

## Microbial metabolism of oil sludge: Isolation & characterization of Chrysene degrading Bacteria

Deepali Rajwade and P. K. Saluja

Department of Botany & Biotechnology, Govt. D.B. Girl's College  
Raipur-492001 (India)

### ABSTRACT

Oily sludge generated by oil refining industry is notified a hazardous waste and its management and safe disposal pose a major challenge for refineries. The objective of this study is to assess the ability of the technique of bioremediation to reduce hydrocarbon content of the oil sludge. Experiments were undertaken for remediation of sludge contaminated soil with indigenous micro flora. After 30 day period the oil sludge was analyzed gravimetrically for its oil content by Soxhlet Apparatus. The reduction in individual fractions like alkanes, poly-aromatic hydrocarbons (PAH) and NSO was determined by silica gel column. Microbial isolates from the soil were tested for their sludge degrading capacity. Out of 9 isolates obtained in pure culture 4 were selected for further study based on their biosurfactant production and catechol dioxygenase enzyme activity. Morphological & biochemical characterization of the isolates was done for preliminary identification and on that basis the four isolates were identified as *Pseudomonas sp.*, *Mycobacterium sp.*, *Flavobacter sp.*, and *Rhodococcus sp.* Isolates were grown on Mineral Salt's Medium with chrysene as carbon source. The isolates were found to utilize chrysene and thus have potential to be utilized for bioremediation technology.

**Key words :** Biosurfactant, Bioremediation, Catechol dioxygenase, Chrysene.

**P**etroleum hydrocarbons are naturally occurring chemicals exploited by humans for wide range of purposes, such as the fuelling of vehicles and heating of homes<sup>3</sup>. The petrochemical industry generates a series of liquid effluents during the petroleum – refining process. These effluents must be treated through depuration processes. The oil refinery sludge's

that result from this depuration process have a high content of petroleum-derived hydrocarbons<sup>15</sup> thus it is potentially dangerous waste product. It gets accumulated over a period of time in storage tanks & pose several environmental & operational problems for refineries & marketing installation. Contamination of the environment with petroleum hydrocarbons,

consisting of aliphatics (cyclics and acyclics), simple aromatics, and polynuclear aromatic hydrocarbons (PAHs) is, therefore, a cause of serious concern worldwide<sup>2</sup>. In addition, PAHs are often toxic and carcinogenic, hence, a threat to plant, animal, and human health<sup>4, 10, 11, 12, 13, 14, 16, 17</sup>. Simply dumping these wastes or burning them with no previous treatment has serious environmental consequences and presents a risk to both ecosystems and human health<sup>1</sup>.

Bioremediation is a treatment technology for the cleanup of polluted sites that use a range of methodologies to enhance the natural biodegradation of hydrocarbons. This treatment method is not only highly efficient for hydrocarbons including PAHs, but also cost effective. Numerous micro-organisms, including bacteria, fungi and yeasts are known for their ability to degrade hydrocarbons. Bioremediation has been shown to be effective in removing some PAH compounds<sup>19</sup>. The degradation of naphthalene using immobilized *Pseudomonas* spp. in several different matrices has been studied previously<sup>8, 9</sup>. However bioremediation is considered to be limited in its application as other PAHs are recalcitrant to microbial attack. The microbial degradation of low molecular weights PAHs such as Naphthalene, Phenanthrene has been well documented and biochemical pathways elucidated<sup>5, 6</sup>. However the environmental persistence of PAHs increases as their molecular size increases to four or five benzenes rings like Fluoranthene, Pyrene, benz [a]anthracene, chrysene, benzo [b] fluranthene etc. The present study aims at degradation of chrysene (4 rings) by bacteria.

The aim of the present study is to

isolate effective bacterial strain from oil contaminated sites for bioremediation technology. The study has also focused attention towards the growth potential of the isolates utilizing chrysene as sole carbon source.

#### 1. Soxhlet extraction:

This method, described by Soxhlet (1879), is most commonly used example of a semi-discontinuous method applied to extraction of oil & grease from a given sample. According to this procedure oil and grease from a solid material are extracted by repeated washing with an organic solvent under reflux in special glassware (Fig. 1). A 1:1 mixture of Dichloromethane: Acetone was used as solvent for the extraction.

#### 2. Rotary vacuum evaporator:

After Soxhlet extraction, the boiling flask was connected to the distilling head of rotary vacuum evaporator to leave the oil and grease in the flask. The solvent was collected for reuse. (Fig. 2).

