (1200 mg/L) shown in Fig. 7. Similarly *Micrococcus sp. Entrobacte sp. Pseudomonas sp.* had a higher tolerance to Dicholorovos (1500-1600 mg/L) and the least tolerance was noted for *Staphylococcus sp. Bordetella sp. and Klebsella sp.* (1300 mg/L).

Total Protein Concentration

Total protein content was measured in all supernatant of isolates. According to the Lowry Assay, the protein concentration for the AUG12 was the highest, while AUG8 was the lowest (Fig. 8).

Effect of Temperature

Bacteria required optimum temperature for growth which is important for degradation of pesticides. The isolate *Pseudomonas* sp. AUG12 showed maximum growth at 27°C, 37°C and 40°C and almost no growth was found at 50°C. This might be due to thermal inactivation of protein and enzyme activity. The bacteria showed rapid growth pattern that is within 22hr was measured at 30°C (Fig. 9).

Effect of pH

Bacteria required optimum pH for maximum growth which is important for degradation of pesticides. The isolate *Pseudomonas* sp. AUG12 showed maximum growth at pH 7, 8 and almost no growth was found at pH 5, 6. This might be due to denaturation of protein and enzyme activity. The bacteria showed rapid growth pattern that is within 40 h was absorbed at pH 7 (Fig. 10).

Effect Initial Pesticide Concentration

The growth performance of the *Pseudomonas* sp. AUG12 strain was studies by increasing initial dye concentration (100-800 mg/L). The growth activity was lower at 500 mg/lit and above. It could effective growth up to 100 mg/lit of Malathion and Dicholorovos within 100hr and decreased, when pesticide concentration increase to 800 mg/lit (Fig. 11).

Effect of Carbon Source

Effect of different carbon source such as starch, lactose, sucrose, glucose and maltose were evaluated on growth of *Pseudomonas* sp. AUG12.

Table 2. Colony characteristics, Morphological characteristics of six different Malathion and Dichlorvos degrading isolates grown on nutrient agar at 30°C for 24 h (S, small; M, Moderate; DW, Dirty White; LY, Light Yellow; LC, Light Cream; NP, No Pigment; TL, Translucent; OP, Opaque)

	Colony characterization							
Isolates	Size	Shape	Margin	Elevation	Surface texture	Consistency	Opacity	Pigmentation
AUG-6	M	Round	Entire	Convex	Smooth	Gummy	OP	LY
AUG-8	S	Irregular	Lobed	Low convex	Smooth	watery	TL	NP
AUG-9	M	Irregular	Uneven	Low convex	Smooth	Gummy	OP	NP
AUG-11	M	Round	Even	Convex	Rough	Gummy	OP	DW
AUG-12	S	Uneven	Entire	Flat	Smooth	Gummy	OP	LY
AUG-13	M	Irregular	Lobed	Flat	Rough	Gummy	OP	LC

Table 3. Biochemical characteristics of six different Malathion and Dichlorvos degrading isolates grown on nutrient agar at 30°C for 24 h (R, Red; Y, Yellow)

Biochemical Ch1aracterization	AUG6	AUG8	AUG9	AUG11	AUG12	AUG13
Indole production	-	-	+	-	-	-
Urea hydrolysis	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Gelatin hydrolysis	-	-	-	-	-	-
Citrate utilization	-	+	+	+	+	+
Starch hydrolysis	-	+	+	-	-	+
Motility test	+	-	+	+	+	-
MR test	-	-	-	-	-	-
VP test	-	-	-	+	-	-
Arginine hydrolysis	-	-	-	-	-	-
Casein utilization	+	-	-	-	-	-
TSI	Y	R/Y	R/Y	Y	R/Y	R/Y
Sugar test	+	+	+	+	+	+
H_2S test	-	-	-	-	-	-
Co_2 test	-	-	-	-	-	-
Gram staining	+	-	-	-	-	-
Identified cultures	*Staphylococcus sp.*	*Micrococcus sp.*	*Entrobacte sp.*	*Bordetella sp.*	*Pseudomonas sp.*	*Klebsella sp.*

