

IDENTIFICATION AND GROWTH CHARACTERIZATION OF A NOVEL 2,4-D (DICHLOROPHENOXYACETIC ACID) DEGRADING BACTERIUM *MARIBACTER* SP AMSU ISOLATED FROM AQUACULTURE EFFLUENT

SANKARALINGAM, S.¹ – NITHYANAND, P.² – KARUTHAPANDIYAN, S.T.² – PALAVESAM, A.¹ –
RAMASUBBURAYAN, R.¹ – IMMANUEL, G.^{1*}

¹Centre for Marine Science and Technology, M.S.University,
Rajakkamangalam- 629 502, Kanyakumari District, Tamilnadu, India

²Department of Biotechnology, Alagappa University,
Karaikudi, Tamilnadu, India

*Corresponding author
e-mail: gimmas@gmail.com

(Received 1st November 2009; accepted 29th January 2013)

Abstract. A bacterial isolate *Maribacter* sp AMSU, capable to degrade the herbicide 2,4-D (dichlorophenoxyacetic acid) was isolated from aquaculture effluent by enrichment culture technique. This isolate exhibited substantial growth in mineral salt medium supplemented with 2,4-D as a sole source of carbon and energy. Based on the morphological, cultural and biochemical characteristics, the isolate was identified as *Maribacter* sp according to Bergy's manual. The isolated bacterial strain was further identified by sequence analysis of its 16S rRNA gene. Growth curve experiment on 2,4-D degradation indicated that 50 to 200mg/100ml stimulated the bacterial growth during 24 to 48 h of incubation. However the bacterial growth was sustained and a significant death was observed with culture media containing 800mg/100ml 2,4-D and above. At regular time intervals the optical density was measured at 600nm. Growth of the isolate was determined by viable cell enumeration immediately after inoculation. The growth of *Maribacter* sp in medium containing 50mg/100ml to 200mg/100ml 2,4-D was not varied much during 24 to 48h, and the total viable count of 121 and 144 × 10⁴ CFU.ml⁻¹ were observed, respectively. Media optimization results indicated that lactose (carbon) and ammonium chloride (nitrogen) added medium at pH 7 and 35 °C found to be suitable for the better growth of *Maribacter* sp. But lead added medium inhibited the growth of *Maribacter* sp. Degradation of 2,4D in culture supernatant was determined by using HPLC and the result obtained was discussed.

Keywords: biodegradation, dichlorophenoxyacetic acid (2,4-D), pesticides, *Maribacter* sp.

Introduction

In India, alarming levels of pesticides have been reported in air, water, soil as well as in foods and biological materials (Viswanathan, 1985; Agarwal et al., 1986; Nawab et al., 2003). The most important pollutants among the toxicants in India are organo chlorine and organo phosphorus pesticides. The later type is tending to replace the previous type to its fast degradation in the environment (Jiries et al., 2002). One way to enhance the break down of these chemicals is bioaugmentation by inoculation of microorganisms that are known to readily metabolize these chemicals (Ramadan et al., 1990).

Metabolically versatile bacterial species namely *Arthrobacter* sp and *Pseudomonas* sp can degrade a wide range of compounds including aromatic compounds (Sanni and Ajesebuter, 2003; Igbinsosa, 2007). A series of growth curve experiment was performed with specific doses of pesticides (Malathion, Methamidophos, Cartap and

Cypermethrin) in order to determine the viable count of *Pseudomonas* sp and to verify whether they could utilize these compounds for their growth (Jilani and Altafkhani, 2004). As growth kinetic studies provide an evidence of mineralization potential of organisms, such studies were conducted by several researchers while performing pesticide degradation using isolated strains of microbes (Karpouzas et al., 2000). Previous reports revealed that the microbial degradation process detoxifies the pesticide contaminations and it can be effectively used to overcome the pollution problems (Bhadhade et al., 2002). Soil bacteria with the ability to degrade several pesticides have been isolated from soil showing enhanced biodegradation, which includes a metamitron degrading bacterium, *Rhodococcus* sp (Parekh et al., 1994); chlorpyrifos- degrading *Flavobacterium* sp (Mallick et al., 1999); atrazine degrading bacterium *Pseudomonas* sp (Ralebits et al., 2002) and an idopropane degrading bacterium *Arthrobacter* sp (Mercadier et al., 2002). 2,4-D is one of the most commonly used herbicides in agriculture and gardening and it exhibits serious ecological effects. Regardless, it has toxic effects on birds, beneficial insects, soil annelids, and non- target plants. It also showed negative impacts on aquatic life, especially algae, small invertebrates, amphibians and fishes, particularly in their juvenile stages (Cox, 1999). Ingestion of high doses of 2,4-D have shown acute toxicity in lower mammals and humans and most of the strains have been well characterized (Fulthorpe et al., 1996). The catabolic pathway of 2,4-D mineralization by *Ralstonia eutropha* Jmp134 was investigated by Leveau et al (1999), Don and Pemberton (1981) and more recent studies have also elucidated the kinetics of 2,4-D degradation. The kinetics of cell growth and biodegradation of 2,4-D of microorganisms growing on herbicide was also reported by Muller et al (2001) and Fuchslin (2003). However no studies have been carried out on screening the 2,4-D degrading bacteria from aquaculture effluent and hence the present work was undertaken.

Materials and methods

Chemicals

Commercial grade 2,4-D was purchased from Sigma- Aldrich co, Bangalore, India, with purity higher than 98% and used throughout the experiment, because it would closely represent the nature of the active compound.

