STUDIES ON THE DEGRADATION OF DIBENZOTHIOPHENE (DBT) BY A MARINE MICROORGANISM AND ITS ROLE IN THE MINERALIZATION OF CRUDE OIL COMPONENTS.

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DOCTOR OF PHILOSOPHY

in

MARINE BIOTECHNOLOGY

by

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2000

CERTIFICATE

This is to certify that the thesis entitled **"Studies on the degradation of Dibenzothiophene (DBT) by a marine microorganism and its role in the mineralization of crude oil components"** submitted by Ms. Janneth Rodrigues for the award of the degree of Doctor of Philosophy in Marine Biotechnology is based on the results of investigations carried out by the candidate under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma of any University or Institute. The material obtained from other sources has been duly acknowledged in the thesis.

Certified Copy based on the reports of external Examiners. No corrections were required in the thesis as per reports.

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Boards of Viva Voce Examination.

STATEMENT

I hereby state that this thesis for the Ph.D. degree on **"Studies on the degradation of Dibenzothiophene (DBT) by a marine microorganism and its role in the mineralization of crude oil components"** is my original contribution and that the thesis and any part thereof has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive study of its kind from this area. The literature pertaining to the problem investigated has been duly cited. Facilities availed from other sources are duly acknowledged.

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ABBREVIATIONS

HC	hydrocarbon
PAH(s)	polyaromatic hydrocarbon(s)
NSO	nitrogen-sulphur-oxygen
ASW	artificial seawater
TLC	thin layer chromatography
UV	ultra-violet
GC	gas chromatography
IR	infra-red
BHCO	Bombay High crude oil
λ_{max}	wavelength of maximum light absorbance
rpm	revolutions per minute
g	gram(s)
mg	milligram(s)
m	metre(s)
nm	nanometer
nmoles	nanomoles
A	absorbance
h	hour(s)
min	minute(s)
ml	millilitre(s)
l	litre(s)
M	molar
mM	millimolar
µM	micromolar
w/v	weight by volume
v/v	volume by volume
°C	degree Centigrade
Phe	phenanthrene
Nah	naphthalene
DBT	dibenzothiophene
Flo	Fluorene
DMF	dimethyl formamide
ONGC	Oil and Natural Gas Commission

CONTENTS

CHAPTER I

PETROLEUM BIODEGRADATION IN THE MARINE ENVIRONMENT WITH SPECIAL EMPHASIS ON THE MICROBIAL UTILIZATION OF THE ORGANIC SULFUR COMPONENT OF CRUDE OILS : *AN INTRODUCTION AND SURVEY OF LITERATURE.*

Prologue

The oceans and seas are both magnanimous and challenging and seem to exist harmoniously as this massive body of water extending to 70% of the earth's surface. Yet hidden within its mysterious depths is a rich wealth of biologically diverse life populations which help meet the nutritional and food requirements of humankind and besides helps combat the large proportions of human junk which is disposed off into its realms. From amidst its surreal and amazing silences, lie a wealth of information, and the origins of life forms itself, a record of evolution and the answers to this great circle that is life. As man has gradually discovered and has become dependent on fossil fuels as the very fabric of his existence, he has plundered the sea-bed, which has proved to be a rich source of this non-renewable energy form. It is not surprising therefore that the seas should also possess magnificent microbial life forms having the capacity to synthesize enzymes which can make and break the chemical structures present in petroleum. These microbial forms are highly versatile and can live in environments which are largely diverse, stringent and devoid of nutrients and replete with physical and chemical pressures. Pollution has brought with itself a myriad of questions and a need for solutions, the answers already exist within the tapestry that is woven, into a mystery that is the Ocean.

MARINE POLLUTION

The role of humans in decreasing the quality of life in the marine environment has been enormous. The potentially detrimental effects of pollution can directly or indirectly affect all forms of life from beaches to the deepest corners of the ocean. There is infact

only one single rotating ocean, that respects no theoretical limits of territorial waters, and which serves as a common sink for the deposition of pollutants by a population of around 4 billion human consumers and polluters. The diversity in the types, distribution and effects of marine pollutants is almost unlimited, and they can be traced to cities, oil drilling, agriculture, shipping, landmining and nuclear tests, etc.

One of the most important and widespread pollutants in the ocean is **petroleum** or crude oil, a complex mixture of hydrocarbons, that is refined to yield not only fuels, but raw materials for making plastics, synthetic fibers, rubber, fertilizers, and countless other products.

PETROLEUM

Origin and Distribution in the sea.

Although the bulk of the oceanographic hydrocarbons that cause public concern is anthropogenic and originates in the geological strata, a considerable amount is formed annually by growing plants, including land plants (Eglington *et al.,* 1962) and fresh water and marine algae (Oro & Noaomer, 1966).

Fossil petroleum itself arises from thermal diagenesis of ancient plant material in fine grained sedimentary rocks. Another natural source of hydrocarbons are the seeps that are found on the ocean floor (Wilson *et al.,* 1974).

Human activity has greatly increased these natural processes as petroleum has been brought to the surface and transported around the world as an energy supply.