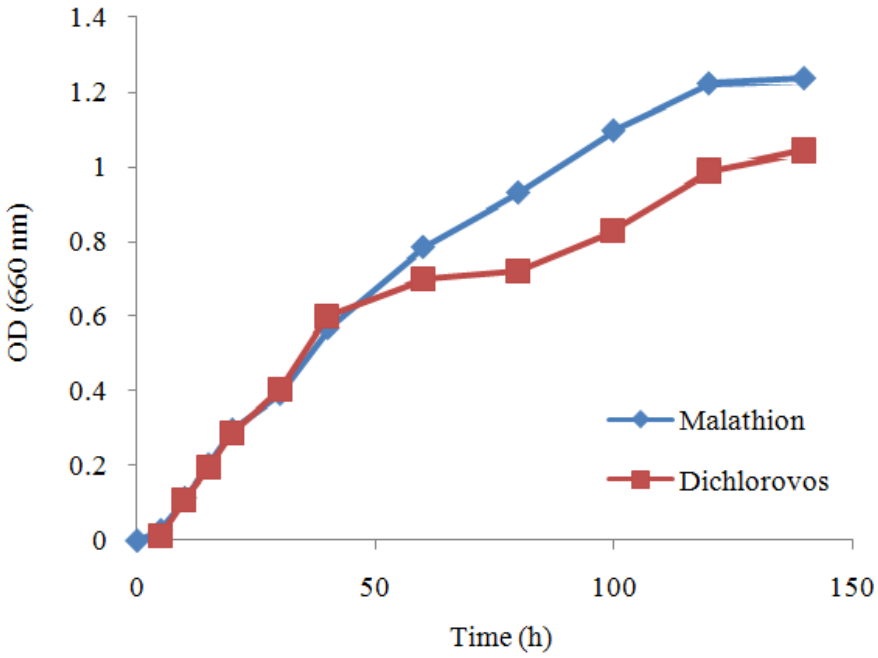

Fig. 2. Bacterial growth by *Micrococcus* sp. AUG8, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

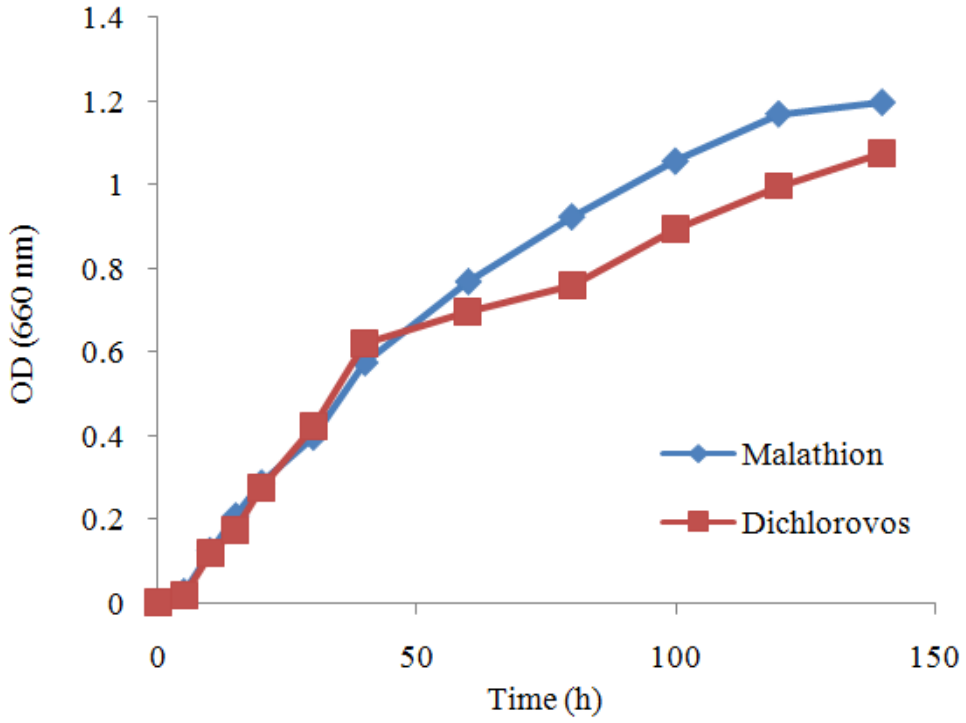

Fig. 3. Bacterial growth by *Entrobacte* sp. AUG9, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

