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Screening For Diclofenac Degrading Microorganisms And Identification Of its Degradation Products.

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Abstract: *Diclofenac bioaccumulation has been a cause for concern as reported by various Environment and Health agencies. Five environmental samples screened for diclofenac degraders, yielded 34 isolates, of which two bacterial isolates belonging to the Alcaligenes and Brevudimonas genera and two fungal isolates, were used to form a consortium. The consortia was used for degradation studies, where 75.90% of Diclofenac degradation occurred in 12 hrs, This is in contrast to the individual isolates which could initiate degradation only after 24 hrs of incubation . Degradation profile by MS analysis after 120 hrs showed peak with retention time of 15.27 mins which could be identified as ,2-[(2,6-dichlo1-(2,6-dichlorophenyl)-2-indolinon*

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I. Introduction

Though pharmaceuticals are a large and diverse group of medicinal compounds used for the diagnosis, cure, treatment, or prevention of diseases in humans and animals. **(Corcoran, J. 2010)** its pharmaceutically active compounds (PhACs) is excreted from the human or animal body within days to reach the aquatic environment via urine and faeces. Within such systems, PhACs have been observed in the ng/l to the low µg/l range, which usually does not have acute toxic effects on aquatic fauna and flora, but can lead to long-term effects due to bioaccumulation, additive and synergistic effects of mixing different PhACs .Thus an increasing number of pharmaceuticals have found their way into varied ecosystems, as many of them are not fully degraded in wastewater treatment plants (WWTPs), with some undergoing partial biodegradation to remain dissolved in the water, or bind to biosolids within the sewage sludge **(Trudeau et al., 2005)**

Diclofenac is a widely used non-steroidal anti-inflammatory drug (NSAID) which has been frequently reported in wastewater effluents, surface water, ground water and even drinking water. NSAID are known to inhibit the activity of cyclooxygenase, an enzyme present in many species of the animal kingdom, responsible for the synthesis of prostanoids **(Corcoran, J. 2010)**.

Though, a variety of organisms play a role in decreasing the environment toxic levels of xenobiotics Diclofenac conversion is restricted to specific group(s) of microorganisms. **Groning, J. et al 2007** reported that within Biofilms that are adapted to diclofenac, high levels of Gram-negative bacteria, especially those belonging to the Cytophaga-Flavobacterium group and the γ-Proteobacteria have been detectd and these organisms have been utilized within oxidation ponds and zoogleal mass to initiate degradation.

Zhang et al. (2010) studied the elimination of pharmaceutically active compounds by white rot fungi which are capable of degrading a broad range of xenobiotic pollutants and reported that crude lignin peroxidase (LiP) enzyme from *Phanerochaete chrysosporium* can be used to degrade several xenobiotics as high removal efficiencies are achieved Besides this, organisms belonging to the *Trametes* genera have been screened for their ability to degrade and detoxify xenobiotics, mainly by the virtue of the laccase enzyme. Laccases are oxidoreductases belonging to the multinuclear copper-containing oxidases that catalyse the mono electronic oxidation of substrates at the expense of molecular oxygen (**Riva, S. 2006**) and thus they can be of significant use in initiating the breakdown of the moiety.

Thus though the lignolytic bacteria like *Trametes versicolor*, white rot fungus *Phanerochete chrysosporium*, *Phanerochete sordida YK-624*, and *Epicoccum nigrum* have been reported for their potential to degrade various pharmaceutical entities very few studies deals with reduction of toxicity of diclofenac through in vitro and in vivo studies. The study thus aims at a the objective of decreasing the toxicity of Diclofenac, by screening for microorganisms existing in natural environments and further employing in vitro systems to assess its degradation products

Thus, obtaining consortia of organisms compatible to each other that can be introduced within various aquatic milieu to form a stable entity that can prevent the bioaccumulation if not degradation of Diclofenac to mitigate Diclofenac effects on aquatic life forms would be a promising approach in assessing the PBT of this active pharmaceutical.

II. Materials And Methods

1.1 Enrichment and screening of Diclofenac Degrading Organisms

Sample Collection:

Soil samples (200g) from the lower layer of the soil profile were collected in clean and dry polythene bags from Bhavan's College Garden and Tower of Silence, Hughes Road. 50ml of Effluents samples discharged by "FAM Interiors" of New Bombay Chemicals Ltd., Chemical Effluent from Outskirts of Bhiwandi industrial estate and an Industrial Effluent Sample (Name withheld on request) obtained from a company producing diclofenac were collected in clean bottle to assess the ability of the inherent flora to degrade the active ingredient of Diclofenac.

Enrichment:

1.0 gm/ml of respective soil/effluent samples were suspended in sterile 10ml of sterile saline, vortexed and allowed to settle. The resulting suspension was transferred to 100ml of screening media (M9 media) which contained 10 ml of Solution-I, 2 ml of Solution-II, 2ml of Solution-III and 86 ml of Solution-IV at pH7.4.prepared as follows

Solution-I: Na_2HPO_4- 0.6g, NaCl-0.5g, KH_2PO_4- 0.3g, NH_4Cl-0.1g, D/W-10ml.
Solution-II: Glucose-0.1g, D/W-10ml.
Solution-III: 1M $MgSO_4.7H_2O$ - 0.25g, D/W - 10ml.
Solution-IV: $CaCl_2$ - 0.11g, D/W - 10ml.

