Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2011, 1 (4):41-49

ISSN: 2248 –9215

Characterization of Alkyl sulphatase required for the biodegradation of Sodium Dodecyl Sulphate (SDS)

Ambily, P. S.[1] and M. S. Jisha[2*]

School of Biosciences, Mahatma Gandhi University, Kottayam

ABSTRACT

The anionic surfactant Sodium Dodecyl Sulphate (SDS), the core components of detergent and cosmetic product formulations contribute significantly to the pollution profile of sewage and wastewaters of all kinds. In this study, 44 SDS degrading strains were isolated by soil enrichment methods and the utilization efficiency was assessed by methylene Blue Active Substances (MBAS) assay and HPLC method. The most efficient isolate was identified as Pseudomonas aeruginosa MTCC 10311 based on phenotypic features and 16 S rDNA typing. The alkyl sulphatase enzyme responsible for the primary degradation of SDS degradation showed enzyme activity 0.0546 U/mg in the crude extract ,which on purification by streptomycin sulphate, dialysis, DEAE cellulose and sephadex G200 gave enzyme units of 3.04 U/mg. The partially purified enzyme could give a maximum SDS degradation of 75% at 4 % of enzyme concentration. Non denaturing PAGE at various stages of purification confirmed that sephadex200 step was particularly effective. The purity of enzyme confirmed by the presence of single band in native PAGE and the molecular mass of the native enzyme was around 77 kDa. Single band was observed in the zymogram of alkyl sulphatase. The optimum p^H ,temperature and enzyme concentration of enzyme activity was 7.5,30^{0}c and 4%

Key Words: Anionic surfactant, *Pseudomonas aeruginosa MTCC* 10311, Methylene Blue Active Substances (MBAS) assay, zymogram.

INTRODUCTION

Society's ever-expanding utilization of materials, energy and space is accompanied by an increasing flux of anthropogenic organic chemicals into the environment [2]. Surfactants are

the largest class of compounds present in raw domestic wastewater. They are used in household and industrial laundry and cleaning operations. The ever-increasing demand of surfactant since the middle of this century is causing great concern about its role in environmental pollution. Studies about the degradation and transformation of such compounds and the proper processing of the effluents containing them are essential for the safe and clean environment.

Sodium dodecyl sulphate (SDS), an anionic surfactant, has been used extensively due to its low cost and excellent foaming properties. The molecule has a tail of 12 carbon atoms, attached to a sulphate group giving it amphiphilic properties required of a detergent. The extensive disposal and toxicity of SDS necessitates its effective remediation. Biodegradation of surfactants is most often performed by soil or aquatic microorganisms and leads to generation of water and carbon dioxide gas [15]. Several species of *Pseudomonas* are known to biodegrade SDS. In fact, many surfactants and their degradation products have been found worldwide in waste water discharges, sewage treatment, plant effluents, natural water and sediments [21]. Because many surfactants are ubiquitous [22, 18], the potential toxic effects of these chemicals have attracted much research attention in the past several decades [10].

Biodegradation is initiated by alkyl sulphatase enzymes which hydrolyses inorganic sulphate from its ester linkage with alcohols, the later being readily assimilated through normal metabolic pathways [17] .The study about the enzymatic characteristic of bacteria involved in Sodium Dodecyl Sulphate (SDS) biodegradation can be exploited for the development of a biosensor for the early detection of detergent contamination in the environment. The present paper describes the purification and characterization of alkylsulphatase from *Pseudomonas aeruginosa* MTCC 10311.