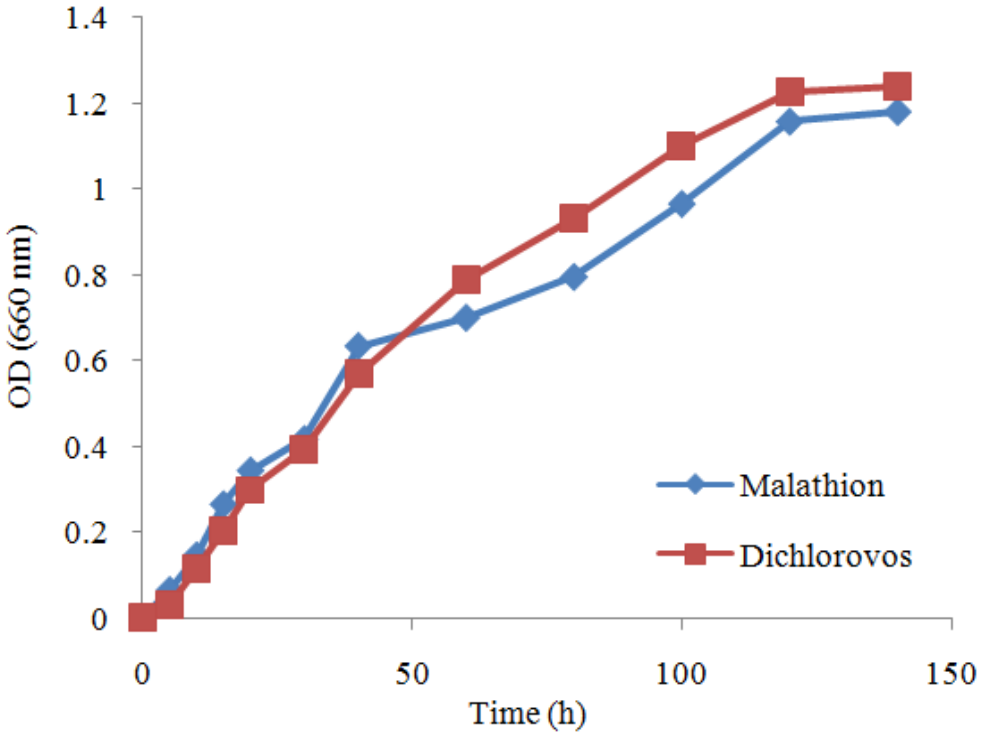

Fig. 4. Bacterial growth by *Bordetella* sp. AUG11, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

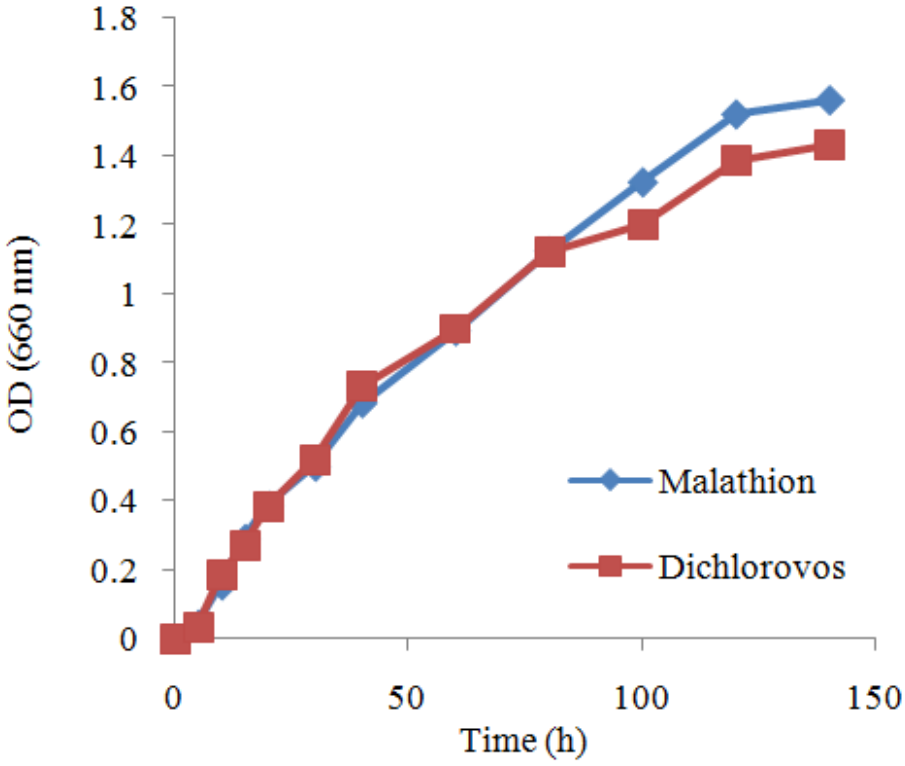

Fig. 5. Bacterial growth by *Pseudomonas* sp. AUG12, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