The first enrichment flask was supplemented with diclofenac at 50,000 times the environmental concentration i.e. 5µg/ml. The flasks were incubated at 28°C under shaker conditions for 48-96 hours after which the 5ml of the aliquot was transferred to sterile M9 media containing increasing concentrations of Diclofenac such that subsequent enrichments were attained at diclofenac concentrations of 10µg/ml, 20µg/ml, 50µg/ml respectively.

Isolation:

Gram staining and plating out of the enrichments, were carried out after 48 to 96 hrs of incubation. Enriched cultures were isolated on M9 agar and Rose Bengal Chloramphenicol Agar supplemented with the concentration of Diclofenac similar to that in the respective enrichments. Representative bacterial and fungal isolates thus obtained were checked for their purity, gram nature and growth on M9 + 1000µg/ml Diclofenac. Short listed isolates which could tolerate 1000µg/ml Diclofenac in a chemically defined medium were selected for further studies.

1.2 Degradation Studies

The degradation ability of the isolates, were assessed in 50 ml of M9 Mineral media containing 1000µg/ml Diclofenac wherein 0.1% wet wt/vol of each isolate was used as an inoculums . The flasks were incubated at RT on shaker for 96 hrs and aliquots of metabolic filtrates were drawn at time intervals of 2hrs, 24hrs, 48hrs, 72hrs and 96hrs, to assess the degradation potential of the isolates.

At each time interval, the metabolic filtrates were centrifuged for 20minutes in swing arm centrifuge, and the supernatant separated from the cellular biomass .Both the aliquots were preserved at 4°C (not more than 24 hrs) for estimation of residual concentration of Diclofenac both in the supernatant as well as that accumulated within the biomass

2. Estimation of residual concentration of diclofenac

2.1 By Spectrophotometeric Method (Agrawal, Y.K. 1991).

The residual concentration of Diclofenac within the metabolic filtrate and within the sonicated biomass was determined by the method of Agrawal et al method, using 0.2% ferric chloride and 0.5% 2,2-bipyridine 200µg/ml of standard Diclofenac Sodium stock (Sigma, Aldrich) was prepared in D/W and used to prepare a calibration curve at the concentration of 10µg/ml - 80µg/ml such that a total volume of 1ml was attained within a series of volumetric flasks using distilled water as the diluent. To each flask, 1 ml 0.2% ferric chloride (w/v) solution prepared in alcohol and 1 ml of 0.5% 2,2-bipyridine (w/v) solution prepared in alcohol were added, followed by dilution to 10 ml with distilled water. The flasks were stopper and kept in a boiling water-bath for 25 min. The solutions were then cooled to room temperature and the absorbance was measured at 520 nm against the reagent blank.

Similarly, the residual diclofenac within the metabolic filtrate of each isolate obtained were assessed by extrapolating the absorbance attained within the calibration curve. The bioaccumulated diclofenac within the biomass was determined by taking 10ml of the aliquot. After centrifugation, the cell pellet was weighed and resuspended in 5ml of sterile saline and sonicated for 15seconds. The disruption of the cell mass was further

enhanced by vortexing for 1min. The cell debris were separated by centrifugation and the supernatant similarly assayed for Diclofenac concentration.

2.2 By HPLC Analysis (Bennet, J. et al)

Detection of Diclofenac within the metabolic filtrate after 2hrs, 4hrs, 8hrs, 12hrs, 24hrs of incubation was assessed using Shimadzu HPLC system coupled to UV detection system at 282nm, with data collection system of 'My GC'. The mobile phase consisted of Acetonitrile: KH_2PO_4 buffer [7:3 v/v], at 0.01M at a pH adjusted to 5.8. The mobile phase was filtered through 0.45µ filter and sonicated for 20mins before use. 20µl of the sample were loaded into the 25cm long Kromasil RP C18 column, with particle size of 5µm and an internal diameter of 4.6mm and the solvent flow rate adjusted to 1ml/min. Under these conditions, Std Diclofenac Sodium has a flow rate of 2.3mins, which was used to detect its presence, while the area under the peak was used to calculate its concentration using a standard diclofenac concentration of 100µg/ml.

III. Preparation Of Microbial Consortia

In order to assess the potential of the organisms in degradation studies, bulk production of each isolate was carried out.

3.1 For Degradation Studies

Each of the short listed fungal isolates *P.chrysosporium,* isolate SI-5 and bacterial isolates BM1 and BM5; were inoculated separately in M9 Broth with 200µg/ml of Diclofenac and incubated for 4 days on shaker. The broth was centrifuged and the cell pellets were washed with sterile saline in order to remove residual amounts of Diclofenac.

For the time course degradation studies 0.1% wt/vol of each of the four shortlisted isolates was inoculated in 100 ml of Mineral M9 media containing 1000µg/ml Diclofenac. Aliquots of metabolic filtrates were drawn at time intervals to assess its degradation potential.