Isolation and maintenance of 2,4-D degrading bacterium

The bacterial species used in this study was isolated from aquaculture effluent. The bacterial strain was initially screened by the enrichment technique (Igbinsosa, 2007). The effluent sample was collected aseptically from the aquaculture farming area nearby Rajakkamangalam estuary, Kanyakumari District, Tamilnadu, India. Then it was transported to the laboratory immediately for further studies. The effluent sample was exposed to high (1000mg/100ml) concentration of 2,4-D and this particular concentration used to obtain only the organisms which were capable to withstand. Then the screened bacterial strain was picked up and streaked on nutrient agar plate and restreaked in nutrient agar slants for further studies. The isolated bacterial strain was further cultured in 50ml nutrient broth containing 0.2 % 2,4-D and kept in an incubator shaker at 35°C for 24 h. Subsequently 0.2 ml of this culture was streaked on mineral salt agar (MSM) g/l of ((NH₄)₂ SO₄ 1.0; Na₂ HPO₄ 2.1; MgSO₄ 0.01; CaCl₂ 2H₂O 0.1;

FeSO₄ 7H₂O 0.001; CuSO₄ 0.04 and NaMOO₄ 0.002) supplemented with 0.2 % 2,4-D plates and incubated at 35°C for 24 h. Individual colonies were sub cultured on nutrient agar plates containing 0.2 % 2,4-D until the pure cultures were isolated (Jilani and Altafkhani, 2004). After incubation, the isolated colonies were identified following the methods described in Bergey's manual of Determinative Bacteriology (Altschul et al., 1997).

Strain identification

Genomic DNA extraction

The bacterial isolate was grown in 2ml Zobell Marine Broth (HiMedia cat #) overnight at 27° C. The culture was centrifuged at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE and lysozyme was added to this to a final concentration of 8 mg/ml and incubated for 1h at 37°C. To this tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K from 20 mg/ml were added and again incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol was precipitated. Finally the DNA pellet was resuspended in sterile distilled water.

Amplification of 16S rDNA gene

Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16SrDNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5' ACGGCTACCTTGTTACGACTT 3'. Polymerase chain reaction was performed in a 50 µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5µM, 1.5 mM MgCl₂, and each deoxynucleoside triphosphate at a concentration of 50 µM, as well as 1 U of *Taq* polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 min at 95 °C, there were 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min and then a final extension step consisting of 5 min at 72 °C; Mastercycler Personal (ependorf, Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel in 1X TAE.

Cloning and sequencing of 16S rDNA gene

The amplified product (1,500-bp) was purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rDNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea).

Nucleotide sequence analysis

The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul et al., 1997). Multiple sequence analysis was carried out using CLUSTALX (Thompson et al., 1997) and further NJ plot (Perrière and Gouy, 1996) and PhyloDRAW were employed for constructing

phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was also performed.

Growth kinetics of 2,4-D degrading bacterium

The tolerance of bacterium was studied by the method of Gupta et al (1994). The mineral salt medium was used to investigate the degradation potential of isolated bacterial strain of *Maribacter* sp. One loopful of isolated bacterial strain was inoculated into freshly prepared nutrient broth containing 0.2 % 2,4-D as the sole source of carbon. For the determination of growth kinetics, aliquots (2.5ml) of 24 h culture inoculated into eight flasks containing 100ml mineral salt medium supplemented with 2,4-D. The concentrations tested were 50, 100, 150, 200, 400, 600, 800, and 1000mg/100ml. The culture was incubated in shaker at 35°C for 24 h. Turbidity was used as an index of biodegradation potential. Three replicates were maintained for each concentration of 2,4-D through out the experimental studies. The lowest concentration of 2,4-D which inhibited the growth of the organism was considered as MIC. At regular time intervals the optical density was measured at 600nm (Mussarat et al., 2000). The growth curve was obtained by plotting the optical density as a function of time.

Enumeration of viable cell count

The viable cell colonies were counted by the method described by Collins and Lyne (1985). The isolated bacterial culture was enumerated with and without addition of 0.2 % 2,4-D. Aliquots of 1ml of 24 h culture was inoculated into 50 ml mineral salt medium (Broth) containing different concentrations of 2,4-D and growth was tested. Then 1ml of the bacterial sample from each concentration was drawn at regular intervals and serial dilutions were performed using 9 ml of sterile blank. Appropriate dilutions of bacterial sample were plated in triplicate nutrient agar plates and were incubated at 35°C for 24h. After inoculation, growth of the isolated bacterial strain was studied with the help of microscope by viable cell enumeration.

Optimization of media components for the growth of Maribacter sp

Effect of different carbon sources

The effect of carbon sources on growth of *Maribacter* sp was individually determined by using different carbon sources such as glucose, sucrose, fructose, lactose and maltose supplemented in the basal medium at 0.5 % concentration each (Kotresha and Vidyasagar, 2008). Here 0.1 % culture broth was inoculated in to the culture media supplied with 0.2 % 2,4-D and growth was measured at every 6 h interval.

Effect of different nitrogen sources

The effect of nitrogen source on growth of *Maribacter* sp was individually determined by using various nitrogen sources such as ammonium chloride, yeast extract, peptone, sodium nitrate and potassium nitrate supplemented in the basal medium at a concentration of 0.5 % each (Kotresha and Vidyasagar, 2008). The culture medium was supplied with 0.2 % 2,4-D, inoculated with 0.1 % culture broth and the growth was measured at every 6 h interval.

Effect of different pH

The effect of optimum pH on degradation of 2,4-D by the experimental microorganism was determined by culturing the bacterium in 0.2 % 2,4-D supplemented medium with different pH (5, 6, 7, 8 and 9) and the growth was measured at every 6 h interval.

Effect of different temperature

The effect of temperature on degradation of 2,4-D was studied by altering the incubation temperature of the culture medium for the growth of *Maribacter* sp. In this experiment 0.1 % culture broth was inoculated in different temperature (20, 30, 35, 40, and 50 °C) range and growth was measured at every 6 h interval (Margesin and Schinner, 1997).

Effect of different heavy metals

The effect of heavy metals on degradation of 2,4-D was determined by using various types of metals namely lead, zinc, copper, cadmium, ferrous iron, mercury and nickel (Kotresha and Vidyasagar, 2008). For the determination of degradation, aliquets (2.5ml) of 24 h culture was inoculated into 100ml mineral salt medium supplemented with 0.2% 2,4-D and 0.1 % individual heavy metals. After 24 h of incubation, the degradation was studied by Turbidometric method (O.D).