MATERIALS AND METHODS

Isolation of SDS degrading bacteria

Bacteria capable of utilizing Sodium Dodecyl Sulphate (SDS) as sole source of carbon were isolated from surfactant contaminated soil (Meenachil river basin, Kottayam, Kerala) by enrichment in mineral salt medium(MSM). Isolation was done by enrichment of the soil extract progressively with SDS. The composition of the mineral salt SDS medium (MSSM) used was (grams per liter) KH_2PO_4, 1.5, K_2HPO_4, 3.5; $MgCl_2.6H_2O$, 0.; NaCl, 0.5; Na_2SO_4, 0.14. The medium also contained the following trace elements (1 ml of stock) (grams per liter): $FeCl_2.6H_2O$, 0.24; $CoCl_2.6H_2O$, 0.040; $CuSO_4.5H_2O$, 0.06, $MnCl_2.4H_2O$, 0.03; $ZnSO_2.7H_2O$, 0.31; and $NaMoO_2.2H_2O$, 0 Incubation was carried out at room temperature (30 ± 2 ^{0}C) for 24 hours with shaking at 150 rpm. At the final concentration of 2100mg/litre of SDS there was only 9 strains that could grow in MSSM and the most efficient strain was selected. MBAS assay (Hayashi, 1976) and HPLC were used for the determination of SDS reduction The selected cultures were purified by repeated streaking and were stored at 20^0 C in 50 Mm KH_2PO_4 buffer at p^H 7.2 containing 20% glycerol .Working cultures were maintained by sub culturing every two weeks on mineral salt agar slants containing SDS

Inoculum preparation

One loopful of the culture was inoculated to a 50 ml of mineral salt SDS medium.The flasks were incubated overnight at room temperature (30± 2^0C) at 150 rpm .From the culture the cells were harvested by centrifugation. The pellets were collected and diluted using physiological saline (0.86% NaCl) till the OD becomes 1.This was used as the inoculum for further studies.

Quantification of SDS by MBAS Assay

The concentrations of SDS were checked by the Methylene Blue Active Substances (MBAS) assay as described by Hayashi [6] with minor modifications. The method is based on the formation of a complex between the anionic surfactant and an excess of the cationic dye methylene blue , followed by the extraction of the complex (but not excess dye) into the chloroform and measurement of the absorption of the blue chloroform layer. Alioquots of methylene blue solution (0.1ml), 0.4ml of 0.825Mm phosphate buffer (pH 7.2), and an appropriate volume of sample (up to 1ml, in triplicate) were mixed in acid-washed, optically matched glass tubes (12cm by 1cm (internal diameter) and vortexed intermittently five times for 3 seconds each time. Chloroform was added to each tube and the contents were vigorously vortexed for 5 seconds. The tubes were allowed to stand at 4^0C for 5 minutes, followed by centrifugation at 2,000 rpm for 4 minutes. The tubes were allowed to warm to room temperature, and the absorbance of the chloroform layer was measured at 655nm, against an appropriate blank.

HPLC analysis of SDS degradation

The biodegradation was confirmed by isocratic HPLC (Shimadzu) analysis using reverse phase C-18 column equipped with SPD20 A UV detector (220 nm).The mobile phase gradient of acetonitrile –water (95:5) was conducted at a flow rate of 1ml/min. Data acquisition and processing was performed by using LC solution system software.(Shimadzu)

16S rDNA sequence analysis

The selected isolate was identified by conducting biochemical test as per Bergey's manual and confirmed by 16srDNA sequencing.DNA extraction was carried out as described by Pitcher *et al*., [13]. Amplification of 16S rDNA was performed using 8f – (5 AGAGTTTGATCCTGGCTCAG 3) and 1492r 5TACGGATACCTTGTTAGCACTT 3) primers. The total volume of PCR reaction mixture was 50 µl, comprising 200 µM dNTPs, 50 µM each primer, 1x PCR buffer, 3 U Taq DNA polymerase and 100 ng genomic DNA. The thermo cycling procedure involved an initial denaturation at 94^0C for 4 min, followed by 35 cycles 94^0C for 1 min, 52^0C for 1 min, and 72^0C for 2 min, and a final extension at 72^0C for 8 min. The gel purified 16S rDNA was ligated to PCR-TRAP cloning vector and transformed in *E.coli*. The sequence of the insert was determined using the automated DNA sequencing service provided at Institute of Microbial Technology (IMTECH), Chandigarh, India. The sequences were analyzed using the gapped BLASTn (www. ncbi.nlm.nig.gov) search algorithm and aligned to their nearest neighbors. The sequence was deposited in the NCBI gene bank data base (HM 214777).