4. Identification of Degradation Products By GC-MS (Kosjek, T. Et Al 2007,Puppo, M.D.1991).

Sample Preparation

The aliquots withdrawn at different time intervals during degradation studies by the above mentioned consortia were acidified with 1 ml of 5N phosphoric acid (final pH 1.5-2) and then vortex for 3 min. 5 ml of toluene was then added vortexed once again and the organic layer transferred to a clean tube. Equal volumes of ether and methanol were then added and the liquid phase transferred into an ependoff tubes and evaporated to dryness overnight in oven at 50°C. The precipitate obtained was reconstituted in 20 µl of Toluene and 1µl loaded for GCMS analysis

The degradation products were determined by GC-MS on Hewlett Packard hp G1800A GCD System, fitted with a HP5 30m x 0.25mm capillary column. The carrier gas used was Helium at a constant velocity of 0.7ml/min. Injection was performed in the split less mode at an injection temperature of 100°C. The GC oven temperature was maintained at an initial temperature of 100°C with a rate of increase of 10°C/min up to 250°C and maintained at 250°C with a hold up time of 7 minutes followed by an increase of 30°C/min up to 280°C.

Mass spectra were obtained at electro energy of 70eV. The detection was performed in full scan mode in the mass range of 45 to 425mu for study of degradation products and in SIM mode with characteristic ions for each compound for quantitative analysis of Diclofenac Sodium

5. Taxonomical identification of the bacterial isolates (Bergey,D.H. et al 1989, Washington C. et al 1997).

Biochemical identification of the bacterial isolates as per Bergeys Manual was based on, its morphological and cultural characteristics, the ability of the isolates to utilize or oxidize different carbon sources like glucose, mannitol, maltose, functionality of different enzymes, ability to grow at different salt concentrations along with its oxidative or fermentative mode of metabolism assessed to classify the bacterial isolate into its respective genera.

IV. Results And Discussion

1. Primary screening.

Pharmaceuticals designed to interfere with biological systems, on reaching their environmental compartments in significant concentrations, can affect various species specially the unicellular flora, which evolve to tolerate the active ingredient through a process involving phenotypic and genotypic adaptations. (Kümmerer 2001) Thus, some bacterial species can survive in the most hostile environments and degrade the

recalcitrant xenobiotics. These and their potentially fast growth are possible reasons why they are frequently not seen as a toxicant's target, but rather as a tool for bioremediation (**Diaz, E. 2008**). Since for most recalcitrant veterinary drugs material, the final resting compartment within the ecosystem is soil (**Diaz, E. 2008**), various garden soil and Tower of Silence soil samples were screened with the aim of isolating diclofenac degraders.

An another route of entry of pharmaceuticals into the environment are from patients consuming the NSAID, where the pharmaceutical may enter as the parent compound or as metabolites; via direct release into the waste water system from manufacturing, hospitals, or domestic discharges and via leaching from terrestrial depositions (e.g., solid waste landfills) (**Boonstra, S. 2008**). Considering the burial practices in the Zoroastrian religion and the source of Diclofenac being the corpse, soil from their burial ground was sampled as it increases the probability of isolating Diclofenac degraders.

Additionally the presence of diclofenac in chemical effluents from various sources results in the persistence of tolerant microbial communities which can metabolize these xenobiotics. In order to isolate such microorganisms; effluents discharged by "FAM Interiors" of New Bombay Chemicals Ltd., along Chemical Effluent from outskirts of Bhiwandi Outskirts and Industrial Effluent Sample (Name withheld on request) from a company manufacturing diclofenac were sampled using chemically defined medium containing increasing concentrations of diclofenac

On chemically defined media, the growth of various heterotrophic flora was poor such that from all the six samples assessed a total of 55 morphologically distinct isolates could form colonies on M9 Agar and Rose Bengal Chloramphenicol Agar (RBC)**.** This may be due to the dual factor of use of a chemically nutritionally exacting medium along with the inoculum sample which due to its chemical nature could not support a heavy load. Of the 55 Operational Taxonomical Units (OTU) obtained, 34 OTU isolates (64%) showed Diclofenac tolerating capacity at concentration of 100µg/ml which is 50,000 times the amount of LOEC Urrea (2010) reported that white rot fungus *Phanerochete.* ch*rysosporium* and *Trametes.versicolor* due to their ability to produce laccase and peroxidase enzyme have capabilities to degrade various xenobiotic compounds and thus were used as standard strains.

With decrease in concentrations of Diclofenac i.e. 50µg/ml the bacterial % recovery was high, while in case of fungal isolates, the fungal % recovery was low at initial concentrations and but increased with an increase in Diclofenac concentrations. This may be due to the fact that bacteria are less likely than fungi to have the capacity to use xenobiotics as sole carbon sources at higher concentration; needing a supplemental carbon source to sustain growth, as their degradative potential is co-metabolic

Degradation Studies

The 34 isolates comprising of 56.6% bacteria and 43.63% fungi could tolerate diclofenac at 100ug/ml also and thus were screened for their ability to degrade Diclofenac Sodium, at time intervals of 2hrs, 24 hrs, 48hrs and 96hrs respectively .Estimation of the residual diclofenac was undertaken using Agarwal method based on its ability to detect ferrous ions, produced by reduction of ferric ions by diclofenac, which gives a colored product in presence of 2,2-bipyridine**.** Of the 34 isolates screened for Diclofenac degradation, maximum (18) of the isolates could initiate Diclofenac degradation within 24hrs ,while 6 of the isolates could initiate degradation of Diclofenac in 48hrs, and they were further shortlisted based on their rate of degradation, Metabolic filterate of 6 of the isolates showed no diclofenac in the metabolic filterate after 2 hrs of incubation as detected by the method of Agrawal et al **(Fig 1)** . Such a result for a recalcitrant molecule indicates to the ability of the isolate to bioaccumulate the molecule more than degradation and thus the presence of this molecule was estimated within the cellular biomass. As expected all the 6 isolates showed bioaccumulated concentration of diclofenac within a range of 105mg% to 198mg% in their cellular biomass, though 2 isolates SI-12 and SI-14 could initiate bioaccumulation after 24 hrs.