#### 3. Silica gel column:

The fractionation of oil & grease extracted from the oil sludge was done using silica gel column. Hexane, Benzene & Methanol were respectively used for extraction of aliphatics, aromatics & NSO fraction.

#### 4. Estimation of Nitrogen by Kjeldahl method:

Kjeldahl method was used to estimate the amount of nitrogen present in the residue left after extraction.

5. *Determination of organic matter by Walkley & Black method (modified):*

The organic matter present in residue left after Soxhlet Extraction is digested with excess  $K_2Cr_2O_7$  &  $H_2SO_4$ , & the residual unutilized dichromate is titrated with Ferrous ammonium sulphate.

6. *Estimation of Inorganic Phosphorus:*

The phosphorus present in the sample reacts with ammonium molybdate & forms complex hetro poly acid, which gets reduced to a complex of blue colour in the presence of  $SnCl_2$ . The absorption is measured at 690 nm.

7. *Isolation of strains:*

Bacterial strains were isolated from petroleum contaminated soil on Nutrient agar medium. Enrichment of the hydrocarbon degrading bacteria was done by streaking the isolates on Mineral Salt Medium plates with crude oil spread on it.

8. *Biosurfactant production & Surface tension measurement:*

Isolates were inoculated in Mineral salt medium in 250 ml conical flasks & incubated for 48 hours in orbital shaker at 150 rpm at 37p C. Surface tension of cell-free broths (isolates for biosurfactant production) was measured by Du Nuoy ring detachment method (Zajic & Stefens 1984) using Fischer Autotensiomat Modal -21(Fischer Scientific & Co. USA).

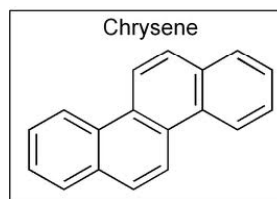
9. *Enzyme assay:*

A qualitative pH indicator assay was

done for presence of catechol dioxygenase.

10. *Poly aromatic Hydrocarbon:*

Chrysene ( $C_{18}H_{12}$ ) was the model PAH used in this study. It is a medium sized PAH molecule with a molecular weight of 228 & log  $K_{ow}$  of 5.86. It is relatively insoluble in water ( $1.3 \times 10^{-5}$ ) & tends to be persistent in the soil environment. It is car-cinogenic in nature<sup>7</sup>.



11. *Cultivation of isolates in Chrysene as sole carbon source:*

Bacterial isolates were tested for growth on chrysene as sole carbon source on MSM plates.

The four isolates were separately inoculated in 150 mL conical flasks containing MSM medium. Chrysene was provided as sole carbon source at the final concentration of 200 ppm. The flasks were incubated in an orbital shaker incubator for 15 days at 37°C.

12. *Characterization of Bacterial isolates:*

Biochemical characterization of the isolates was done using Hi-Media Kit KB001.

13. *Growth on Jenson's medium:*

Isolates were streaked on Jenson's Agar plates & incubated at 37°C.

#### 14. Starch hydrolysis:

Starch agar plates were inoculated with the culture & inoculated for 5 days at 28p C, then flooded with dilute iodine solution. Hydrolysis was indicated by clear zone around the growth.

#### 15. Gram staining:

Gram staining was performed for the isolates.

#### 16. Growth of isolates on *Pseudomonas* Isolation agar:

Isolates were streaked on *Pseudomonas* isolation agar plates & incubated for 24 hours.

#### 17. Catalase test:

Isolates were streaked on nutrient agar medium & incubated for 24 hours at 37p C. After incubation, hydrogen peroxide was added on colonies. Bubbling represents positive catalase test.

#### 18. Gas chromatography:

A Perkin- Elmer gas chromatograph equipped with a flame ionization detector (FID) was used with hydrogen as carrier gas. The injection port was maintained at a temperature of 280°C, & the detector temperature of 300°C. The oven temperature was programmed from 60°C to 280°C (Fig. 4). A fused silica capillary column (25 m x 1 mm i.d.) was used for all determinations. The injection port was glass-lined & allowed to split or splitless injection with volume of 5µL. A split ratio of 1:30 was used. Data acquisition & handling were computer assisted.