Extraction and purification of alkyl sulphatase from *Pseudomonas aeruginosa* MTCC 10311

Stage I: Extraction of crude extract

Isolates grown on a mineral salt medium supplemented with SDS were harvested by centrifugation (10,000 rpm).This pellet were collected and resuspended in 0.1 ml of 100Mm phosphate buffer (pH7.5) containing 0.58 M sucrose and 10Mm EDTA and lysed by incubating with 4 mg of lysozyme (sigma) in 0.1 ml of phosphate-sucrose –EDTA buffer for 60 minutes at 37^0 C. Triton X-100 (0.1 ml of 10% v/v) was added to complete lysis (Lee et al. 1996). The centrifuged cell free lysate was subjected to purification.The universal protease inhibitor, α_2 macroglobulin, was added to the extract to a final concentration of 0.5 mg/ml, and the whole mixture was stirred for 5 min in ice [11].

Alkylsulfatase assay

Alkylsulfatase activity in cell extracts was assayed by incubating 50 µl partially purified enzyme (0.14 mg ml-1 protein) of isolate *Pseudomonas aeruginosa* with 450 µl of 50 Mm Tris-HCl and 500 µl of 100 Mm SDS. The loss of the substrate was measured by MBAS assay as described before. The disappearance of SDS was linear until 15 min under the assay conditions. Total enzyme activity was determined from the initial rates of SDS disappearance. One unit of enzyme was defined as the amount of enzyme which converted 1 µl of SDS per minute under assay conditions [16].

Protein assays

Protein was routinely determined by Lowry's method with BSA as standard and measuring the absorbance of sample at 280 nm

Stage: 2 Removal of nucleic acid

The cell free extract from stage I was treated with sufficient streptomycin sulphate (5% w/v) to give a final concentration of 1 mg of antibiotic /mg of protein. After stirring for 10 min at 4^0C ,precipitated nucleic acid was removed by centrifugation as before.The clear supernatant was dialyzed overnight against 100 Mm tris /HCl, pH 7.5 (3×5 liters) [6].

Stage 3: DEAE-cellulose (DE 52) column chromatography

The nucleic acid –free extract from stage 2 (35 ml) was applied to DEAE-cellulose (sigma, USA) column (1.5 ×30 cm) that had been pre-equilibrated with column buffer.(10Mm Tris/HCl containing 0.5 M, pH 7.5). The column was washed with column buffer(50 ml) followed by adding NaCl (0.8 M, total volume 200ml) in column buffer. The flow rate was adjusted to 5ml/hr and 2 ml fraction was collected and assayed for protein and enzyme activity. Fractions with the highest specific activity (fractions 64-70) were combined and dialyzed for 2 h(3×5 liters column buffer) [1].

Stage 4: Sephadex G-200 exclusion column

The pooled fractions from stage 3 (10 ml) were applied to a Sephadex G-200 column that had been pre-equilibrated with column buffer. The equilibration and elution buffer was 50 Mm Na H_2PO_4 containing 0.15 M NaCl [20].

Determination of the molecular mass of the enzyme - Native Polyacrylamide gel electrophoresis (Native PAGE)

The purified enzyme was subjected to electrophoretic studies to confirm purity using polyacrylamide.