HPLC analyses

Degradation of 2,4-D in culture supernatant was also determined by using HPLC (model No SPD70 AVP, SHIMADZU). For this 100 ml of supernatant was taken from growing bacterial cultures and transferred in to 1.5 ml micro centrifuge tubes. These were then centrifuged at 15,000 rpm for 10 mins and the supernatant was collected, pooled and stored at frozen condition for HPLC studies. After incubation, samples were warmed at 35 °C for 5 to 10 min. and 70 µl was transferred in to a 1.8ml amber auto sampler vial containing 730 µl HPLC grade H₂O and 50 mg dissolved in 200 ml acetonitrile: 80% H₂O. Samples were eluted at a flow rate of 1.0 ml/min with 0.025 % H₃PO₄ – acetonitrile gradient. Absorbance of eluted compounds was monitored at 210 nm. Sample retention time was compared to known prepared standard and analyzed using identical protocols.

Results and discussion

Isolation and identification of 2,4-D degrading bacteria

A bacterium was isolated from the aquaculture effluent sample that was able to withstand high concentration of 2,4-D (1000mg/100/ml). On the basis of morphological, cultural and biochemical characteristics of the isolated bacteria was identified as *Maribacter* sp (Table 1).

Table 1. Morphological, cultural and biochemical tests for identification of the strain

Tests	Strain <i>Maribacter</i> sp AMSU
Morphological tests	
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Convex
Surface	Smooth and shiny
Pigment	-
Opacity	Opaque
Gram's reaction	
Cell shape	Rods
Size (µm)	Length: 1-2µ, Width: ~ 1µ
Arrangement	Singles
Spores(s)	-
Position	-
Shape	-
Sporangia	-
Motility	+
Physiological tests	
Growth at temperatures	
4°C	-
15°C	W
20°C	+
25°C	+
30°C	+
37°C	+
42°C	+
52°C	-
Growth at pH	
pH 4.0	+
pH 5.0	+
pH 6.0	+
pH 7.0	+
pH 8.0	+
pH 9.0	+
Growth at NaCl (%)	
2.0	+
5.0	+
7.0	-
10.0	-
Growth under anaerobic conditions	
Biochemical tests	
Strain <i>Maribacter</i> sp AMSU	
Growth in MacConkey agar	+
Indole test	-
Methyl red test	-
Voges Prauskauer	-
Citrate utilization	+
H ₂ S Production	-
Gas from glucose	+
Acid from glucose	+
Acid from lactose	+
Casein hydrolysis	-
Esculin hydrolysis	-
Gelatin hydrolysis	-
Starch hydrolysis	-

Table 1. cont.

Tests	Strain <i>Maribacter</i> sp AMSU
Morphological tests	
Urea hydrolysis	+
Nitrate reduction	+
Catalase test	W
Oxidase test	-
Lysine decarboxylase	-
Arginine dihydrolase	W
Ornithine decarboxylase	+
Tween 80 hydrolysis	-
Tween 40 hydrolysis	-
Phosphatase test	-
Lecithinase	-
ONPG test	-
DNase	-
Tyrosine	-
Hippurate	-

The isolated bacterial strain was described as Gram mucoid rods. It is a sugar fermenting bacterium and a non-acid gas producer. The results on phylogenetic studies revealed that the 16S rDNA of the strain *Maribacter* sp AMSU had 93% similarity with the nearest match in the Genbank (accession number – EU624432) (*Fig.1.* – Phylogenetic tree). This result is identical with the earlier report of Mussarat et al (Mussarat et al., 2000), Who reported that two *Pseudomonas* sp isolated from agricultural soil contaminated with 2,4-D, capable of utilizing 2,4-D as a source of carbon.

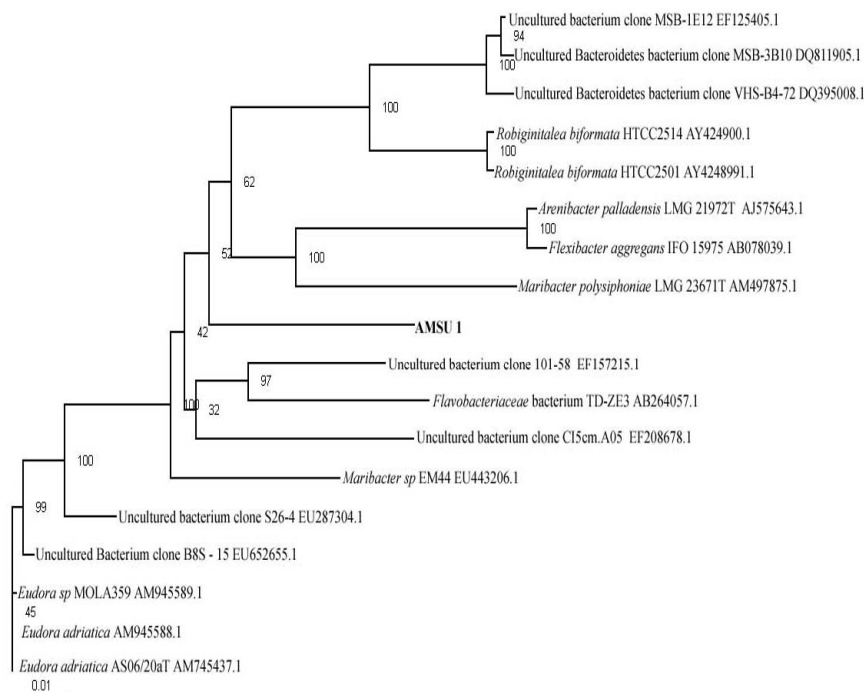
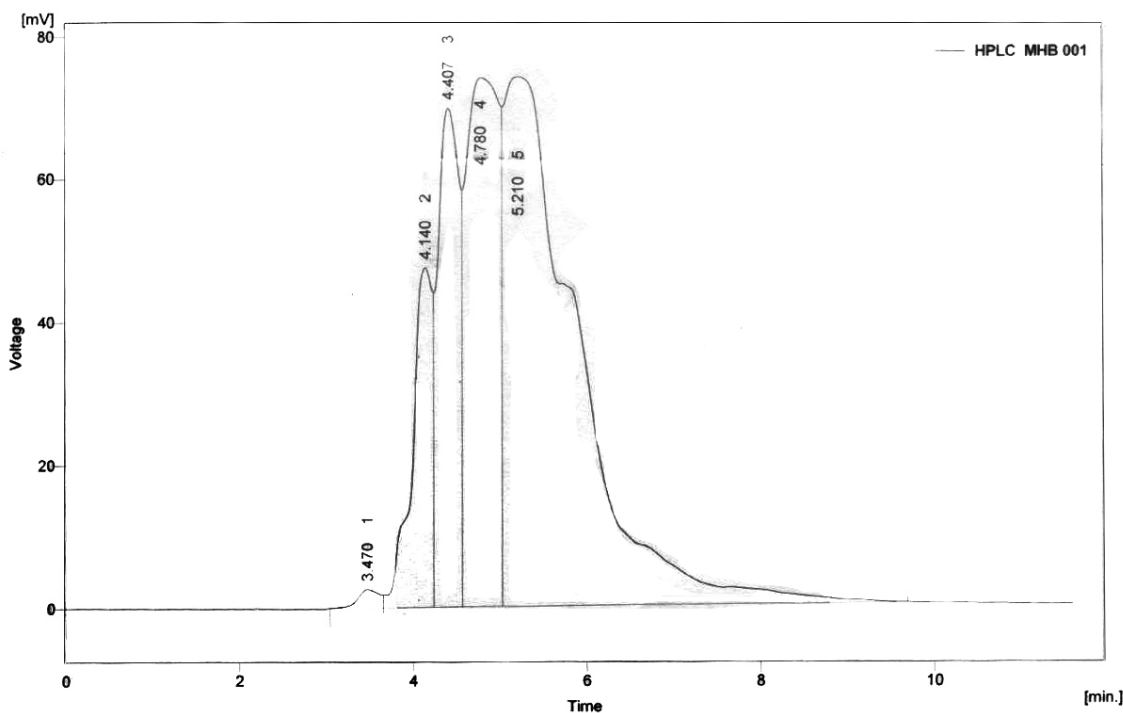