Evironmental impacts occur at all stages of oil and gas production. They are the result of prospecting activities, of the physical impacts due to installation of rigs, of the

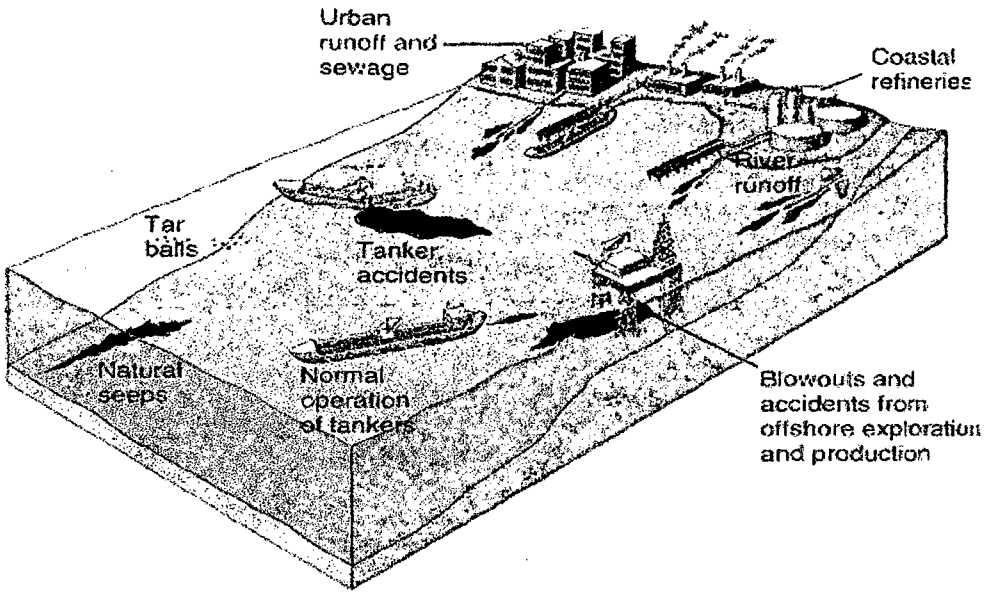


Fig 1.1: Sources of oil in the marine environment (Castro and Huber, 1997).

Table 1.1: **ANNUAL GLOBAL OIL DISCHARGES TO THE SEA**
(Mathiesen,1994)

SOURCE	AMOUNT(Mt)
Industrial waste(rivers and urban runoffs)	1.48
Refineries, terminals	0.03
Offshore production	0.05
Tankers:Operational	0.114
Tankers: Accidental	0.16
Other Shipping*	0.35
Natural Sources	0.025
Total	2.434

* Non tanker accidents, bilge waste & fuel oil, dry docking.

operational discharges when production starts and of accidental spills (Nihoul & Ducrotoy, 1993) (Fig. 1.1 and Table 1.1). Large amounts of crude oil and derivatives like fuel, heating and lubricating oil are present in the run off from coastal cities and rivers. Infrequent but potentially disastrous are the blow outs of offshore rigs used in the extraction of oil. The underwater blowout of an exploration well in the Gulf of Mexico (1979) spilled atleast half a million tons of oil over a period of 9 months.

Marine pollution by ships is strictly controlled by the International Convention for the Prevention of Pollution from ships (MARPOL) (Castro & Huber, 1997). Yet, it is the operational discharges from the ships and the wastes from the rivers and urban runoffs which form the largest source of oil pollution in the ocean. Since the oil is spread over a number of locations, the effects of operational pollution seem less dramatic than those accidents involving oil but in certain areas they rise to a number of chronic pollution problems (Nihoul & Ducrotoy, 1993). Most petroleum therefore is found around the shipping lanes and close to large ports, many of which are situated in estuaries (Atlas, 1981).

The oil spills that result from the sinking or collision of supertankers have the most devastating effects on the marine environment. Large catastrophic quantities of crude oil enter the sea after a major tanker accident. Since the first wreck of a super tanker in 1969 (the *Torrey Canyon* oil spill in the English Channel) there have been over 40 major marine oil spills (Fig. 1.2). Major oil spills, have heavily contaminated marine shore lines, as in the case of the *Exxon Valdez* spill, causing severe localized ecological damage to the near-shore community (Atlas, 1995).