11.76% of the isolates allowed diclofenac accumulation within 2 hrs of exposure, while 10 isolates including the standard *Trametes spps* allowed diclofenac accumulation within 24 hrs of exposure, Surprisingly 3 isolates F7, F2 and SI 5 showed initiation of diclofenac degradation without any bioaccumulation indicating their true degradative capabilities and thus were shortlisted for further screening studies. Additionally two isolates SI-7 and BM4 showed no accumulation along with capability of degradation and thus were shortlisted for further studies.

In the current study, the concentration of diclofenac (1000µg/ml) used was higher than the environmentally relevant concentrations of diclofenac, thus testing the efficacy of the microbial consortia to degrade high concentrations of diclofenac.

Thus assessment of the degradative capabilities of the isolates along with the capability to prevent bioaccumulation shortlisted 3 islolates (SI-5 a fungi and bacterial strains BM1 and BM5) along with the standand *Phenerocheate* strain for further studies and consortia development .

2. Degradation of diclofenac as assessed by HPLC method

Agarwal method used to detect diclofenac though a spectrophotometric assay fails wrt to its specificity as well as its sensitivity. Thus the degradative potential of the isolates were confirmed using HPLC. Kasperek. R, reported the retention time of Diclofenac when dissolved in methanol as 2.4mins, while in the study undertaken diclofenac when estimated in metabolic filtrate, shows a retention time of 2.3 mins when used as the internal reference. The residual diclofenac concentrations within the metabolic filterate of the isolates after 24 hrs of incubation were estimated using the calculated area under the peak method at an RT of 2.3mins in comparision with that obtained using a standard diclofenac load of 100µg/ml, the results of which are indicated in **Fig.2.**

In contrast to the results obtained with Agarwal method which was not very quantitative, analysis by HPLC proved its specific nature of the estimation. Lignolytic isolate *P.chrysosporium MTCC 787* proved to have degradative capability of 73.44% in contrast to our isolates which after 24 hours could individually degrade diclofenac quite efficiently, specially isolate BM5 which showed degradation efficiency of 91.9684%, though isolate SI-5 and isolate BM1 showed 76.72% degradation and 72.78 %degradation respectively **Table 1.**

3. Time course diclofenac degradation studies by consortia

Based on the individual degradation studies, an equivalent consortia was prepared using isolates *P. chrysosporium MTCC 787*, isolates SI-5, BM5 and BM1, to assess its degradative potential. Degradation wrt to time indicated that the efficiency of degradation increased with the increase in time such that maximum degradation of Diclofenac (75.9023%) as detected by HPLC was observed at 12 hrs after which a sudden drop in the degradation was observed **(Fig 3)** This may be due to accumulation of toxic compounds within the media. During the course of a similar study Quintana et al.(2002) found no degradation of diclofenac over 28 days either when it was the sole source of carbon nor when an external carbon source was added to achieve co-metabolic degradation. Batch elimination tests using an activated sludge system and membrane bioreactors also revealed very poor degradability of diclofenac in comparison with other pharmaceuticals. Gröning et al. (2007) showed that under aerobic conditions biofilms of river sediment extensively transformed diclofenac to p-benzoquinone imine of 5- OH diclofenac, which was not further degraded, in an apparent co-metabolic process (Groning, J., 2007).

In contrast Urrea et al. (2009), carried out similar time-course degradation experiments of diclofenac where 10µg/ml of diclofenac was added in a chemically defined medium, supporting the growth of the white rot fungi *Trametes. versicolor*. The results obtained on inoculation of such low concentrations of diclofenac exhibited almost complete diclofenac removal (94%) after the first hour of incubation such that after 4 hours no diclofenac was detected in the liquid medium. This fast removal was not in accordance with previous reports by Urrea et al. (2008) regarding degradation of environmental pollutants by *T. versicolor* pellets in the same experimental conditions, in which a lag phase of several hours was observed. In the current study, the concentration of diclofenac (100µg/ml) used was higher than the environmentally relevant concentrations of diclofenac, which could have caused accumulation of diclofenac and /or its metabolized byproducts that could be toxic.