Figure 1. Phylogenetic relationship of *Maribacter* sp AMSU with closely related bacterial genera

Mineralization of 2,4-D

HPLC (Fig. 2 & 3) analyses indicated that, 2,4-D was detected in medium containing 0.2% 2,4-D. The present result inferred that the retention time of the control medium recorded was 3.479 (min). Whereas the medium inoculated with bacterial strain supplemented with nutritional sources and 2,4 - D inferred almost same level of retention time with two peaks of 2.400 and 3.670 (min).



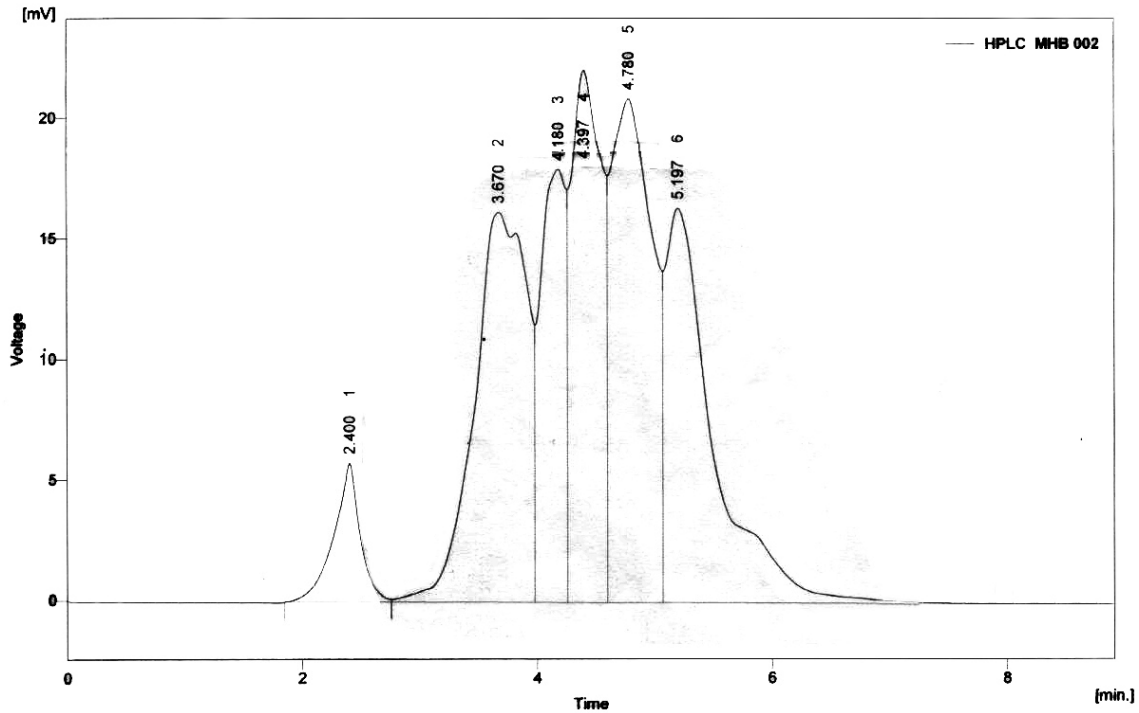
Result Table (Uncal – HPCL MHB 001)

	Reten. Time (min)	Area [mV.s]	Height [mV]	Area [%]	Height [%]	WO5 (min)
1	3.470	43.039	2.498	0.5	0.9	0.30
2	4.140	763.023	47.363	9.2	17.7	0.23
3	4.407	1203.213	69.638	14.5	26.0	0.33
4	4.780	1933.568	73.922	23.3	27.6	0.46
5	5.210	4354.028	73.994	52.5	27.7	0.92
	Total	8296.871	267.415	100.0	100.0	

Figure 2. HPLC profile of 2, 4-D supplied medium (control)

Here the results imply that 2,4-D was converted into Dichloro phenol and acetic acid. This present results was supported by the earlier studies of Christian et al (1999), who have reported that aerobic degradation of mecoprop and dichloroprop were detected by using HPLC. Under anaerobic conditions, the concentrations of 2,4-D decreased exponentially and 2,4-D was the main metabolite of anaerobic degradation, only traces of 4-chlorophenol detected in the media. Rice et al (2005) also reported that 2,4,5-T and 2,4-D contaminated soil samples were analysed in HPLC. They observed that 2,4-D and 2,4,5-T were observed in 73.8mg/Kg and 23.4mg/Kg, respectively. During the process of degradation, the bacteria was greatly stressed and as a result, the growth was delayed in the medium supplemented with high concentration of 2, 4-D. Growth curve

experiment with different concentrations of 2,4-D indicated that the concentration of 50 mg to 200 mg/100ml provoked an increase in bacterial growth during 24 to 48 h of incubation, whereas in medium added with 800 mg/100ml, the growth was sustained. But in medium supplemented with 1000mg/100ml 2,4-D, the growth of the organism was arrested and significant death was observed (Fig. 4). Further the results indicated that in 200mg/100ml 2,4-D, the growth of *Maribacter* sp was not affected much and it was compared with control experiment.