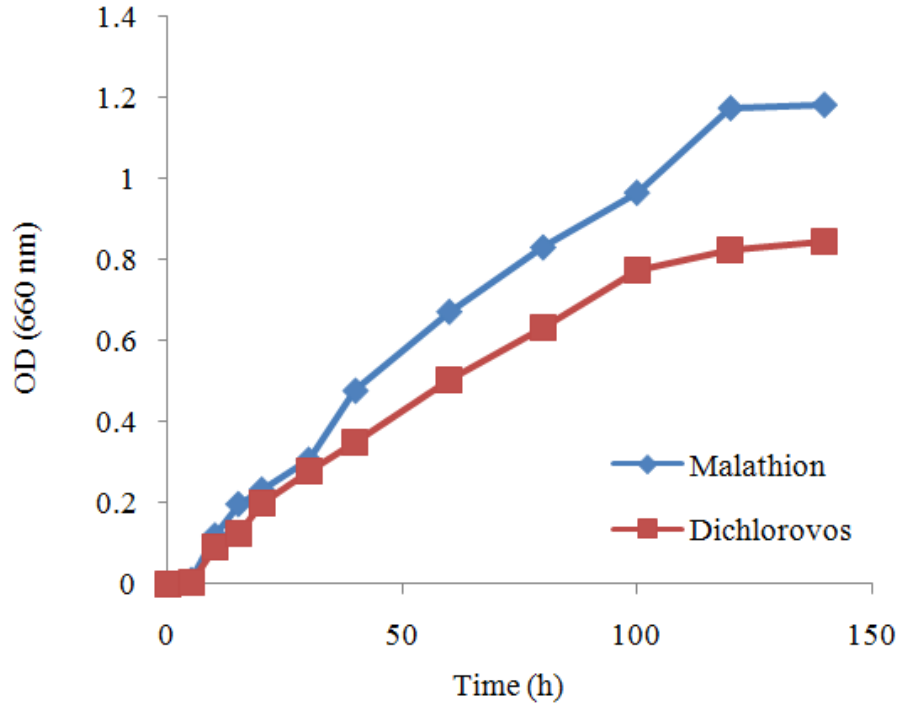

Fig. 6. Bacterial growth by *Klebsella* sp. AUG13, in mineral salt medium supplemented with Malathion and Dichlorvos as a sole carbon source during biodegradation studies

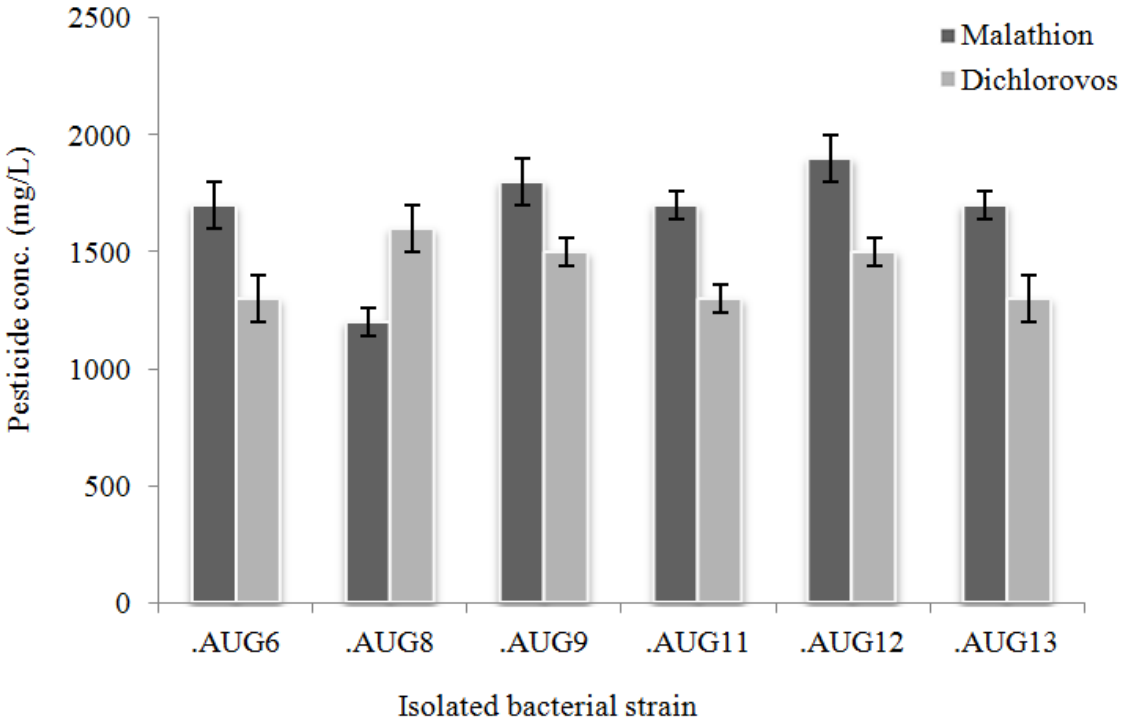

Fig. 7. Bar graph showing growth of the isolated bacterial strains on Minimal Salt (MS) agar plates supplemented with various concentrations (100-2,000 mg/L) of Malathion and Dichlorvos