4. Identification of diclofenac degradation products

Commonly used pharmaceuticals have been detected in wastewater, surface water, and even in drinking water. The factors affecting their occurrence in the environment are their overall consumption and the fate of individual compound in the human or animal body, in wastewater treatment plants, and within the aquatic environment itself. Thus, besides consumption or information on the biodegradability and metabolic pathways, the conjugation, de-conjugation, sorption, persistence, the occurrence and fate of the pharmaceuticals and their degradation products, in the environment, are required to be predicted. Since diclofenac is a heat stable moiety, the degradation products after 12hrs and 120hrs by the consortia in the study undertaken were identified by GC-MS,the results of which are shown in **Fig.4 A, B, C**

Kosjek. T., reported that diclofenac can be detected at an retention time of beyond 30mins by GC while its retention time obtained in our study was 15.55mins. The 12 hr aliquot though showing a number of unidentifable peaks could not detect any peak at 15.27mins, implying metabolism of of the active fraction. Additionally increase in the incubation period upto 120 hrs concomitantly increased the number of peaks with one peak at retention time of 15.27 mins which could be identified as ,2-[(2,6-dichlo1-(2,6-dichlorophenyl)-2-indolinon by MS analysis **Fig.5**. This is very similar compound to 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one, a reported degradation product of Diclofenac , indicating that active degradation of diclofenac to identifable product could be obtained through use of our consortia

2-[(2,6-dichlo1-(2,6-dichlorophenyl)-2-indolinon has molecular weight of 278.13 and molecular formula $C_{14}H_9C_{l2}NO$. and is a nontoxic compound, proving that the metabolism of this xenobiotic molecule by the consortia would not lead to formation of toxic bi products.

Identification of bacterial isolates

The bacterial isolates short listed on the basis of their morphologically distinct characteristics as well as their ability to degrade Diclofenac, which was confirmed by HPLC and GCMS analysis.were gram negative aerobic short rods, non fermentors of glucose, maltose and xylose, with salt tolerance of 3%NaCl but differing in their abilities to convert nitrate to nitrite.

Groning et al. (2007) in his studies on transformation of diclofenac by the indigenous microflora of river sediments reported the conversion of Diclofenac by a restricted group(s) of microorganisms mainly belonging to the Alcaligenes and Stenotrophomonas spps. Groning (2007) reported the ability of Cytophaga-Flavobacterium group and the γ-Proteobacteria(13) within the biofilms, to adapt to diclofenac. The bacterial isolates obtained from environmental samples in our study identified on the basis of their biochemical characteristics also belonged to the ***Alcaligenes*** genera and ***Brevudimonas*** genera respectively **Table.3.** and further classification based on its 16s RNA profile may help to identify its species type

Our study thus showed that the microbial consortia that consisted of isolates belonging to ***Alcaligenes*** and ***Brevudimonas*** genera and two fungal isolates one of which was Phanerocheate were capable of degrading diclofenac in 12 hours by 75.90%efficiency. This was evident by degradation studies and estimating the residual diclofenac concentrations by HPLC and further identification of the degradation products by GC-MS.

Thus we suggest the screening of more of those microbial isolates, capable of degrading the recalcitrant compounds that escape the chemical and biological treatment in effluent treatment plants and enter natural water bodies though in low amounts, eventually resulting in their accumulation. It is also essential to consider the future risk assessments of degradation products when such studies are undertaken

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Table.1 Estimation of diclofenac degradation within metabolic filtrate by Agrawal method.

Residual Diclofenac In Metabolic Filtrate				**Residual diclofenac within cellular biomass (mg %)wet wt**	
Isolates Nos	2hrs	24 hrs	48hrs	2hrs	24 hrs
Phanerochete chrysosporium MTCC 787	**++**	**+**	**-**	**-**	**++**
Trametes versicolorNCIM 1086	+	-	-	-	++
SI 3	+	-	-	-	166.25
SI 5	**+**	**-**	**-**	**-**	**-**
SI 7	**++**	**+**	**-**	**-**	**-**
SI 10	+	-	-	-	27.2
SI 11	+	-	-	-	193.4
F1	++	-	-	-	-
F2	**+**	**-**	**-**	**14.58**	**-**
F3	-	-	-	105.0	-
F4	-	-	-	145.8	-
F5	-	-	-	125.34	-
F6	++	+	-	174.07	-
F7	**+**	**-**	**-**	**-**	**-**
F8	-	-	-	135	-
SI 4	++	+	-	-	70
SI 6	+	-	-	-	35
SI 8	+	-	-	-	245.3
SI 9	+	-	-	-	119.4
SI 12	-	-	-	-	143.2
SI 13	**-**	**-**	**+**	**-**	**24**
SI 14	-	-	-	-	198.6
BM1	**-**	**-**	**+**	**6.27**	**-**
BM 2	-	-	+	39.52	-
BM 3	++	+	-	86.95	-
BM 4	++	+	-	-	-
BM 5	**-**	**-**	**+**	**6.68**	**-**
BM 6	++	+	-	86.6	-
BM 7	-	-	+	233.3	-
BM 8	++	+	-	158.3	-
BM 9	++	+	-	38.5	-
BM 10	++	+	-	172	-
BM11	-	-	+	6.4	-
BM12	++	+	-	136.4	-

Key: - Diclofenac negative + Trace quantities of Diclofenac ++ Diclofenac positive (But below 10µg/ml)

Fig.2 Calibration curve of Diclofenac by Agrawal method.