Zymogram of alkyl sulphatase extracted from *Pseudomonas aeruginosa*

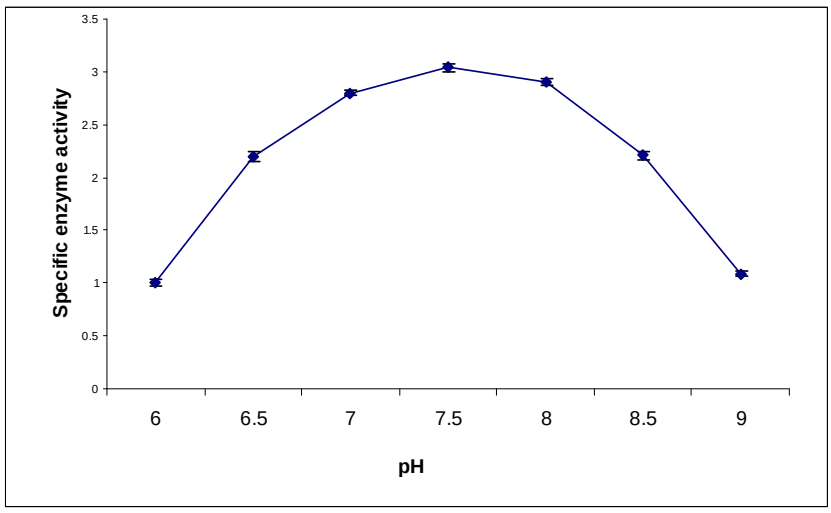
Non–denaturing polyacrylamide gel electrophoresis of extracts of cells grown on SDS, followed by incubation of gel at 30^0C in solution containing 10 Mm SDS and 20 Mm –$BaCl_2$ in 20Mm Tris-HCl, pH 7.8 [5].

The optimum pH and temperature were determined [19].

RESULTS AND DISCUSSION

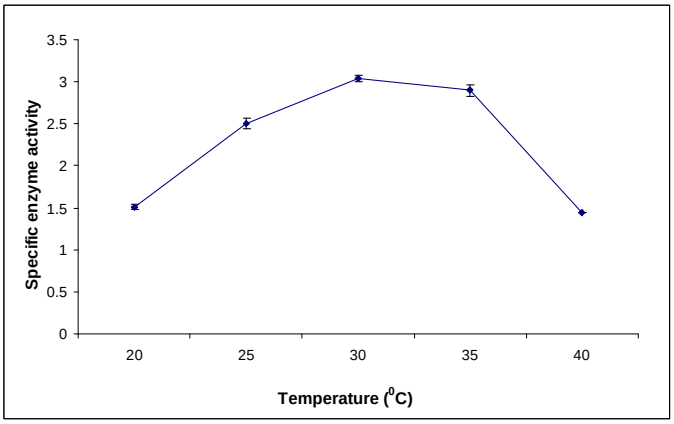
Pseudomonas aeruginosa (MTCC 10311) was isolated from detergent contaminated soil for their ability to utilize SDS as the sole carbon source. The isolate was capable of degrading 96% of SDS at 48 hours of incubation period (Fig 1). The results of BLAST search of 16S r RNA compared with the available 16S r DNA sequence in the gene bank database indicated that this organism belongs to *Pseudomonas aeruginosa*. The Gene bank accession number for the 16S r DNA sequence generated in this study was HM 214777. The optimum condition for degradation was 37ºC and p^H 7.5.

Fig :1 Effect of p^H on the activity of alkyl sulphatase extracted from *Pseudomonas aeruginosa*



HPLC analysis showed that the area of biodegradation of SDS in mineral salt medium was decreased from 56156126 to 432439 (2.758min) within 48 hours of incubation (Fig 2). Schleheck *et al*.[18]suggested that HPLC is a superior technique for surfactant identification. HPLC analysis indicated that the area of biodegradation of sodium dodecyl benzene sulphonate reduced from 144216 to 67312 within 15 days of incubation time by *Stenotrophomonas maltophilia* biofilm [4].

Fig :2 Effect of temperature on the activity of the alkyl sulphatase extracted from *Pseudomonas aeruginosa*



Cell crude extract retained >95% activity during the storage at 4^0 C for about 4 days but thereafter activity decreased rapidly (<15% in 10 days). In contrast, inclusion of the protease inhibitor, α_2 macroglobulin (0.5 mg/ml) produced marked stabilization of the enzyme activity. (Table: 1)This inclusion was made in all subsequent experiment including purification of enzyme from crude extracts. The same observation was found in the study of alkyl sulphatase from *Coryneform* B1a [11].