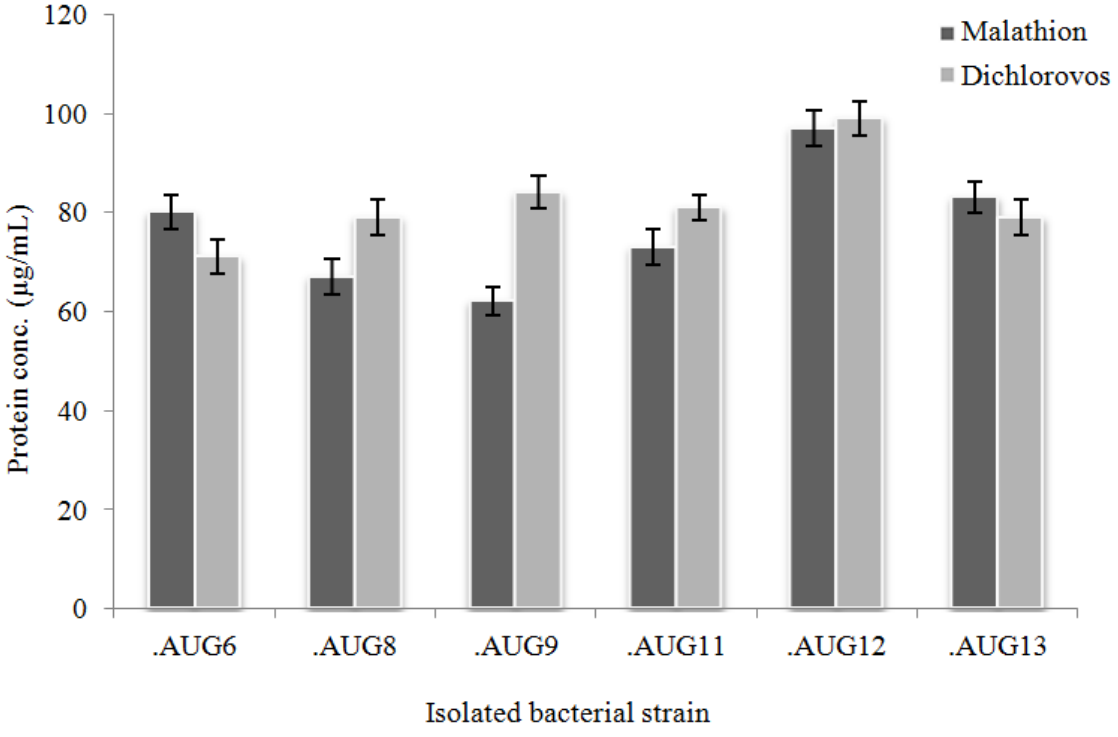

Fig. 8. Bar graph showing concentration of total protein produced by isolated bacterial strains on Minimal Salt (MS) agar plates supplemented with various concentrations (100 mg/Lit) of Malathion and Dichlorvos

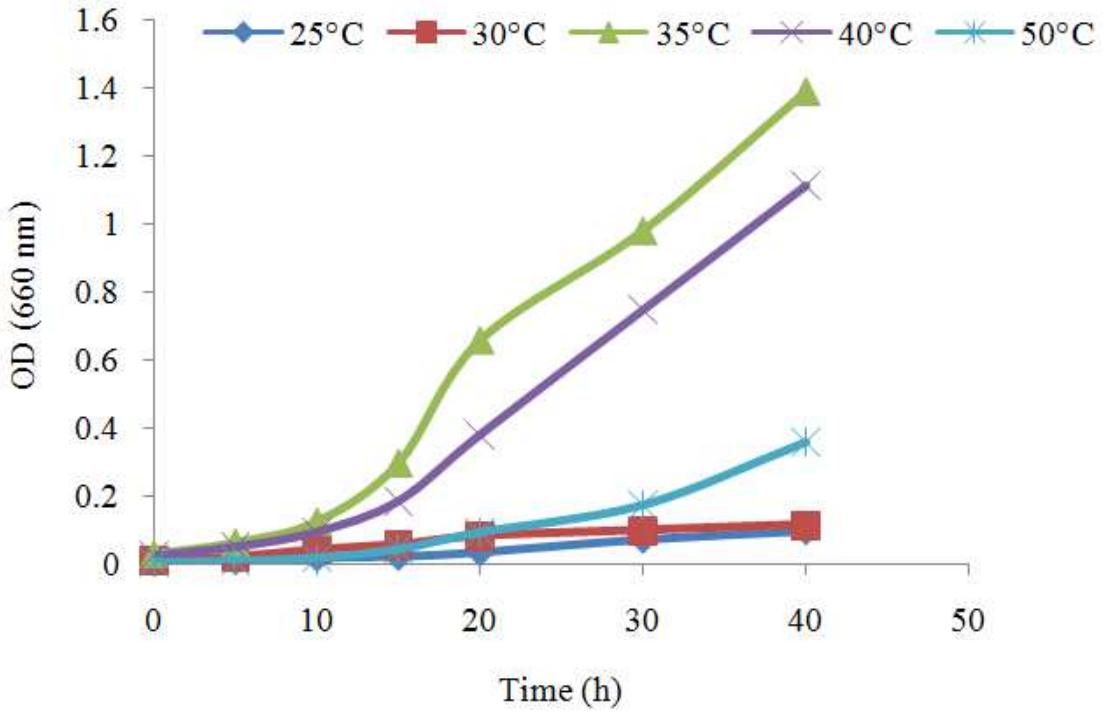

Fig. 9. Effect of temperature on the growth of *Pseudomonas* sp. AUG12 bacteria in Minimal Salt broth containing Malathion