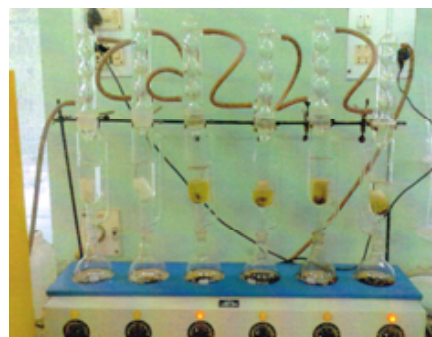


Fig. 1. Soxhlet extraction

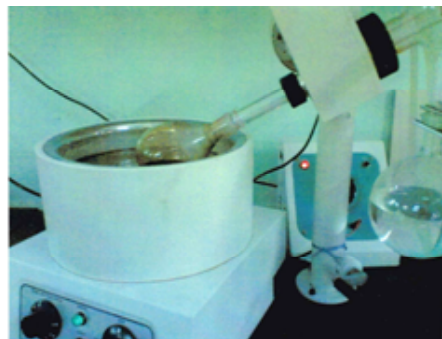


Fig 2 Rotary Vacuum Evaporator

#### 1. Characterization of oil sludge:

Oil & grease were extracted from the oil sludge using Soxhlet Apparatus. Solvent was evaporated in a rotary vacuum evaporator & oil % estimated gravimetrically. Organic carbon, nitrogen, & inorganic phosphorus content of residue left after extraction from the sludge was estimated. Oil % was found to be 45.77% in the oil sludge before degradation (Table-2). After 30 days degradation with the four isolates similar procedure was repeated to check the decrease in oil content (Table-2).

#### 2. Fractionation of oil:

The oil & grease extracted from the

oil sludge was fractionated using silica gel column. Hexane, Benzene & Methanol were the three solvents used for extraction of alkanes, aromatics & NSO fraction. (Table-3).

### 3. Biosurfactant production:

The isolates were tested for biosurfactant production (Fig. 13). Surface tension of cell-free broths was measured to check it (Table-4).

### 4. Gas Chromatographic analysis of chrysene degradation:

The isolates were streaked on MSM plates on which 50 ppm chrysene was spread & incubated for 24 hours at 37°C. growth after incubation indicated that the isolates are capable of utilizing chrysene as carbon source (Fig. 5,6,7,8). Gas chromatograph was used to analyse decrease in chrysene content in liquid cultures (Table-5).

### 5. Identification of Bacterial isolates:

Biochemical characterization of the isolates was done using Hi-Media Kit KB001 (Table-6).

### 6. Test for Catechol 1,2 Dioxygenase:

The isolates were tested for the presence of ring cleavage enzyme Catechol 1,2 dioxygenase which plays a central role in the pathway for catabolism of many aromatic growth substrates. The change of colour from red to orange by the isolates indicates the formation of cis,cis- muconate from catechol in the presence of enzyme catechol 1,2 dioxygenase. On the basis of orange colour formation, isolates were found to be metabolically active in intermediate products formation (Fig. 9).

### 7. Test for Catechol 2,3 dioxygenase :

The change in colour from yellow to brown by the isolates as compared to the control indicates the presence of enzyme catechol 2,3 dioxygenase . The brown colour indicates the formation of 2-hydroxymuconic semi aldehyde which is the intermediate product of aromatic metabolism (Fig. 10).

### 8. Amylase test:

The isolates were streaked on starch agar & then flooded with iodine after incubation. Clearing of zone around the growth indicates positive test (Fig. 2).