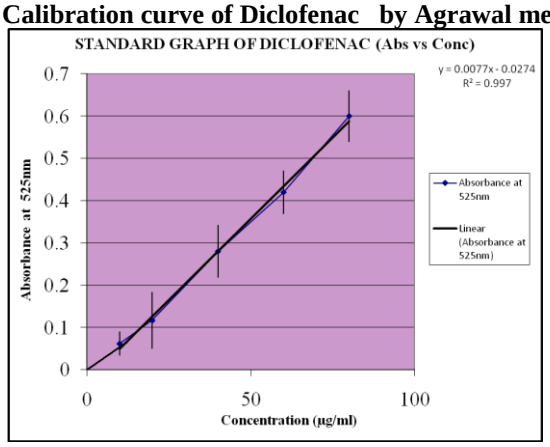
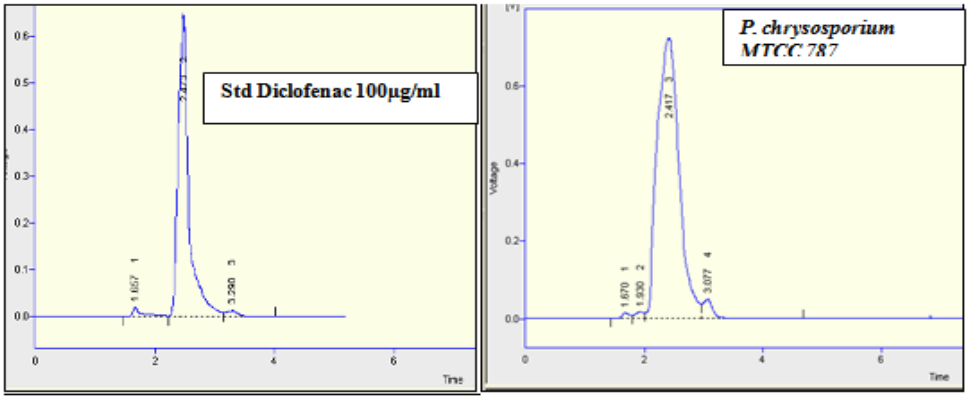


Fig.2 Estimation of diclofenac by HPLC method



Result Table (Uncal - D:\KAVITA\ALIQUOTS\START INJECTIONS\DFS 100PPM WITH M9)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.657	243.497	19.589	2.9	2.9	0.10
2	2.473	7980.931	647.234	94.9	95.3	0.16
3	3.290	185.061	12.396	2.2	1.8	0.25
	Total	8409.488	679.219	100.0	100.0	

Result Table (Uncal - D:\KAVITA\CULTURE 1\CULTURE 1;24HRS)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.653	235.601	32.007	1.0	2.4	0.15
2	1.913	528.774	24.645	2.3	1.8	0.41
3	2.383	21195.408	1250.061	93.8	92.7	0.23
4	3.187	630.335	42.090	2.8	3.1	0.22
	Total	22590.117	1348.803	100.0	100.0	

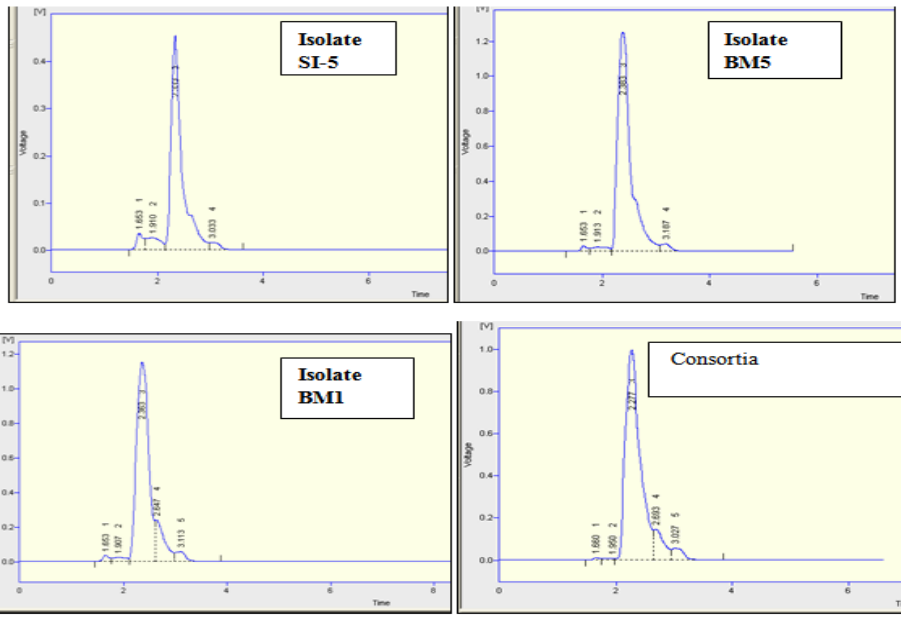


Table.2 Diclofenac Degradation efficiency by shortlisted isolates estimated by HPLC method.