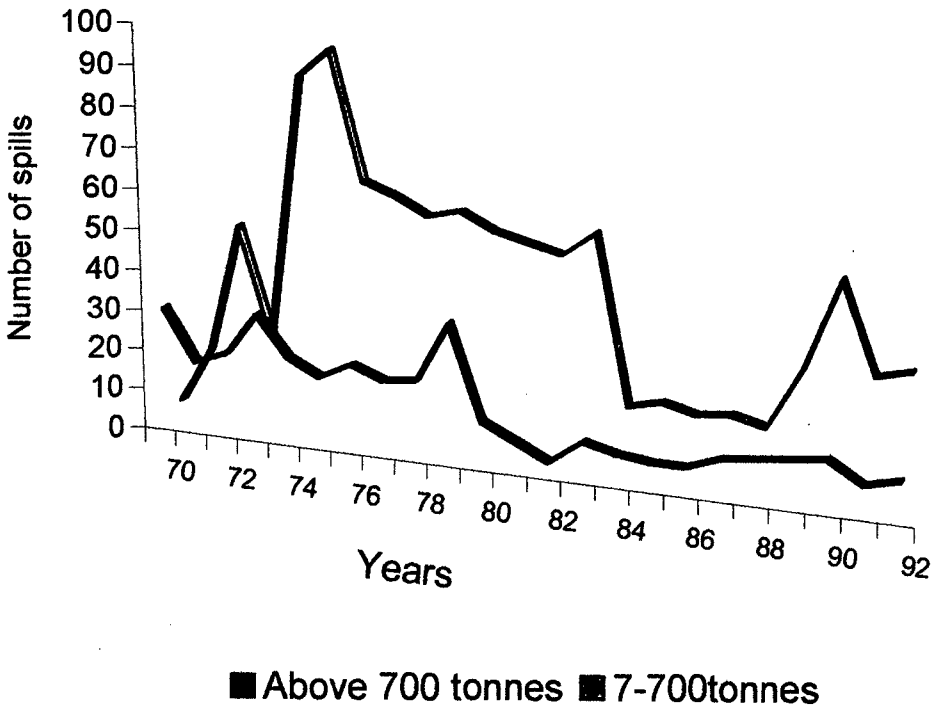


Fig.1.2 Worldwide accidental oil pollution from tankers. Source : Intertanko, Mathiesen, (1994).

The effects of an oil spill depend on the following (Nihoul & Ducrotoy, 1993);

(i) The **type** and **state** of oil, and especially its composition (degree of weathering, mousse formation and biodegradation).

(ii)The **receiving** environment (e.g. sheltered and low energy habitats or land locked seas).

The susceptibility of the organisms: animals who live at the water surface, such as marine mammals and sea birds are especially vulnerable.

(iii) The oil **clean up** activities: Mechanical recovery is the biologically preferred option but it requires easy access and good weather conditions. Dispersants can be preferable in certain circumstances to prevent oil from reaching sensitive areas but a compromise has to be found between the advantages and the toxicity of the dispersant being used. **Bioremediation**, which is accomplished by adding exogenous microbial populations or stimulating indigenous ones attempts to raise the rates of degradation without resorting to the addition of toxic chemical dispersants (Atlas, 1995) and could provide an environmentally friendly alternative.

Petroleum Biodegradation

Petroleum is a very complex mixture of tens of thousands of compounds that differ markedly in their volatility, solubility and susceptibility to biodegradation. Biodegradation of hydrocarbons (HC's) by natural populations of microbes represents one of the primary mechanisms by which petroleum is eliminated from the marine environment. But, for oil to be degraded by these microbial life forms, the HC's in petroleum have to be made amenable to attack by the microbial enzyme systems (Leahy

& Colwell, 1990). The quantitative and qualitative aspects of biodegradation depends on; the nature and amount of oil or HC's present, the ambient seasonal environmental conditions, and the composition of the autochthonous microbial community.

Composition of the indigenous microbial community

Since HC's are natural products as well as anthropogenic pollutants it is not surprising that petroleum-degrading ability is common among marine bacteria (Atlas, 1981). Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons (Leahy & Colwell, 1990). In waters which have not been polluted by HC's, HC using bacteria make up 1% or less of the bacterial population, whereas in most chronically polluted systems they make up 10% or more of the population (Vestal *et al.*, 1984). The presence of oil selectively enriches the HC using microbes and amplifies the genes in the population, which code for the degradation of HC's (Leahy and Colwell, 1990). A large number of reports reviewed by Colwell & Walker (1977), Atlas (1981), Floodgate (1984), Cooney (1984), Bossert and Bartha (1984), have shown that the numbers of the HC utilizing microorganisms and their proportion in the heterotrophic community increase upon exposure to petroleum and other HC pollutants. The indigenous microbial communities contain microbial populations of different taxonomic relationships, which are capable of degradation, and these include genera like *Pseudomonas, Achromobacter, Flavobacterium, Acinetobacter, Vibrio, Bacillus, Arthrobacter, Nocardia, Corynebacterium and Micrococus* (Floodgate, 1984; Atlas, 1981).

Environmental factors affecting biodegradation of petroleum

Environmental conditions strongly influence the expression of an organism's metabolic capabilities. These factors were reviewed by Atlas (1981), Cooney (1984) and Leahy & Colwell (1990). The principal factors are temperature, nutrients and oxygen.

(i) Temperature: Hydrocarbon degradation can occur over a wide range of temperatures and psychotrophic, mesophilic and thermophilic hydrocarbon utilizing microorganisms have been isolated. Zobell (1973) & Traxler (1973) reported hydrocarbon degradation below 0°C. Klug and Markovetz (1967) & Mateles *et al.*, (1967) reported hydrocarbon degradation at 70°C.

Walker and Colwell (1974), using model petroleum incubated with estuarine water, found slower but more extensive biodegradation at 0°C than at higher temperatures. Decreased toxicity of hydrocarbons at lower temperatures was hypothesized to explain the more extensive growth at lower temperatures. Temperature is often not the major limiting factor except when it relates to the other factors such as the physical state of oil or whether water is available for microbial growth (Atlas, 1981).

Contradictorily, low temperatures, by depressing the volatilization of the smaller alkanes, increase the degree of toxicity, thus depressing the oxidative activity (Atlas & Bartha, 1972; Atlas, 1975).

It can be concluded that the range of temperature changes in seawater will cause variation in the rate of oil biodegradation, but the process will continue year round. However, in extremely cold environments degradation will be markedly slower, because of factors such as the change in viscosity and the retention of potentially toxic light hydrocarbons, the effect of which is greater than a temperature mediated drop in

enzyme activity (Atlas, 1981; Floodgate, 1984).

(ii) Nutrients: Petroleum itself contains virtually no nitrogen and phosphorous and oil added to the sea simply increases the carbon and energy supply. Therefore in oil polluted waters, the available nitrogen and phosphorous severely limit the extent of hydrocarbon degradation (Floodgate, 1984). Indigenous bacterial populations can use the addition of nitrogen and phosphorous containing fertilizers to stimulate microbial hydrocarbon degradation.