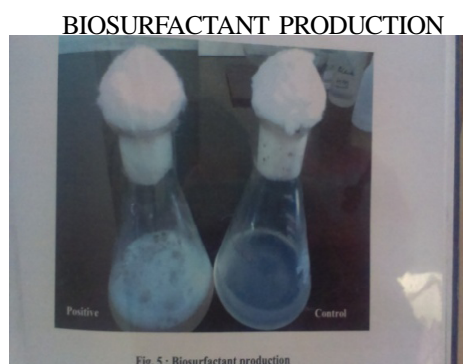


Fig. 3

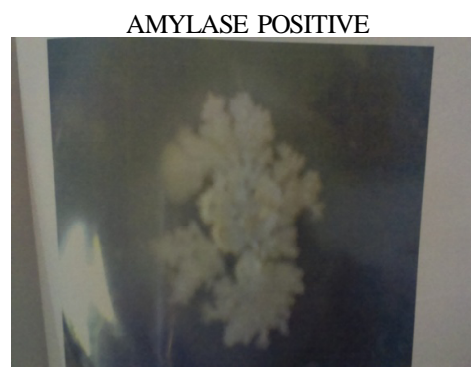


Fig. 4

ISOLATE 1 ON CHRYSENE

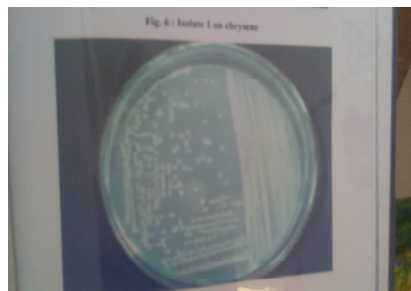


Fig. 5

ISOLATE 4 ON CHRYSENE

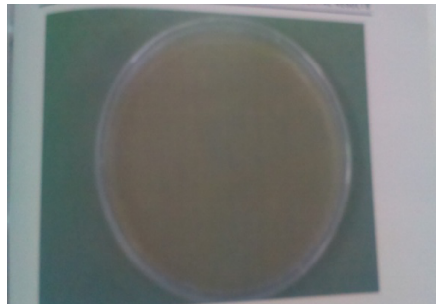


Fig 8

ISOLATE 2 ON CHRYSENE

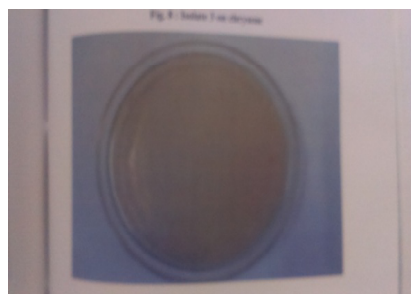


Fig 6

CATECHOL1, 2 DIOXYGENASE

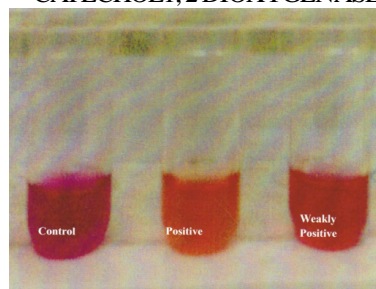


Fig 9

ISOLATE 3 ON CHRYSENE



Fig 7

CATECHOL2, 3 DIOXYGENASE

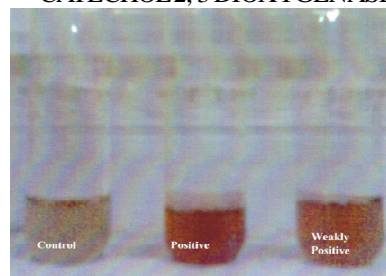


Fig 10

Table-1. Characterization of residue left after extraction of oil from oil sludge

Organic carbon	2.346%
Organic matter	4.044%
Nitrogen	0.154%
Phosphorus	24.02mg/kg

Table-2. Oil & Grease % after Soxhlet Extraction  
Oil & grease %

Oil sludge	45.77%
Control	40.44%
Oil sludge + Isolate 1	28.13%
Oil sludge + isolate 2	30.77%
Oil sludge + Isolate 3	25.36%
Oil sludge + Isolate 4	31.78%