Isolates	Area under the peak	Concentarion of diclofenac in metabolic filtrate	% Degradation
100µg/ml Std diclofenac	7980.931	100µg/ml	-
P. chrysosporium MTCC 787	21195.408	265.575 µg/ml	73.4425%
SI-5	18573.538	232.723 µg/ml	76.7277%
BM5	6410.013	80.316 µg/ml	91.9684%
BM1	21718.673	272.132 µg/ml	72.7868%
Consortium	17437		75.90%

Fig.3 Time course diclofenac degradation curve

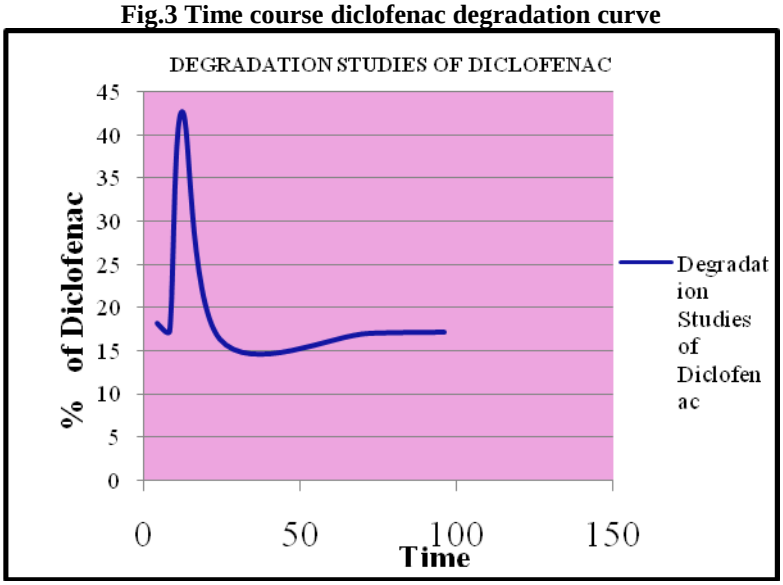


Fig.4A GC of Diclofenac Sodium salt within the Control flask.

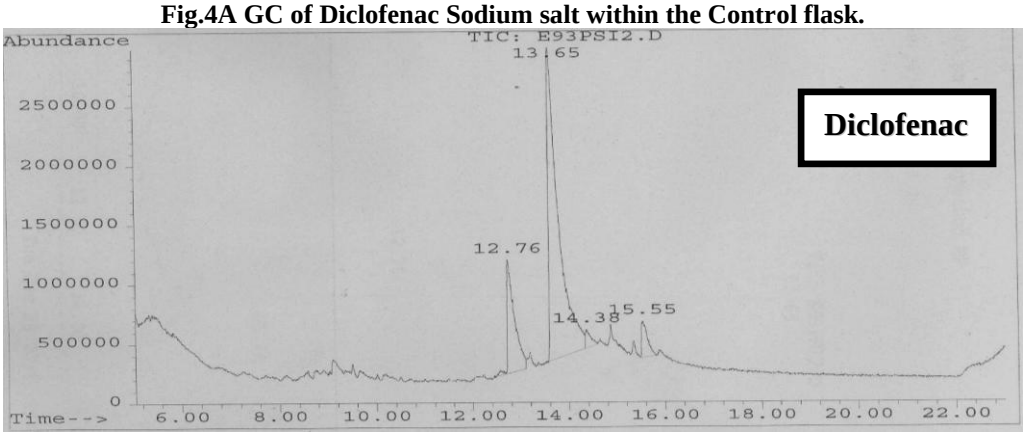


Fig.4B GC of 12 hrs metabolic filtrate extract set up for degradation of diclofenac.

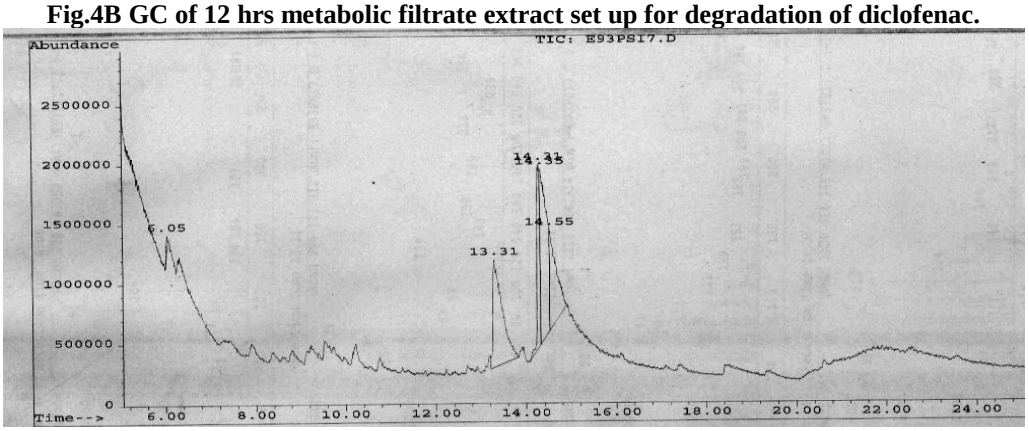


Fig.4C GC of 120 hrs metabolic filtrate extract set up for degradation of diclofenac.