(iii) Oxygen: The importance of oxygen for hydrocarbon degradation is indicated by the fact that major degradative pathways for both saturated and aromatic hydrocarbons involve oxygenases and molecular oxygen (Atlas, 1981). Oxygen is usually plentiful in the upper parts of the water column where petroleum is most often found and is rarely rate limiting in open waters. Besides oxygenases have a high affinity oxygen and therefore will continue to function even when the concentration of dissolved oxygen is low (Floodgate, 1984).

Physical aspects of petroleum degradation

Biodegradation of a given hydrocarbon depends on its dispersion state and is optimum when this water insoluble substrate is dissolved, solubilised or emulsified. (Betrand, 1993). Availability of an increased surface area should accelerate biodegradation (Gatellier *et al.*, 1973). Oil is dispersed in the marine environment in 4 different states; films, solutions, emulsions and tar balls (Atlas, 1981). Dispersion is a function of the seawater temperatures, the wind velocities, wave agitation and photo oxidation. Large masses of mousse and tar ball persist because of the low surface to volume ratio and the

fact that tar has chemical structures which are not readily attacked by microbial enzymes make it an unsuitable substrate for micro-organisms (Leahy and Colwell, 1990).

Chemical aspects of Petroleum degradation

Petroleum is an extremely complex mixture of hydrocarbons. It can be fractionated into several classes based on related structures. These include, saturate or aliphatic, aromatic, asphaltic or polar; this fraction may be further separated out into the NSO or nitrogen, sulfur and oxygen-containing component (Westlake *et al.*, 1974).

Several studies have been performed to determine the metabolic pathways for degradation of these compounds. The greater the complexity of the hydrocarbon structure, i.e. the higher the number of methyl branched constituents or condensed aromatic rings, the slower the rates of degradation (Atlas, 1995). Microbial degradation has been shown to occur by attack on aliphatic or light aromatic fractions of the oil with the high molecular weight aromatics, resins and asphaltenes considered to be more recalcitrant or exhibiting very low rates of biodegradation (Herbes & Schwall, 1978). The initial steps for biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required.

(a) Saturate Fraction:

Hydrocarbons within the saturate fraction include n-alkanes, branched alkanes and cycloalkanes (naphthenes) and their susceptibility to degradation is in the order; n-alkanes > branched alkanes > cycloalkanes (Atlas, 1981). The alkanes are oxidized to alcohol's, followed by aldehyde and carboxylic acids. Further degradation of

carboxylic acids proceeds by β-oxidation with the subsequent formation of 2- carbon unit shorter fatty acids and acetyl CoA with eventual liberation of CO_2.

(b) Aromatic Fraction:

Bacteria oxidize aromatic hydrocarbons by incorporating two atoms of molecular oxygen into the substrate to form a dihydrodiol with *cis* configuration (Gibson *et al.*, 1977). This reaction is catalyzed by a dioxygenase which is a multicomponent enzyme system consisting of a flavoprotein, an iron sulfur protein and a ferrodoxin (Crutcher and Geary, 1979; Yeh *et al.*, 1977). Further oxidation of the *cis* hydrodiols leads to the formation of catechols that are substrates for another dioxygenase that brings about enzymatic fission of the aromatic ring (Dagley, 1975). Fungi, like mammalian enzyme systems form *trans*-diols, whereas bacteria almost always form *cis*-diols. Light aromatic hydrocarbons are subject to evaporation and microbial degradation in a dissolved state. Polycyclic aromatic hydrocarbons (PAH) contain C and H atoms arranged in a series of adjoining six membered rings and many occur with a variety of alkyl substituents or as heterocyclic molecules containing N, O or S (Cerniglia, 1992). PAH's undergo metabolic activation that induces toxic mutagenic or carcinogenic properties (Heidelberger, 1975; Sims & Grover, 1974, 1981).

(c) Asphaltenic Fraction:

The metabolic pathways for the degradation of the asphaltic components of petroleum are least understood, as these are complex structures, which are difficult to analyze with current chemical technology. The elucidation of the biochemical fate of asphaltic petroleum compounds is a major challenge for research on petroleum biodegradation (Atlas, 1981).

During the biodegradation of petroleum hydrocarbons by microorganisms, Jobson *et al.*, (1972) and Zajic *et al.*, (1974) reported an increase in the polar nitrogen-sulfur-oxygen fraction. Walker and Colwell (1974), found an increase in the high boiling n-alkanes after the microbial degradation of the oil. These compounds cannot be analytically resolved by gas chromatography. Because petroleum is a mixture of compounds that degrade at different rates, the rate of oil loss tends to become lower as the recalcitrant substances form a greater proportion of the residue. Low rates are found when the substrate contains a high proportion of condensed polyaromatic compounds, condensed cycloparaffins, asphaltenes and resin compounds, while a high proportion of medium length n-alkanes makes for quicker loss (Floodgate, 1984). The localization of HC utilizing bacteria, which utilize this highly refractile fraction of oil, has received considerable attention because of the possibility of using their biodegradative potential in the treatment of oil spills.

SULFUR CONTENT OF PETROLEUM

Types of sulfur in petroleum/crude oil

Sulfur is the third most abundant element present in petroleum after carbon and hydrogen. The sulfur content of petroleum from different sources (Table 1.2) ranges from 0.025% to more than 5% (Monticello & Finnerty, 1985), although values as high as 13.95 % have been reported (Rall *et al.*, 1972).