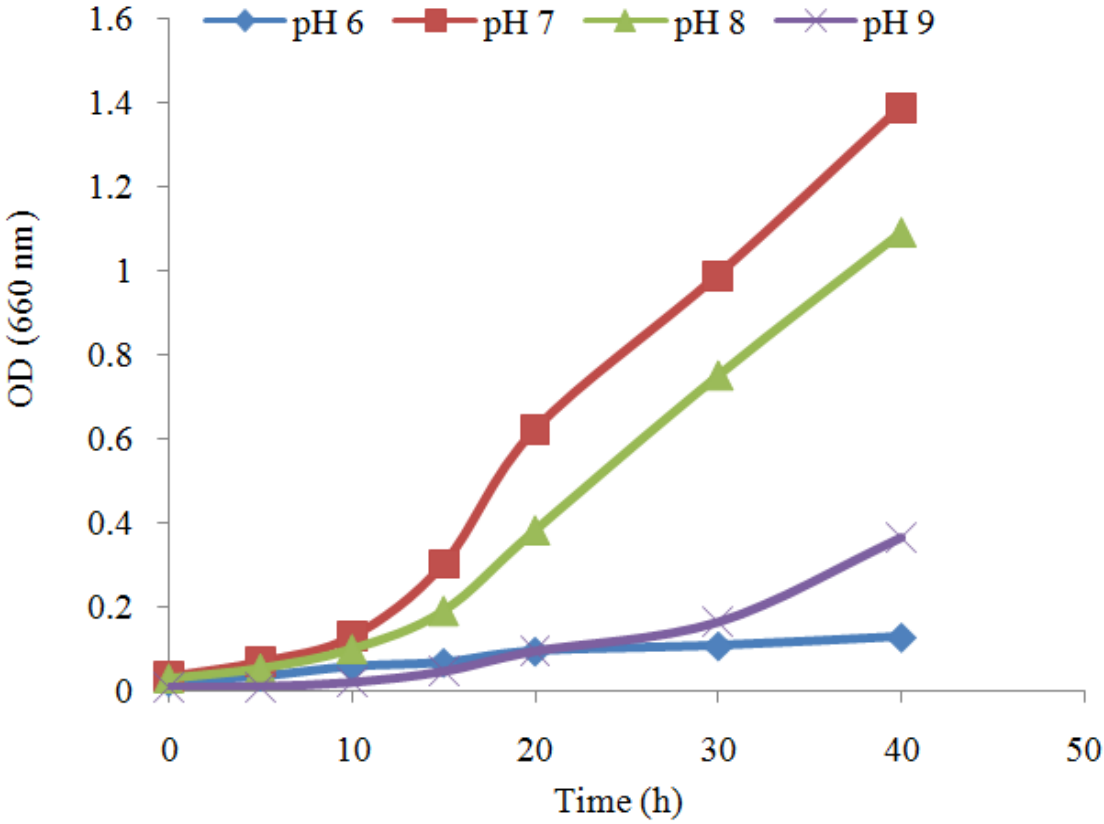

Fig. 10. Effect of pH on the growth of *Pseudomonas* sp. AUG12 bacteria in Minimal Salt broth containing Malathion