Table: 1 Percentage of enzyme activity during the storage at 4º C

Sample	DAYS						
	2	4	6	8	10	20	30
Crude enzyme	98±0.3	95± 0.2	60± 0.4	43±0.3	13±0.1	5±0.2	1±0.05
Dialysed Crude enzyme	70±.0.9	25±0.3	12±0.4	2±0.2	-	-	-
Crude enzyme+ α_2 Macroglobulin	99±0.2	98±0.4	98±0.3	97±0.6	95±0.4	90±0.3	54±1

The alkyl sulphatase was extracted from the purified crude cell free lysate after disruption of the cells. Problems due to the presence of mucous substance such as caroteinoides, lipids and various surface active components, which were released from the cells during the disruption, were overcome by pretreating the lysate with streptomycin –sulphate and DEAE cellulose [8]. The treatment of crude extracts with 0.5 mg of streptomycin –sulphate /mg of protein increased specific activity to 262 % of the starting value, and simultaneously decreased the total protein to 71% of the initial amount. This observation is consistent with earlier findings of Matts et al. [11] where the treatment of streptomycin sulphate /mg of protein increased enzyme activity to 120-200 % of the starting value and simultaneously decreased total protein to 77 % of the initial amount .The increase in the enzyme activity attributed to removal of proteinaceous inhibitor

DEAE cellulose chromatography was used to remove the α_2 macroglobulin from the alkyl sulphatase .Multiple bands were visible in this stage due to the removal of α_2 macroglobulin The purification profile of enzyme is given in table. 2.

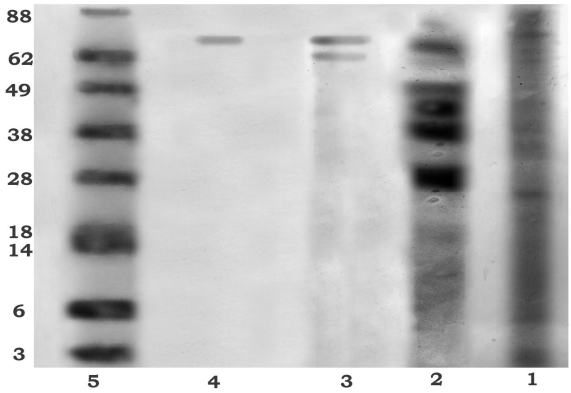
Table: 2 Purification profile of the alkyl sulphatase extracted from *Pseudomonas aeruginosa*

Step	Vol (ml)	Protein (mg)	Total protein (mg)	Total enzyme activity(u)	Sp ac- (U/mg)	[a] % of recovery	[b]Purification(fold)
Cell extract	30	6	180	10	0.0546	100	1
Streptomycin sulphate treated	35	4.26	147	21.2	0.1441	212	2.63
DEAE-cellulose	10	3	30	11.30	0.376	113	6.886
Sephadex G-200	40	0.041	1.64	5	3.04	50	55.677