In addition to elemental sulfur, sulfate, sulfite, thiosulfate and sulfide more than 200 sulfur containing organic compounds have been identified from crude oils (Rall *et al.*, 1972). The organic sulfur present in petroleum crude generally predicts the quality of

TABLE NO. 1.2: ORGANIC SULFUR CONTENT OF SOME CRUDE OILS
(Source : Monticello & Finnerty, 1985)

SOURCE	% SULFUR
1)INDIAN CRUDES	
Bombay High A	0.13
Bombay High B	0.16
2)PERSIAN GULF CRUDES	
Iranian Crude	1.41
Dubai Crude	1.89
Basrah Crude	2.18
Kuwait Crude	2.56
3)AFRICA NORTH	0.18
4)NORTH AMERICA CRUDES	
Mississipi	1.6
West Texas	0.05-5.0
California	1.0
East Texas	0.26
5) SOUTH AMERICA	
Venezuela	1.7
6) CANADA	0.44
7) FAR EAST	0.10

the crude oil. These include sulfide, thiols, thiophenes, substituted benzo- and dibenzothiophenes and many considerably complex molecules (Willey *et al.*, 1981). The sulfur content in crude oil fractions generally increases in the sequence; saturates < aromatic < resins < asphaltenes. The sulfur compounds in the high boiling range fractions (the asphaltenes) are predominantly benzo- and dibenzothiophene linkages (Speight & Moschopedis, 1982). Victor Meyer made the original discovery of thiophene and its homologues in coal tar in 1882. Challenger (1959) and Hartough & Meisel (1954) have reviewed the early work, which lead to the identification of benzothiophene, dibenzothiophene and naphthobenzothiophenes and various alkylated thiophene ring systems in coal and lignite tar as well as in shale oils and petroleum.

In addition to possible carcinogenic and mutagenic properties, these compounds have shown a high potential for biological accumulation and toxicity (Ensley, 1984).

Dibenzothiophene as a Model system

High levels of dibenzothiophene have been found in a marine isopod after exposure to an oil spill. In addition, the biological magnification of DBT has been demonstrated with a laboratory ecosystem and field studies (Gundlach, *et al.*, 1983). Sulfur containing hydrocarbons have proved especially refractory to microbial and physical degradation following contamination of terrestrial and marine environments (Atlas, 1975).

An extensive analysis (Gundlach *et al.*, 1983) of the fate of oil from the *Amoco Cadiz* wreck (off the coast of Northern France in 1978) showed that alkylated DBT and phenanthrene compounds persisted years longer than other hydrocarbon fractions and

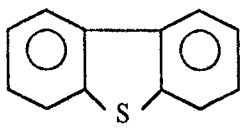
erve as a major aromatic marker in the sediment. Dibenzothiophenes were recognized be among the compounds that were most resistant to biodegradation in sediments ntaining oil from the *Amoco Cadiz* spill (Boehm *et al.*, 1981). Dibenzothiophene like mpounds persist at the sites of surface spills after more easily degradable compounds ve disappeared (Atlas, 1975; Fedorak & Westlake, 1983).

e microbial degradation of heterocyclic aromatic compounds results in dispersion d mineralization of these molecules in the environment. Fedorak and Westlake 983) have reported that microorganisms found in seawater can degrade enzothiophene present in Prudhoe Bay crude oil. The recalcitrance of the nzothiophenes, relative to the other aromatic compounds found in crude oil that are enable to analysis by gas chromatography has also been observed in other studies rthou *et al.*, 1981; Boehm *et al.*, 1982; Teal *et al.*, 1978). Its recalcitrance ntributes to the potential of condensed thiophenes to accumulate in the tissues of llfish in the marine environments that become contaminated with crude oil (Laseter *al.*, 1981; Ogata & Fujisawa, 1983). This has led to the suggestion that enzothiophenes might serve as oil pollution markers (Friocourt *et al.*, 1982; Ogata & isawa, 1985). The physico-chemical characteristics of this sulfur heterocycle are en in Table 1.3.

hways for biodegradation of dibenzothiophene

re have been numerous biodegradation studies using DBT as a model substrate. ce the initial studies of Kodama *et al.*, (1968), with *Pseudomonas* strains there have en several reports of bacterial dioxygenation, oxidation and cleavage of one of the enzene rings of DBT to yield 3-hydroxy-2-formyl benzothiophene (HFBT) without

TABLE NO.1.3: PHYSICO CHEMICAL CHARACTERS OF DIBENZOTHIOPHENE

APPEARANCE	CRYSTALLINE
COLOR	CREAMISH WHITE
NATURE	REFRACTILE
MOLECULAR WEIGHT	184.26 gm/mol
STRUCTURE	
MANUFACTURER	MERCK-SCHUCHARDT
PURITY	98 %
MELTING POINT	95°C - 98°C
SOLUBILITY A) WATER B) ORGANIC SOLVENT	 PARTIAL OR IMMISCIBLE SOLUBLE
MAX. ABSORBANCE IN : A) $CHCl_3$ B) CCl_4 C) ETHANOL D) DIMETHYLFORMAMIDE	λ_{max}(nm) 252, 287nm 264, 289nm 255, 286nm 269, 286nm
TLC SOLVENT SYSTEM $CHCl_3$: CH_3OH 100 : 5	Rf VALUE 0.98
GAS CHROMATOGRAPHY FID PROG RATE:60-300°C@6°C/min	Rt VALUE 21 minutes