Table-3. Fractions collected by Silica Gel Column

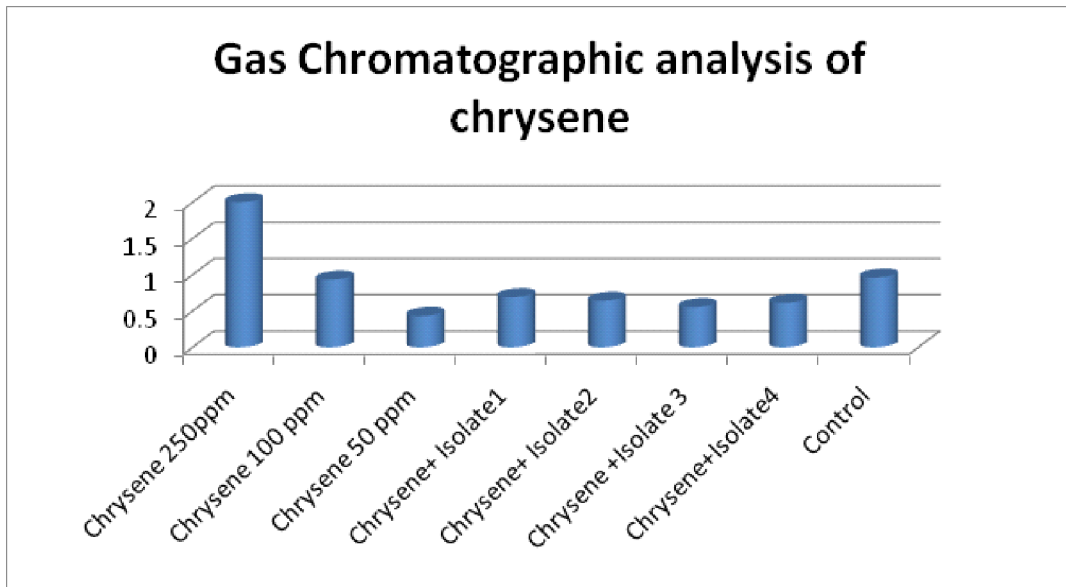
Sample	Alkanes	Aromatics	NSO
Sludge	44.7%	38%	12.78%
Control	42%	36.8%	12.86%
Sludge+ Isolate 1	30%	21.6%	10.4%
Sludge + Isolate 2	34.5%	19.6%	11.7%
Sludge+ Isolate 3	32.8%	28.7%	10.9%
Sludge+ Isolate 4	28.4%	25.9%	13.6%

Table-4. Surface Tension measurement  
Surface tension

Distilled water	73 dyne/cm
Isolate1	47 dyne/cm
Isolate 2	43 dyne/cm
Isolate 3	32 dyne/cm
Isolate4	40 dyne/cm
Control	71 dyne/cm

Table-5. Analysis of Chrysene degradation by Gas Chromatography

Component	Time	Area( $\mu$ V/sec)	Height ( $\mu$ V)	Area%
Chrysene 250ppm	29.10	1808793.02	223987.44	2
Chrysene 100 ppm	29.04	746253.89	144847.38	0.93
Chrysene 50 ppm	29.978	389895.07	95757.13	0.43
Chrysene+ Isolate 1	29.012	649383.49	130233.32	0.69
Chrysene+ Isolate 2	29.002	562503.37	120419.48	0.64
Chrysene +Isolate 3	29.020	474731.34	111632.72	0.55
Chrysene+Isolate 4	29.015	438825.59	101475.61	0.61
Control	29.039	705257.82	134624.29	0.96



Improper disposal methods and inadequate control of toxic materials have led to widespread contamination of soils, ground, water and surface water bodies. Achieving permanent clean up of these sites is problematic in that some remediation technologies are not always viewed positively by the public or may not be amenable to particular sites. Polycyclic aromatic hydrocarbons (PAHs) are considered as important environmental pollutants<sup>18</sup>. They are exceedingly recalcitrant to degradation due to their inhibitory nature and their very low aqueous solubility<sup>19</sup>.

Current strategies for disposing of heavily contaminated soils include excavation followed by incineration and /or secure land filling. Land farming has also been used for less contaminated soils. Bioremediation often appears the most acceptable remediation technology. Bacteria were isolated from oil contaminated sites. Nine isolates were obtained

and tested for their bio surfactant production. Four isolates were found to be positive and further study was carried out with them. The growth of the four isolates on Chrysene as sole source of carbon indicates that have the capacity to metabolize it. The reduction in surface tension and production of potent bio surfaces observed during their growth is in agreement with the findings of Youssef *et al.*,<sup>20</sup>. Although, there are differences in the relative ability of four isolates to degrade hydrocarbons, all of them exhibit a major advantage for efficient utilization of their potential in natural environment through production of bio surfaces that bring about the emulsification of the hydrocarbon substrate. The ability of those isolates to degrade hydrocarbons in oily sludge suggests that they could be used for the treatment of other oil wastes. Morphological parameters such as size, shape, color and colony textures were used for preliminary selection and identification.