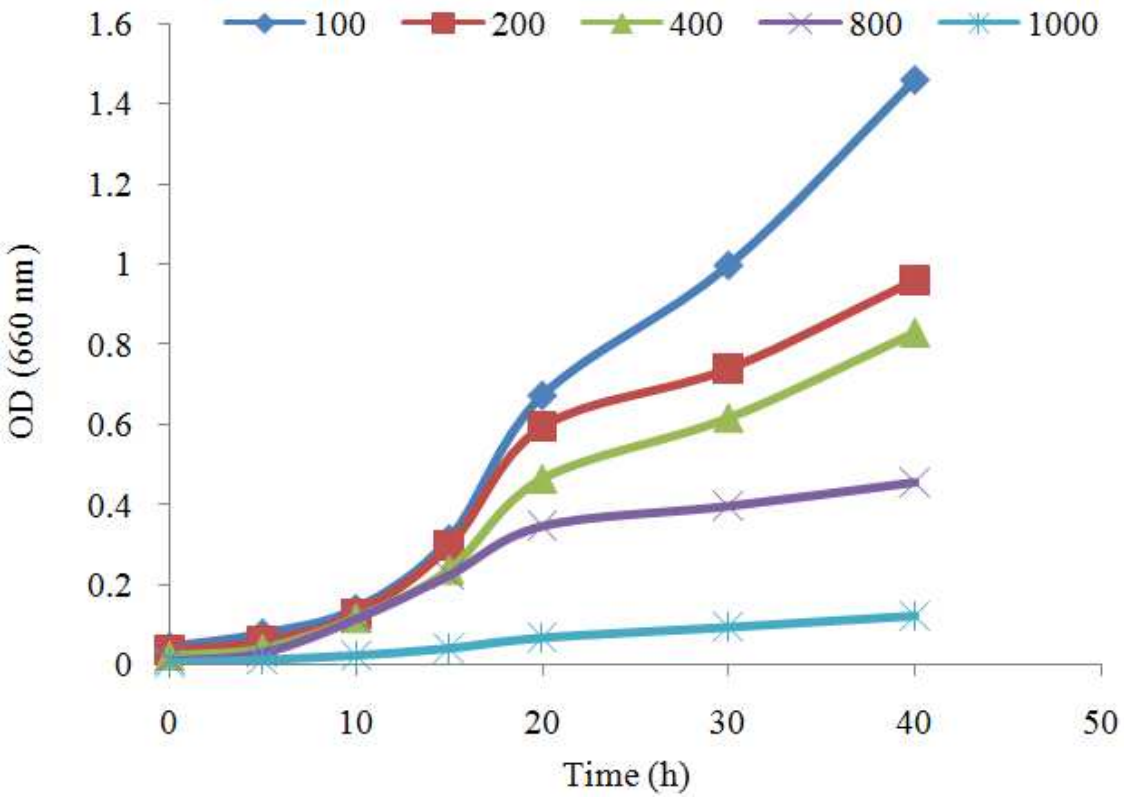

Fig. 11. Effect of initial pesticide concentration on the growth of *Pseudomonas* sp. AUG12 bacteria in Minimal Salt broth containing Malathion

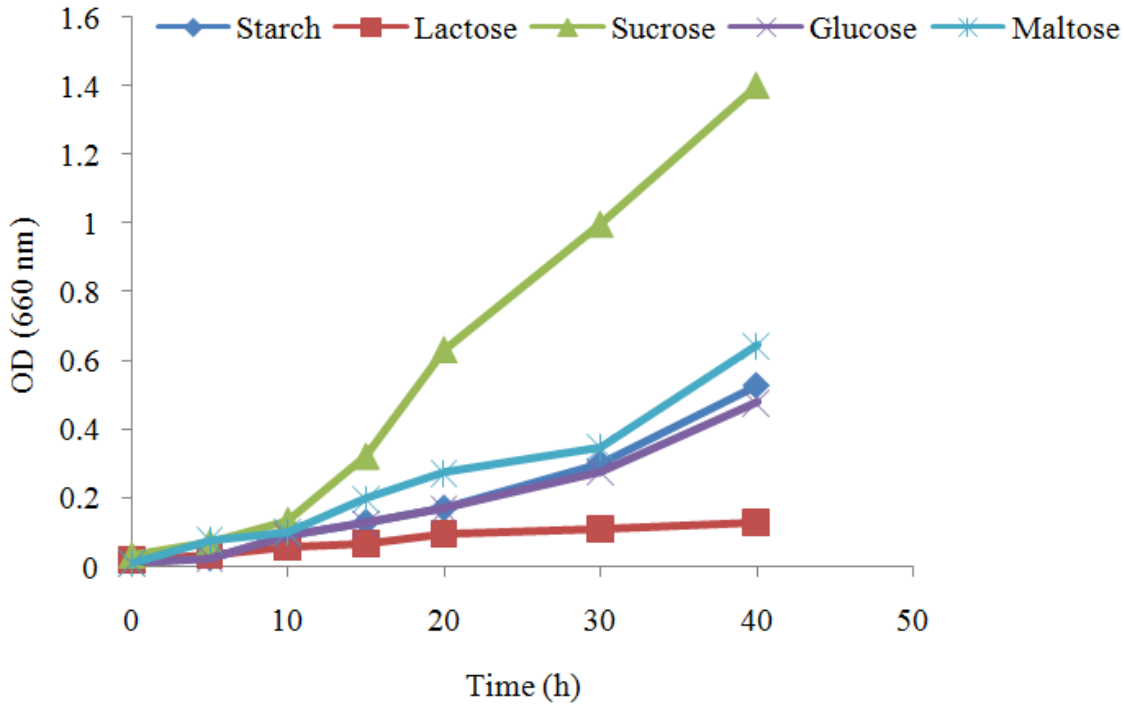

Fig. 12. Effect of various Carbon source on the growth of *Pseudomonas* sp. AUG12 bacteria in Minimal Salt broth containing Malathion