releasing sulfur, by the so called Kodama pathway (1970, 1973) (Fig 1.3 A). Kodama *et al.*, identified intermediates that led to them to conclude that DBT is metabolized by a series of oxidations, analogous to those by which naphthalene is degraded. Such findings were also reported by Laborde & Gibson (1977), Monticello & Finnerty (1985), Mormille & Atlas (1989), Foght & Westlake (1988). The isolation of DBT oxidation products from cultures of this organism revealed the presence of several compounds (Kodama *et al.*, 1970). In addition to 3-hydroxy-2-formyl benzothiophene, the presence of *trans*-4 [2-(3-hydroxy) thionaphthenyl]-2-oxo-3-butenoic acid (*trans*-HTOB) and the hemiacetal form of this compound were also detected (Kodama *et al.*, 1973). Since the hemiacetal form of HTOB can only arise from the *cis* isomer, it was proposed that DBT-diol is oxidized to *cis*-HTOB and *trans*-HTOB arises through the action of *cis-trans* isomerase (Kodama *et al.*, 1973).

Many of these isolates, which utilize this C-destructive pathway, also oxidize the sulfur atom giving DBT sulfoxide and sulfone (Kodama *et al.*, 1973; Kodama *et al.*, 1970; Kropp *et al.*, 1994), which appears to be the dead-end pathway product in these aromatic compound -degrading bacteria (Fig 1.3 A).

Other isolates were reported to use DBT as a sulfur source, by a S-specific metabolic pathway by oxidizing DBT by the '4S' pathway or the sulfoxide-sulfone-sulfonate-sulfate, leaving the 2-hydroxybiphenyl (Gallagher *et al.*, 1993; Izumi *et al.*, 1994; Omori *et al.*, 1992; Campbell, 1993; Grossman, 1996; Kilbane & Bielaga, 1990) (Fig. 1.3 B). This results in removal of sulfur from DBT, leaving the carbon intact. This pathway is potentially useful for removing the sulfur compounds in petroleum i.e. biodesulfurization (BDS), without decreasing the calorific value of the fuel. Kinetic