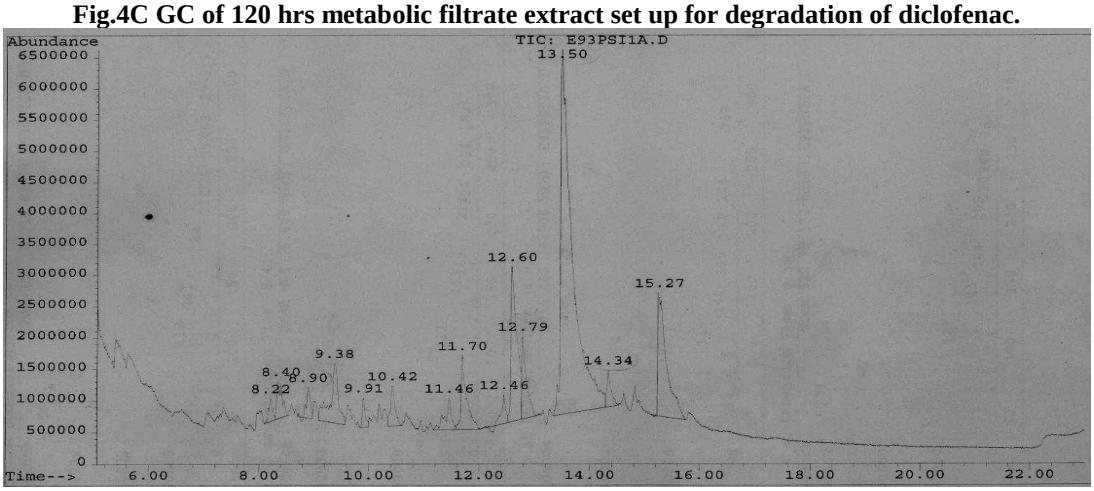
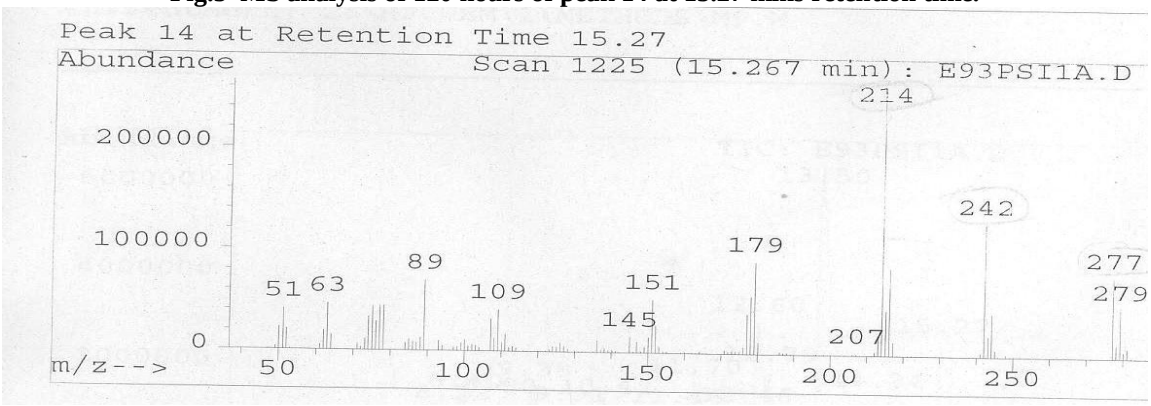
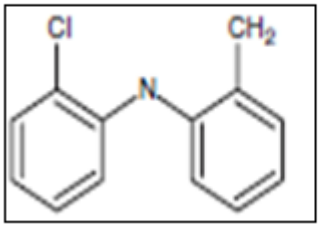
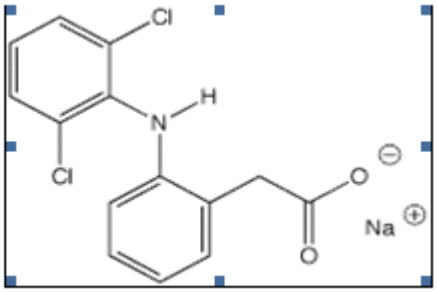


Fig.5 MS analysis of 120 hours of peak 14 at 15.27 mins retention time.





Structure of 2-[(2,6-dichlo1-(2,6-dichlorophenyl)-2-indolinon



Diclofenac sodium salt.
Molecular weight is 318.14
Molecular formula is $C_{14}H_{10}C_{l2}NNaO$

Table.3 Biochemical identification of diclofenac degrading bacterial isolate.

BIOCHEMICALS	Isolates	
	BM5	BM1
Grams nature	Gram negative short rods	Gram negative short rods
Motility	Actively motile	Motile
Oxidase	+	+ (weakly)
Catalase	+	+
Sugar Utilisation		
Maltose	-	-
Xylose	-	-
Salt Tolerance		
3% NaCl	+	+
6% NaCl	-	-
8% NaCl	-	-

10% NaCl	-	-
Without NaCl	+	+
Enzymes		
Gelatinase	-	-
Urease	+ (Weakly)	+ (Weakly)
Lysine Decarboxylase	-	-
Nitratase	-	+
Indole	-	-
Citratase	-	-
PPA	-	-
Metabolism		
TSI Slant	Alkaline	Alkaline
Butt	No change	No change
Gas	-	-
H_2S	-	-
Methyl red	-	-
Voges Proskauer	-	-
Hugh and Leifson		
Glucose	oxidative	Oxidative
Fructose	oxidative	Oxidative
Mannitol	oxidative	Oxidative

Key: Sugar utilisation + acid ++acid and gas – no acid no gas. Enzymes + present - absent

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