Result Table (Uncal – HPLC MHB 002)

	Reten. Time (min)	Area [mV.s]	Height [mV]	Area [%]	Height [%]	WO5 (min)
1	2.400	90.084	5.737	4.0	5.8	0.22
2	3.670	506.222	16.113	22.7	16.3	0.51
3	4.180	265.088	17.896	11.9	18.1	0.28
4	4.397	398.438	22.026	17.8	22.3	0.34
5	4.780	510.052	20.824	22.8	21.1	0.47
6	5.197	462.303	16.285	20.7	16.5	0.37
	Total	2232.187	98.880	100.0	100.0	

Figure 3. HPLC profile of 2, 4-D supplied medium inoculated with *Maribacter* spAMSU

The maximum growth was observed in 200mg/100ml 2,4-D added medium against the minimum growth in 1000mg/ml. These results indicated that high concentrations of 2,4-D become toxic to the organism and therefore this isolate was unable to utilize 2,4-D at this concentration. Further 2,4-D at 50mg to 200mg/100ml did not show any toxic effect to the *Maribacter* sp when compared to those organisms grown in 400mg to 1000mg/100ml. This result is identical with earlier report of Jilani & Altafkhani (2004), who reported that the growth curve experiment with different concentrations (60 to 80mg/100) of cartap pesticides provoked a bacterial growth. Mussarat et al. (2000) have

also reported that two *Pseudomonas* sp isolated from agricultural soil contaminated with 2,4-D capable of utilizing 2,4-D as a source of carbon. Most of the strain can able to grow well in simple minimal media with a single organic compound as carbon and energy source (Palleroni, 1986).

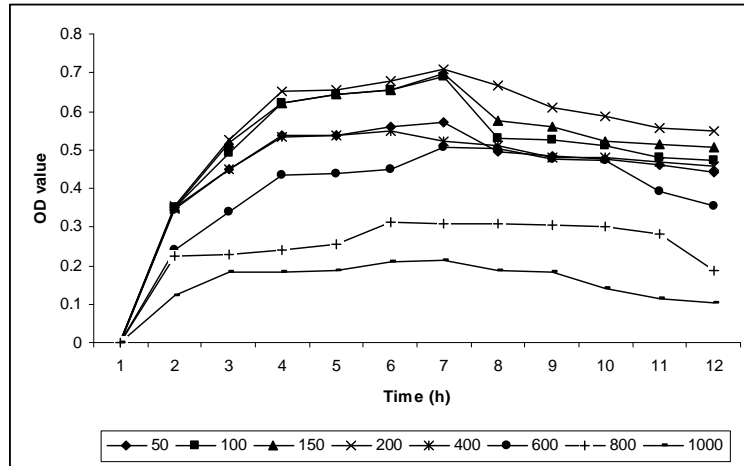


Figure 4. Effect of different concentration of 2,4-D on growth of *Maribacter* sp AMSU

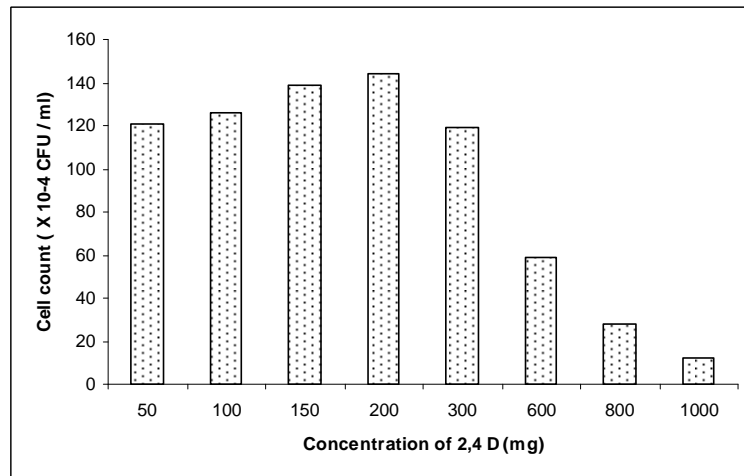


Figure 5. Enumeration of viable cell count of *Maribacter* spAMSU cultured in different concentrations of 2,4-D

Enumeration of viable cell count

The growth of *Maribacter* sp was not varied much in medium containing 50mg/100ml to 200mg/100ml 2,4-D, where the total viable count observed was between 121 and 144 × 10⁻⁴ CFU.ml⁻¹. The results indicated that 2, 4-D concentrations in the range of 50mg to 200 mg/100ml did not affect the growth of the isolated bacterium. However, marked reduction in bacterial count at 24 h was recorded in the medium containing 1000 mg/100ml 2, 4-D. These results indicated that high concentration of 2,4-D inhibited the bacterial growth. During the process of degradation, it was observed

that in the presence of high concentration of 2,4-D, the bacteria was stressed much and in consequence the growth was reduced (Fig. 5). Another believable explanation is that microorganism may need an accumulation period to induce the necessary degradative enzymes to degrade the organic compound and the prolonged log phase observed high concentration of 2,4-D (Mussarat et al., 2000).