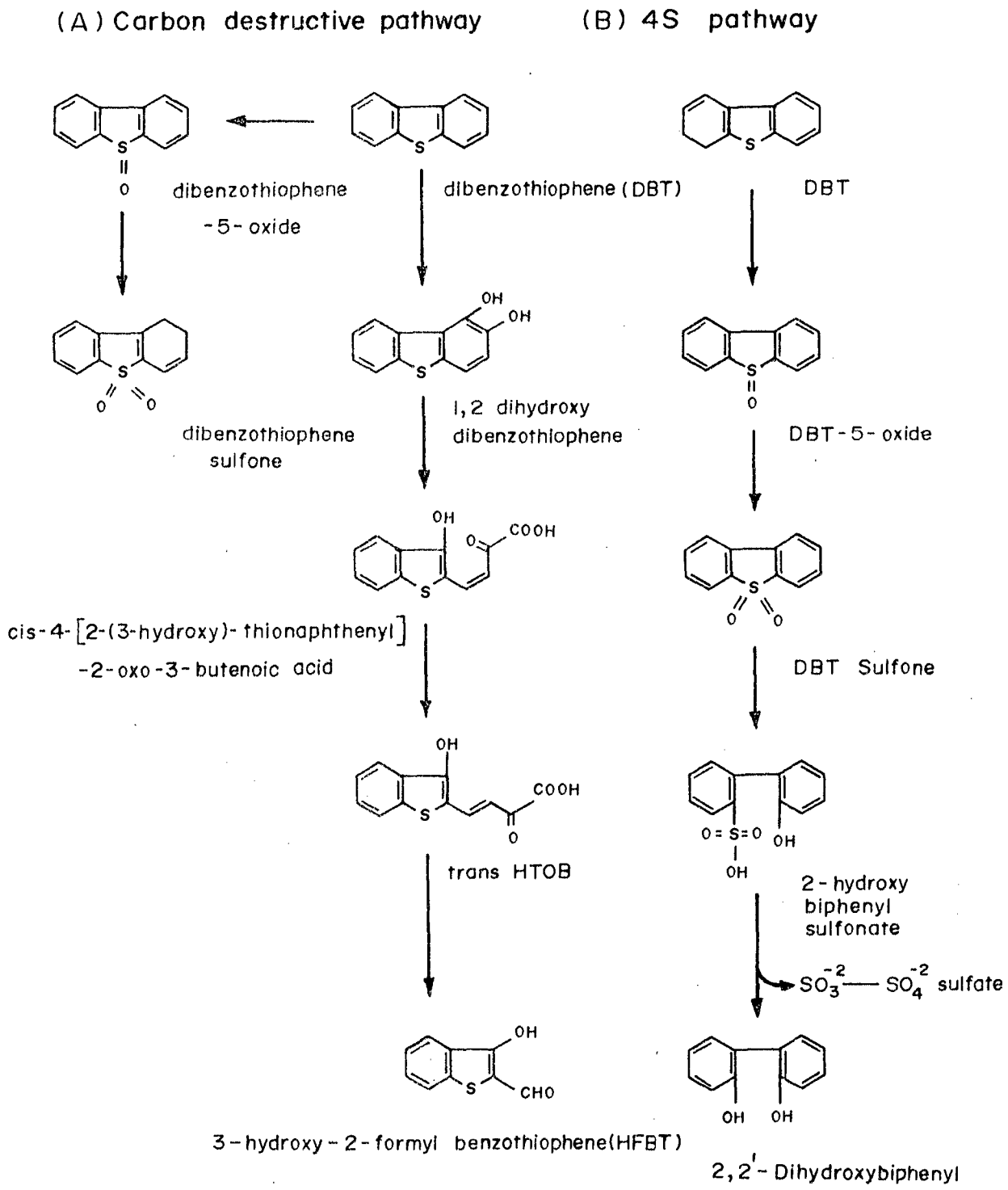


Fig. 1.3 Proposed pathways for the degradation of DBT by the;
(A) Carbon destructive pathway (Kodama et al., 1970)
(B) 4S (sulfoxide-sulfone-sulfonate-sulfate) pathway (Kilbane, 1989)

data of the metabolism of DBT by *Rhodococcus erythropolis*, which uses this pathway, has been reported by Wang & Kraweic (1996).

Total mineralization of DBT via DBT-5-oxide, DBT-sulfone and benzoic acid by a strain of *Brevibacterium* species capable of growing on DBT as a sole source of sulfur, carbon and energy was reported by Van Afferden *et al.*, (1990). These researchers also observed the release of sulfite and subsequent spontaneous oxidation of sulfite to sulfate during the degradation of DBT. This organism oxidized DBT via the sulfoxide and sulfone, released the sulfite and then subsequently degraded the desulfurized hydrocarbon. The only metabolite detected in the degradation of the carbon backbone was benzoic acid.

Molecular studies on desulfurizing microrganisms

Monticello *et al.*, (1985a) have shown that the oxidation of DBT is plasmid mediated in two soil isolates, strains of *P. alcaligenes* and *P. putida*, which were found to harbor a similar 72kb plasmid designated as pDBT2, coding for DBT oxidation and growth on naphthalene and salicylate. Tn5 insertion mutants unable to grow on naphthalene were also incapable of DBT oxidation (Monticello, 1985b). Similar Tn5 insertion mutants of the naphthalene degradative plasmid made by Yen & Gunsalus (1982) were unable to oxidize DBT. These results suggest that the same plasmid coded enzymes oxidize DBT and naphthalene.

Monticello *et al.*, (1985a) studied DBT metabolism by *P. stutzeri* DBT3 and assumed it to be encoded chromosomally, as no plasmid was mediating DBT degradation in this strain. Foght and Westlake (1988) isolated a *Pseudomonas sp.* HL7b that constitutively

co-metabolized DBT via the *trans* ring cleavage product to the pathway end product HFBT. DBT degradation could not be co-related with any of the 4 plasmids present in the strain HL7b. Neither strain DBT2 nor HL7b could use DBT as sole carbon source for growth.

The cloned DBT degradative genes from *P. alcaligenes* DBT2 (on plasmid pC1) were introduced into a spontaneous DBT negative mutant of *Pseudomonas sp.* HL7b and into *P.alcaligenes* strain DM201 (the cured DBT negative derivative of *P. alcaligenes* DBT2). This conjugal transfer yielded tetracycline resistant, DBT positive putative transconjugants. DNA-DNA hybridization using pC1 from these transconjugants as a probe occurred with the positive control pC1 but not with the total genomic DNA from HL7b, HL7bR, DM201 or NAH2.

Denome *et al.*, (1993a) have isolated a soil *Pseudomonas* strain C18 and cloned and sequenced a 9.8 KB fragment that encodes DBT-degrading enzymes in *Pseudomonas* strain C18. Nine open reading frames were identified and designated *dox* ABDEFGHIJ, collectively referred to as the DOX pathway. Their findings indicate that a single genetic pathway controls the metabolism of DBT, naphthalene and phenanthrene in strain C18 and that the DOX sequence encodes a complete upper naphthalene catabolic pathway.

A genomic library of *Rhodococcus* sp. strain IGTS8, which possesses the enzymatic pathway to remove covalently bound sulfur from DBT without breaking the C-C bonds was constructed in cosmid vector pLAFR5 (Denome *et al.*, 1993b). The DNA sequence of a 4.0 kb fragment that carries the genes for this pathway was determined. Frame shift and deletion mutations established that three open reading frames were required

for DBT desulfurization and the genes were designated as *sox* ABC (for sulfur oxidation).

MICROBIAL DESULFURIZATION TECHNOLOGY:

Combustion of low quality fuels with high levels of sulfur results in the release of toxic sulfur dioxides into the atmosphere, one of the major sources of acid rain. About 2.15 million tonnes of SO_2 are contributed annually to the atmosphere in India alone, due to the combustion of such high sulfur fuels (Stoker & Seager, 1976).

As the world supply of low sulfur petroleum decreases, there is an increased interest in the use of heavy crude's, oil sands and oil shale's, which typically have a higher sulfur content compared to conventional crude oils. Environmental restrictions in the United States and other parts of the world require the use of fossil fuels with low sulfur content. To reduce sulfur-related air pollution, the level of sulfur in fuels is regulated and to meet these regulations sulfur must be removed from fuels during the refining process. Low quality fuels therefore have to be subjected to (either pre or post combustion) **desulfurization** processes (Monticello & Finnerty, 1985; Finnerty & Robinson, 1986).