Table-6. Preliminary Identification of isolates:

Parameter	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Gram Staining	Gram positive	Gram negative	Gram negative	Gram positive
Shape	Rods	Pleomorphic	Rods	Cocci in chain
Amylase test	positive	negative	positive	positive
Catalase test	positive	positive	positive	positive
Pseudomonas Isolation Agar	negative	negative	positive	negative
Catechol dioxygenase	positive	positive	positive	positive
Indole	negative	positive	negative	negative
Methyl Red	positive	negative	positive	negative
Citrate utilization	positive	positive	positive	positive
Glucose	negative	negative	negative	positive
Adonitol	negative	negative	negative	negative
Arabinose	negative	negative	negative	negative
Lactose	negative	negative	negative	negative
Sorbitol	negative	positive	negative	negative
Mannitol	negative	negative	negative	positive
Rhamnose	negative	negative	negative	negative
Sucrose	negative	negative	positive	positive
ONPG	positive	negative	positive	negative
Lysine decarboxylase	negative	negative	positive	positive
Ornithine decarboxylase	negative	negative	positive	positive
Urease	negative	negative	negative	negative
Deamination	negative	positive	negative	positive
Nitrate reduction	positive	positive	positive	negative
H <sub>2</sub> S production	negative	negative	negative	positive
Malonate	positive	positive	negative	positive
Esculin hydrolysis	negative	negative	positive	negative
Xylose	negative	negative	negative	positive
Cellobiose	positive	negative	positive	negative
Mellibiose	negative	negative	negative	negative
Saccharose	positive	negative	positive	negative
Trehalose	positive	negative	positive	negative
Oxidase	positive	negative	positive	positive

In the present work, surfactant production by the isolates and their ability to grow and to degrade chrysene is described. Chrysene was used as model contaminant because of its recalcitrance and carcinogenicity. The isolates were identified as *Pseudomonas* sp., *Mycobacterium* sp., *Flavobacter* sp. & *Rhodococcus* sp. on the basis of colony morphology, Gram staining & biochemical tests.

References :

1. Baheri and Meysami (2001). Feasibility study of fungal bioremediation of a flare pit soil using white rot fungi. Chemical and Petroleum Engineering, University of Calgary.
2. Barker, A.V. and M.B. Gretchen (2002). *Sci. World J.*, 2: 407–420.
3. Committee on *In situ* Bioremediation, Water Science and Technology Board, Commission on Engineering and Technical Systems, and National Research Council. (1993). *In Situ Bioremediation: When Does It Work?* National Academy Press: Washington, D.C.
4. Ekpo, M.A. and I.I. Nwankpa (2006). *J. Sustain. Trop. Agric. Res.*, 16: 67–71.
5. Guerin, W.F. and S.A. Boyd. (1992). *Applied Environmental Microbiol.* 58: 1142–1152.
6. Heitkamp, M. A., and C. E. Cerniglia. (1987). *Environ. Toxicol. Chem.* 6: 535-546.
7. IARC. (1983) Monographs on the Evaluation of the carcinogenic Risk of Chemicals to Humans: Polynuclear Aromatic Hydrocarbons. Vol. 32. WHO. Lyon France.
8. Manohar, S., C.K. Kim and T.B. Karegoudar (2001). *Appl. Microbiol. Biotechnol.* 55: 311–316.
9. Manohar, S. and T. B. Karegoudar (1998). *Microbiol. Biotechnol.* 49: 785–792.
10. Offor, U.S., J.A. Onwugbuta-Enyi and L. A. Akonye (2009). *Afr. J. Biotechnol.*, 8: 65–68.
11. Ogbo, E. R. (2009). *Afr. J. Biotechnol.*, 8: 250–253.
12. Ojumu, T. V., O. O. Bello, J.A. Sonibare and B.O. Solomon (2005). *Afr. J. Biotechnol.*, 4: 31–35.
13. Onweremadu, E. U., G. F. Ndukwe and G. F. Okoroafor (2008). *Life Sci. J.*, 5: 62–66.
14. Ort, M. P., S. E. Finger and J. R. Jones (1995). *Environ. Pollut.*, 90: 105–110.
15. Overcash, M.R. and D. Pal. (1979). Design of Land Treatment Systems for Industrial Wastes –Theory and Practice. Ann Arbor Science. Ann Arbor, Michigan.
16. Shara, S. I. and Y. M. Moustafa (2009). *J. Appl. Sci. Res.*, 5: 2406–2411.
17. Siddiqui, S., W. A. Adama and J. Schollion, (2001). *J. Plant Nutr. Soil Sci.*, 164: 631–635.
18. Soniassy, R., P. Sandra and C. Schlett. (1994). *Water Analysis—Organic Micropollutants*. Germany: Hewlett Packard.
19. Wilson, S.C. and K.C. Jones (1993) *Environ. Pollut.* 81: 229-49.
20. Youssef, N.H., K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp and M.J. McInerney (2004). *J. Microbiol. Methods*, 56: 339-347.