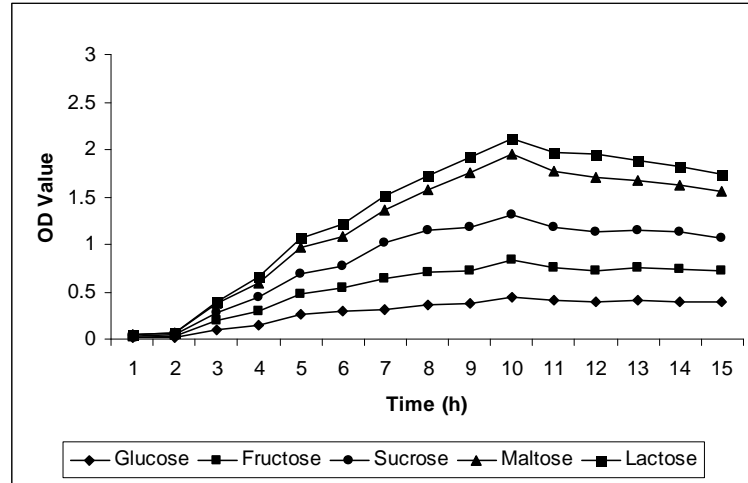


Figure 6. Effect of different carbon sources on growth of *Maribacter sp AMSU*

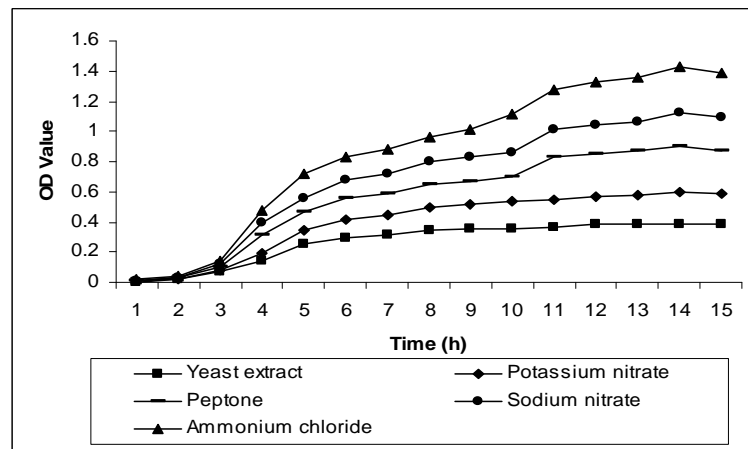


Figure 7. Effect of different nitrogen sources on growth of *Maribacter sp AMSU*

Media optimization

Experimentation on the carbon sources on growth of *Maribacter sp AMSU* indicated that, the growth was maximum in medium supplemented with lactose (Fig. 6). María Celina and Marisa Anahí (2005) have reported that the phenotypic diversity of rhizobia belonging to the main genera of rhizobial bacterial strains that colonize the same soil, by assessing the growth different bacterial strains in the presence of herbicides and the ability of these strains to utilize different energy and carbon sources. Likewise among the nitrogen sources tested, the growth of *Maribacter sp AMSU* was high in medium supplemented with Ammonium chloride (Fig. 7). This report is harmonizing with the

earlier report on Kale et al (2005), who observed that the degradation and growth rate of *A. chroococcum* was not affected at 0.5 – 5 ppm carbofuran in nitrogen supplemented medium. Rice et al (2005) have reported that *Burholderia* sp JR7 B2 and *Burholderia* sp JR7 B3 were able to mineralize 2,4,5-T in the presence of 0.01 % yeast extract.

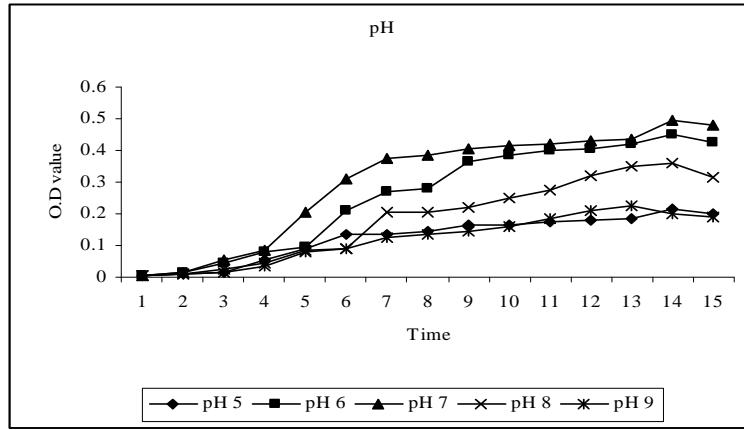


Figure 8. Effect of different pH on growth of *Maribacter* sp AMSU

In the present study, among the media tested with pH 5 to 9, the growth of *Maribacter* sp AMSU was maximum at pH 7 (Fig. 8) and it was found to be optimum for enhancing the 2,4-D degradation. This report is identical with the earlier report of Mussarat et al (2000). They reported that the optimum growth in mineral medium at pH 7 clearly indicated their capability to utilize 2,4-D as source of carbon and energy. Among the temperature (20 to 50 °C) tested, *Maribacter* sp attained maximum growth at 35 °C and the minimum growth was registered at 50 °C (Fig. 9). This result was identical with the earlier report of Cookson (1995), he reported that the microorganisms found effective in bioremediation have been shown to perform well in the temperature range of 10 to 40 °C.