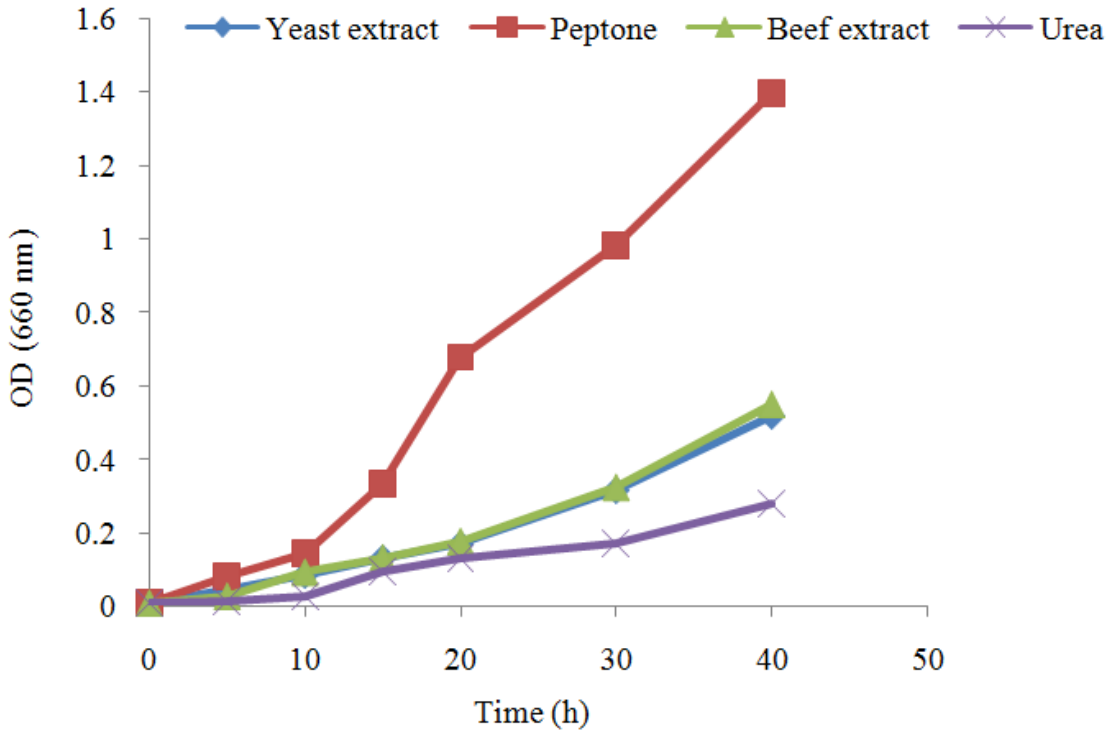

Fig. 13. Effect of various Nitrogen source on the growth of *Pseudomonas* sp. AUG12 bacteria in Minimal Salt broth containing Malathion

It was found that the bacterial strain showed maximum growth in the presence of sucrose and moderate growth was shown in presence of starch, maltose where as negligible growth in the presence of lactose (Fig. 12).

Effect of Nitrogen Source

Different type of nitrogen source such as yeast extract, peptone, urea and beef extract are used to study the growth rate of *Pseudomonas* sp. AUG12. The results showed negligible growth of in the presence of urea, where as moderate growth activity was shown in the presence of yeast extract and beef extract and maximum growth was reported in the presence of peptone (Fig. 13).

Organophosphorous Phosphatase (OPP) Assay

The enzyme *organophosphorous* phosphatase determined by the OPP assay was found to be present in the intracellular fractions of the six selected isolates. The extracellular OPP activity was 0.0079 U in *Pseudomonas* sp. AUG12.

Discussion

To increase the agricultural yield insecticides plays an important role, but only less than 1% of the pesticides are enough to kill the pests, remaining pesticides enter into the ground and surface water and causes environmental pollution and affect human health also (Battaglin and Fairchild, 2002). Thus some of the persistent pesticides were banned and some are modified without damaging to the environment. However, organophosphates are creating a lot of human health problems (Sogorb *et al*., 2004). Different kinds of bacteria present in soil are capable of degrading many persistent pesticides (Ramanathan and Lalithakumari, 1999; El-Deeb *et al*., 2000; Bhadbhade *et al*., 2002; Chen *et al*., 2002).

A total of six isolates were used in this study which were isolated from Gwalior region of Madhya Pradesh. The tentative identification of isolates was done by various biochemical tests. These isolates were tested for the pesticides tolerance against Malathion and Dichlorvos. The tolerant efficiency of isolates was tested by cell plat efficacy test. The growth curve of all Six isolates were measured by spectrophotometry analysis. The total protein content also measured at the end of degradation. The most tolerant strains were selected for the optimization of physicochemical parameter for maximum growth. The maximum growth rate of *Pseudomonas* sp. AUG12 was recorded at pH 7 followed by pH 7 at 30°C. The growth of bacteria was maximum in the presence of sucrose and peptone as a carbon and nitrogen source respectively. The growth of *Pseudomonas* sp. AUG12 was

decreased by increasing the concentration of pesticide. Interestingly, the extracellular OPP activity of *Pseudomonas* sp. AUG12 was very high.

Conclusion

Six bacterial isolates belonging to *Staphylococcus, Micrococcus, Entrobacte, Bordetella, Pseudomonas and Klebsella* genera were isolated and identified, which had efficient degradation capability of the organophosphorus pesticide Malathion and Dichlorvos. Addition of Sucrose and peptone enhanced the growth of the isolated strain *Pseudomonas* sp. AUG12. Results of the good growth of isolates indicated that apart from chemical processes microbial degradation is considered to be one of the main mechanisms of Malathion and Dichlorvos dissipation in water. Moreover, obtained results have implications for the development of a bioremediation strategy of Malathion and Dichlorvos polluted water.

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Author Contributions

Hotam Singh Chaudhary: Development of research idea, supervising research work, analysis of whole data, writing draft of whole manuscript and final approval of manuscript.

Soni Yadav: Isolation and Characterization of Organophosphate Pesticides Degrading Bacteria, compiling and analysis of whole data, reading draft and final approval of manuscript.

Sitansu Kumar Verma: Helping in lab work, compiling and analysis of whole data, reading draft, correcting and final approval of manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved

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