[a] -% of the initial activity
[b-] Specific activity/0.0546

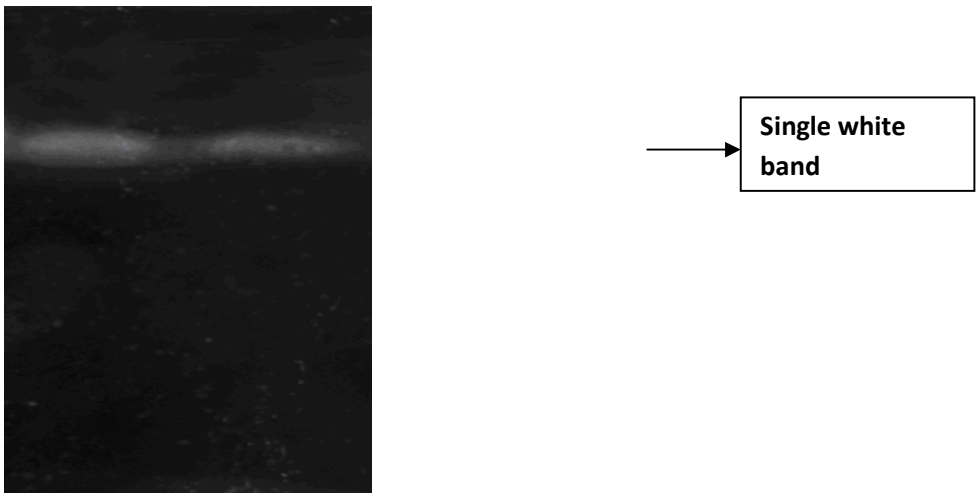
Non denaturing PAGE at various stages of purification confirmed that sephadex G-200 step was particularly effective.(Plate : 1 & 2). The purity of enzyme confirmed by the presence of single band in native PAGE and the molecular mass of the native enzyme was around 77 kDa.The molecular mass of alkyl sulphatase in *Rhodococcus rubber* was found to be 67 kDa [14]. Extracts of *Pseudomonas* sp. strain C12 B cells grown on SDS supplemented basal salt medium in batch culture produced P1 and P2 bands when gels were incubated with SDS. When the same cell extracts were separated on gels and gels were with 2- butyl octyl sulphate instead of SDS, no alkyl sulphatase bands were observed [3].

Plate: 1 Native page of the partially purified Enzyme



Lane 1 ⟶ Crude enzyme
Lane 2 ⟶ Purified with Streptomycin sulphate
Lane 3 ⟶ Purified with DEAE-cellulose
Lane 4 ⟶ Purified with Sephadex G-200
Lane5 ⟶ Sigma ladder

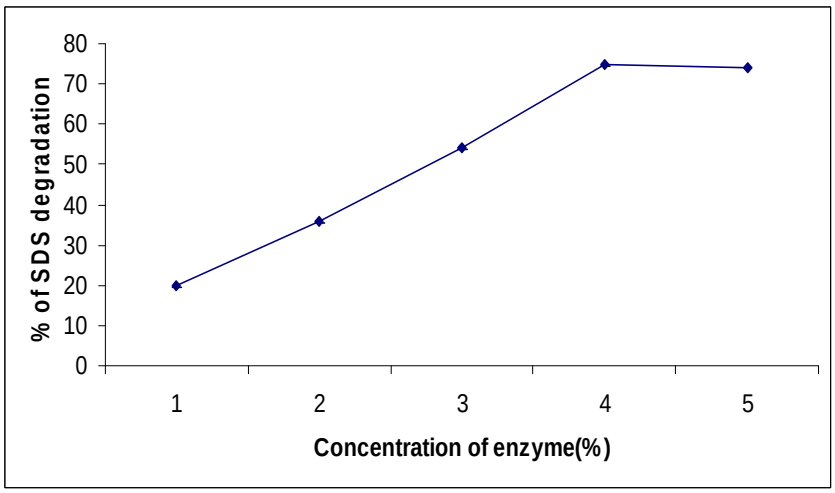
Plate: 2 Zymogram of alkyl sulphatase



The optimum pH for the activity of alkyl sulphatase was 7.5. In most of the reported cases, the optimum P^H for the enzymatic action was in the alkaline range .In *Pseudomonas sp*.,the activity was maximum in the range of 7-7.5 [12]. The optimum temperature for the activity of alkyl sulphatase was found to be 30ºC. *Pseudomonas sp*.and *Rhodococcus rubber* DSM 44541also showed the optimal temperature of 30ºC for maximum enzymatic activity [12,14].

On increasing enzyme concentration the rate of degradation was found to be increased up to 4 % enzyme concentration and was stable thereafter. There was 75% degradation of SDS at 4% enzyme concentration (Fig: 3). Even though the free and immobilized cells could bring about 96% SDS reduction, the partially purified enzyme could bring about only 75% of SDS degradation. This clearly showed that the enzyme is not the only reason for the SDS degradation, but several other factors are also involved in SDS degradation.