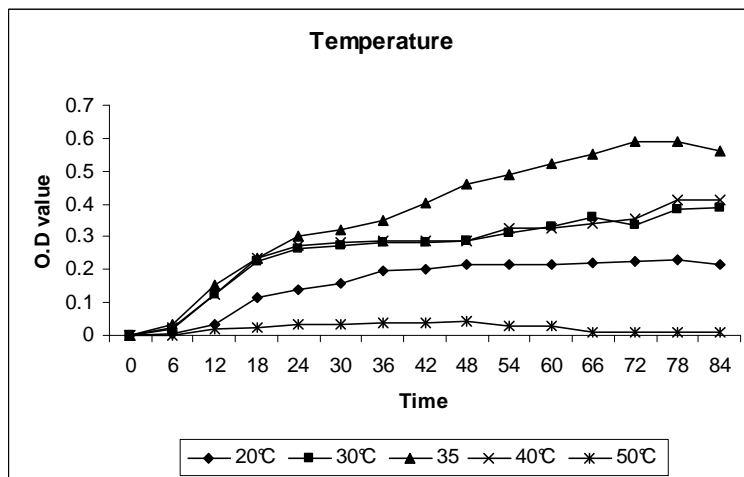


Figure 9. Effect of different temperature on growth of *Maribacter* sp AMSU

The effect of heavy metals revealed that lead has inhibited the growth of *Maribacter* sp more effectively when compared with the other tested heavy metals (Fig. 10). Roane et al. (2001) reported that cadmium added at a level of 60mg /l was found to inhibit the biodegradation of 2,4-D in soil system inoculated with the 2,4-D degrader *Alcaligenes eutrophus*. The impact of copper toxicity on biodegradation of biodegradable polymer and Polyhydroxybutyrate (PHB), have been investigated by Birch and Brandl (1996). The authors found that 8-15mg/l were required to inhibit PHB degradation. Benka-Coker and Ekundayo (1998) investigated the impact of lead, copper and manganese on crude oil biodegradation by a *Micrococcus* sp and *Pseudomonas* sp. This biodegradation was reduced by zinc at low concentration of 0.43g/l and least by manganese at 28.2mg/l. Kuo and Genthener (1996) investigated that the addition of low levels of metals (0.1-2.0mg/l) prolonged the acclimation period and decreased dechlorination and biodegradation process. From these findings it is evident that *Maribacter* sp AMSU could be a better bacterium for the biodegradation of agricultural effluent contaminated with 2,4-D pesticides.

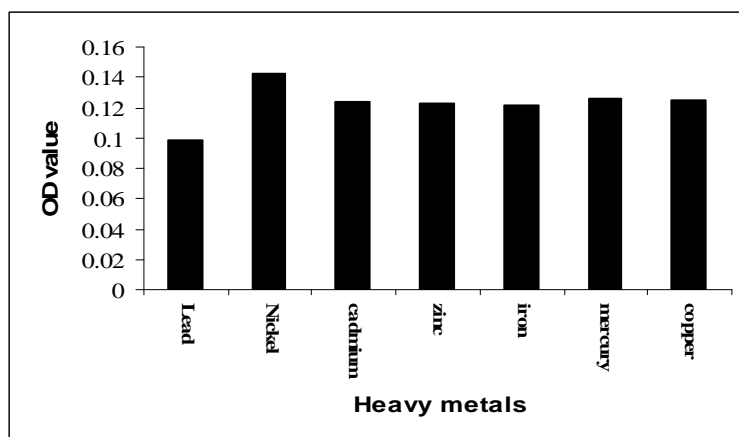


Figure 10. Effect of different heavy metals on degradation of 2,4-D by *Maribacter* sp AMSU

Conclusions

The bioaugmentation of polluted soil has already been studied by many authors. But, in many cases the introduced bacteria fail to degrade the pollutants due to their poor survival or low activity. The present study is the unique report on the isolation of 2,4-D bacteria from aquaculture effluent. The isolated bacterium appears to be a novel and it shares just 93% similarity with the closest match in Genbank. The isolated bacterium showed substantial growth in mineral media containing 2,4-D as a sole source of carbon and energy. During the process of degradation, the growth of the bacterium was high in medium containing 200mg/100ml 2,4-D. While the medium containing 1000mg/100ml the bacterium was greatly stressed. Growth of the isolate was determined by viable cell enumeration immediately after inoculation. The rate of degradation of 2,4-D in culture supernatants was studied by using HPLC. Media optimization results indicated that lactose (carbon) and ammonium chloride (nitrogen) added medium at pH 7.0 and at 35°C temperature found to be suitable for the better growth of *Maribacter* sp. But lead added medium inhibited the growth of *Maribacter* sp.

REFERENCES

- [1] Agarwal, H.C., Mittal, P.K., Menon, K.B., Pillai, M.K.K. (1986): DDT residues in the river Jammu in Delhi, India. – *Water, Air, Soil Poll.* 28: 89-104.
- [2] Altschul, S.F., Madden, T., Schaffer, A.A., Zhang, J., Anang, Z., Miller, W., Lipman, D.J. (1997): Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. – *Nucleic Acid Res.* 25: 3389-3402.
- [3] Benka-Coker, M.D., Ekundayo, J.A. (1998): Effects of heavy metals on growth of species of *Micrococcus* and *Pseudomonas* in a crude oil/mineral salts medium. – *Biores. Technol.* 66: 241-245.
- [4] Bhadhade, B.J., Sarnaik Kanekar, S.S. (2002): Bioremediation of an industrial effluent containing monocrotophos. – *Curr. Microbiol.* 45(5): 346-349.
- [5] Birch, L., Brandl, H. (1996): A rapid method for determination of metal toxicity to the biodegradation of water insoluble polymers. – *Fresen J. Anals Chem.* 354: 760 -762.
- [6] Christian, Z., Christof, B., Thomas, F., Marc, J.F.S., Werner, A., Markus, D.M., Hans, P.E.K. (1999): Fate of herbicides mecoprop, dichloroprop and 2,4-D in aerobic and anaerobic sewage sludge as determined by laboratory batch studies and enantiomer-specific analysis. – *Biodegradation* 10: 271-278.
- [7] Collins, C.H., Lyne, P.M. (1985): *Microbiological methods*. – 5th Edn., Butterworth and Co (Publishers) Ltd.
- [8] Cookson, J.T. (1995): *Bioremediation engineering: design and application*. – New York, USA: McGraw-Hill Publishing Co., 554 pp.
- [9] Cox, C. (1999): 2,4-D: ecological effects. *Herbicides fact sheet*. – *J. Pest. Reform.* 19(3): 14-9.
- [10] Don, R.H., Pemberton, J.M. (1981): Properties of six pesticides degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophs*. – *J. Bacteriol.* 145: 681-686.
- [11] Fuchslin, H.P., Riegg, I., Van der Meer, J.R., Egli, T. (2003): Effect of integrations of a GFP reporter gene on fitness of *Rhastonia eutropha* during growth with 2,4-Dichlorophenoxyacetate. – *Environ. Microbiol.* 5(10): 878-87.
- [12] Fulthorpe, R.R., Rhodes, A.N., Tiedje, J.M. (1996): Pristine soils mineralize 3-chlorobenzoate and 2,4-Dichlorophenoxyacetic acid via different microbial population. – *Appl. Environ. Microbiol.* 62: 1159-1166.
- [13] Gupta, N., Gahlot, R., Lakshminarayana, N., Narula, K. (1994): Pesticide resistance among *Azotobacter chroococum* soil isolates and mutants. – *Microbiol Res.* 149: 391-393.
- [14] Holt, J.G., Noel, R.K., Sneath, P.H.A., Stanley, J.T., Williams, T. (1994): *Bergey's manual of Determinative Bacteriology*. – Vol:1-4, Williams & Wilkins Co., Baltimore, 787 p.
- [15] Igbiosa, O.E. (2007): *Studies on aerobic biodegradation of 2,4-Dichlorophenoxy acetic acid (2,4-D) by petroleum degrading bacteria*. – M.Sc Thesis, Obafemi Awolowa University, Ile-Ife, Nigeria.
- [16] Jilani, S., Altafkhani, M. (2004): Isolation, Characterization and growth response of pesticide degrading bacteria. – *J. Biol. Scie.* 4(1): 15-20.
- [17] Jiries, A.G., AL-Nasir, F.M., Beese, F. (2002): Pesticide and heavy metals residues in water, soil and plants in waste water disposal site near Al-Lajoun Valley, Karak, Jordan. – *Water, Air, Soil Poll.* 133: 97-107.
- [18] Kale, S.P., Murthy, N.B.K., Raghu, K. (1989): Effect of carbofuran, carbaryl, and their metabolites on the growth of *Rhizobium* sp. and *Azotobacter chroococum*. – *Bull. Environ. Cont. Toxicol.* 42(5): 769-772.
- [19] Karpouzias, D., Morgan, G.J.A., Walker, A. (2000): Isolation and characterization of 23 carbofuran-degrading bacteria from soils from distant geographical areas. – *Lett. Appl. Microbiol.* 31: 353-358.

- [20] Kotresha, D., Vidyasagar, G.M. (2008): Isolation and characterization of phenol degrading *Pseudomonas aeruginosa* MTCC 4996. – World J. Microbiol. Biotech. 24: 541-547.
- [21] Kuo, C.W., Genthener, B.R.S. (1996): Effect of added heavy metal ions on biotransformation and biodegradation of 2-chlorophenol and 3-chlorobenzoate in anaerobic bacterial consortia. – Appl. Environ. Microbiol. 62: 2317-2323.
- [22] Leveau, J.H., König Fuchslin, H., Werlen, C., Van der Meer, J.R. (1999): Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP 134 (pJP4) to the herbicide 2,4-Dichlorophenoxyacetate. – Mol. Microbiol. 33: 396-406.
- [23] Mallick, K., Bharati, K., Banerji, A., Shakti, N.A., Sethunathan, N. (1999): Bacterial degradation of chlorifos in pure culture and in soil. – Bull. Environ. Cont. Toxicol. 62: 48-54.
- [24] Margesin, R., Schinner, F. (1997): Effects of temperature on oil degradation by a psychotrophic yeast in liquid culture and in soil. – FEMS Microbiol. Ecol. 24 : 243-249.
- [25] María Celina, Z., Marisa Anahí, G. (2005): Diversity of Rhizobia isolated from an agricultural soil in Argentina based on carbon utilization and effects of herbicides on growth. – Biol. Fert. Soil. 42: 83-88.
- [26] Mercadier, C., Garcia, D., Vega, D., Bastide, J., Coste, C. (2002): Metabolism of iprodione in adapted and nonadapted soils; effect of soil inoculation with iprodione-degrading *Arthrobacter* strain. – Soil Biol. Biochem. 28: 1791-1796.
- [27] Muller, R.H., Kleinstuber, S., Babe, W. (2001): Physical and genetic characteristics of two bacterial strains utilizing phenoxyacetate herbicides. – Microbiol. Res. 156(2): 121-31.
- [28] Mussarat, J., Bano, N., Rao, R.A.K. (2000): Isolation and characterization of 2,4-Dichlorophenoxy acetic acid-catabolizing bacteria and their biodegradation efficiency in soil. – World J. Microbiol. Biotech. 16: 495-497.
- [29] Nawab, A., Allem, A., Malik, A. (2003): Determination of organochlorine pesticides in agriculture soil with special reference to γ -HCH degradation by *Pseudomonas* strains. – Biores. Technol. 88: 41-46.
- [30] Palleroni, N.J. (1986): – In: Krieg NR, Holt JG (eds.), Bergey's Manual of Systematic Bacteriology, Volume 1, Williams and Wilkins, Baltimore.
- [31] Parekh, N.R.A., Walker, S., Roberts, J., Welch, S.J. (1994): Rapid degradation of triazinone herbicide metamitron by a *Rhodococcus* sp. isolated from treated soil. – Appl. Bacteriol. 77: 467-475.
- [32] Perrière, G., Gouy, M. (1996): WWW-Query: An on-line retrieval system for biological sequence banks. – Biochimie. 78: 364-369.
- [33] Ralebits, T.K., Senior, E.H., Van Versevell, T. (2002): Microbial aspects of atrazine degradation in natural environments. – Biodegrad. 13: 11-19.
- [34] Ramadan, M.A., El-Tayeb, O.M., Alexander, M. (1990): Inoculum size as a factor limiting success of inoculation for biodegradation. – Appl. Environ. Microbiol. 56: 1392-1396.
- [35] Rice, J.F., Menn, F.M., Hay, A.G., Sanseverino, J., Sayler, G.S. (2005): Natural selection for 2,4,5-Trichlorophenoxy acetic acid mineralizing bacteria in agent orange contaminated soil. – Biodegradation 16: 501-512.
- [36] Roane, T.M., Josephson, K.L., Pepper, I.L. (2001): Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. – Appl. Environ. Microbiol. 67: 3208-3248.
- [37] Sanni, G.O., Ajesebuter, S.O. (2003): Biodegradation of Escrvo's light crude oil by some species of soil bacteria. – Scientific Focus 4: 87-95.
- [38] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997): The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. – Nucleic Acid Res. 24: 4876-4882.
- [39] Viswanathan, P.N. (1985): Environmental toxicology in India. – Biol. Memb. 11: 88-97.