Fig: 3 Biodegradation of SDS with partially purified alkyl sulphatase from *Pseudomonas aeruginosa*



CONCLUSION

A powerful SDS degrader, *Pseudomonas aeruginosa*, MTCC 10311 was isolated from soil.Alkyl sulphtase enzyme was isolated and purified from *Pseudomonas aeruginosa*. These alkyl sulphatase will be promising tool to conduct further studies to reduce the environmental pollution of anionic surfactant,SDS.

Acknowledgement
The authors are grateful to KSCSTE, Kerala for the financial support.

REFERENCES

[1] V. Abirami , S. A. Meenakshi1, K. Kanthymathy1, R. Bharathidasan, R. Mahalingam ,A. Panneerselvam, *Euro. J. Exp. Bio.*, **2011**, 1 (3):114-123
[2] S.O. Adewoye, *Advances in Appl. Science Research.*, **2010**, 1 (1): 89-95
[3] A.J. Ellis, S.G. Hales, N.G.A. Ur-Rehman, G.F. *White, Appl. Environ. Microbiol.*, **2002,** 68, 31-36.
[4] H. Farzaneh, M. Fereidon, N. Amirmozafari, G. Naser, *Afr J Biotechnol.*, **2010**, 9 (1),55-62.
[5]S.G. Hales, K.S. Dodgson, G.F. White, N. Jones, K.G. Watson, *Appl.Environ.Microbol.* **1985**, 44, 790-800.
[6] K. Hayashi, *Anal Biochem*, 1975. 67,503-506
[7] N . Lee, M. Hwang,.Jung. Y. Kim, K. Min, *Biochem Bioph Res Co.,* **1996**, 218 ,17-21.
[8] D.M. Lee, J.B. Guckert, R.M. Ventullo, D.H. Davidson, S.E. Belanger, *Ecotox Environ safe*, **1997,** 36,288-296.
[9] M .A. Lewis, *Water Res*, **1991,** 25, 101-113.
[10] M.A. Lewis, D. Suprenant, *Ecotox Environ Safe.*, **1983**, 7, 313-322.
[11] P.J. Matts, Graham F, White, W.J. Payne, *Biochem J.*, **1994**, 304,937-943.
[12] W.J. Payne, J.P. Williams, W.R. Mayberry, *Appl Microbiol.*, **1965**, 13, 698-701.
[13] D.G. Pitcher, N.A. Saunders, R.J. Owen, *Lett Appl Microbiol.*,**1989,** 8,151-156.
[14] M. Pogorev**,** K. Faber, *Appl Environ Microbiol.,* **2003**, 69 (5), 2810-2815
[15] D. Schleheck, W. Dong, K. Denger, *Appl. Environ. Microbiol.*, **2000**, 66, 1911-1916.
[16] M.Y. Shukor, W.S.W. Husin, M.F.A. Rahman, N.A. Shamaan. M.A. Syed, *J Environ Biol* **2009**, 30(1), 129-134.
[17] O.R.T. Thomas, G.F. White, *Biotechnol Appl Bioc.*, **1989**, 11, 318-327.
[18] S.H. Venhuis, M. Mehrvar, *Int J Photoenergy, Int J Photoenergy*, **2004**, 6, 115-125.
[19] Vipul Verma, Mrigank Shekhar Avasthi, Abhishek Raj Gupta, Monika Singh and Akhilesh Kushwaha, *Euro. J. Exp. Bio.,* **2011**, 1 (3):107-113
[20] Vengadaramana, A., Balakumar, S, Vasanthy Arasaratnam, *Euro. J. Exp. Bio.,* **2011**, 1 (3):58-69.
[21] G.G. Ying, *Environ. Int.,* **2006**, 32, 417-431.
[22] G.G. Ying, B. Williams, R. Kookana, *Environ Int.* **2002**, 28, 215-226.