Conventional desulfurization or Hydrodesulfurization (HDS), which involves the reaction of petroleum fractions with an inorganic catalyst and hydrogen under conditions of high temperature and pressure to produce hydrogen sulfide and a desulfurized product, is process inconvenient and expensive. The high cost and inherent chemical limitations associated with HDS therefore, makes alternatives to this technology of interest to the petroleum industry.

Biodesulfurization (BDS) has been studied as an alternative to HDS and is a process

that is being developed based on naturally occurring bacteria that can remove organically bound sulfur from petroleum. The application of bacteria that metabolize sulfur heterocylces, to oil desulfurization processes has been considered for many years. Early patents by Strawinski in 1950 and 1951, Zobell in 1953 & Kirshenbaum in 1961, described microbial systems for solubilization of sulfur from oil, but none of these were ever commercialized (Monticello & Finnerty, 1985).

Walker *et al.*, (1975) first reported the oxidation of heterocyclic compounds in conventional crude oil by microbes. Fedorak & Westlake (1983), reported removal of alkyl benzothiophenes and dibenzothiophenes from Prudhoe Bay crude oil by microbes of marine origin. He observed that susceptibility to microbial attack decreased with increasing molecular weight. The low molecular weight heterocyclic sulfur compounds from crude oils were metabolized within 14 days by mixed marine cultures. Finnerty & Hartdegen, (1984) isolated a *Pseudomonas* species that reduced the viscosity of heavy oils by 95-98% and subsequent treatment with DBT co-metabolizing organisms resulted in enhanced desulfurization. But the use of hydrocarbon degradation pathways in all of these microbial systems that attacked DBT, relied on oxidation and mineralization of the carbon skeleton instead of on specific sulfur removal. Therefore although the organically bound sulfur was converted to water soluble, easily removable forms these desulfurization processes resulted in a significant reduction in the fuel value of the desulfurized end product. More recently, bacteria that desulfurize DBT and a variety of other organic sulfur compounds typically present in petroleum oils via the sulfur selective oxidative pathway have been isolated (Campbell, 1993; Grossman, 1996; Izumi *et al.*, 1994; Kilbane & Bielaga 1990; Omori *et al.*, 1992; Van Afferden *et al.*,

1990; Wang & Krawiec, 1994). To be commercially useful, BDS must be able to remove sulfur from fuels. Although considerable research on the desulfurization of model compounds via the sulfur selective oxidative pathway has been reported, little information on the desulfurization of fuel oils has been published. Rhee *et al.*, (1998), have isolated a DBT-desulfurizing bacteria, *Gordona* strain CYKS1 which could reduce the total sulfur content of diesel oils, by 70% (wt/wt) in MDUF (middle distillation unit feed) and 50% (wt/wt) in the LGO (light gas oil). Up to 30% of the total sulfur in the middle distillate fraction of crude oil was removed when treated with *Rhodococcus* sp. strain ECRD-1 (Grossman *et al.*, 1999). Although significant, the degree of desulfurization is not sufficient to meet the required sulfur levels for all fuels. Nonetheless, these systems have good potential for application in BDS of fossil fuels.

Aim and Scope of the Work

From the above discussion we could conclude that organic sulfur compounds and more specifically sulfur heterocycles are carcinogenic and recalcitrant and tend to persist in the marine environment after an oil spill, thereby contributing significantly to environmental hazards after such disasters.

Selection of microbial degraders that are capable of attacking specific hydrocarbon components of crude oil have generally relied on crude oil itself as a substrate for enrichment of hydrocarbon degrading populations from the natural environment. But crude oil is a complex mixture of hydrocarbons varying in their chemical structures and toxicity and in their susceptibility to degradation. Enrichment on such a mixture will select for populations of bacteria, which can attack the more easily utilizable fractions

of the crude, thereby eluding our attempts for selection of the degraders of our choice. Therefore, we specifically used DBT as a model compound, representing the organo-sulfur fraction of crude oil and thus attempted to isolate a microbial system from a marine environment (exposed to hydrocarbon contamination), which exhibited specificity towards the conversion of this S-heterocycle to water-soluble products.

Further we purified and characterized this marine microbial isolate using various biochemical and physiological tests and determined the metabolic pathway that it utilizes to degrade dibenzothiophene and the nature of the enzymes involved in such degradative processes. We also screened for its ability to degrade individual hydrocarbons that are normally present in petroleum. The application of this bacterial system developed on model sulfur containing heterocycle, DBT was then extended to crude oil to verify the bioremediative potential of this marine isolate.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF A DBT DEGRADING MARINE BACTERIUM AND A STUDY OF THE DISTRIBUTION OF DBT DEGRADERS IN THE MARINE ENVIRONMENT.

The sea serves as a global recipient for hydrocarbon pollutants. The bulk of the pollution comes from the creeks and rivers, which are polluted by inland sewer systems and seepage from industry. The ocean is also a rich source of marine microorganisms, which are capable of adapting to, and thereby attacking these toxic moieties. But the sea is not a quiescent or dormant environment and the fact that it is in a state of constant flux and circulation impedes our attempts to isolate and study the distribution of hydrocarbonoclastic microbial species from such a vast medium.

Enrichment of hydrocarbon degraders in areas exposed to oil pollution has been shown following accidental and experimental oil spills (Atlas, 1981). We therefore restricted our search to such exposed marine environments.

Dibenzothiophene (DBT) is a recalcitrant component of the sulfur containing organic fraction of crude oil. Efforts for isolation of a marine bacterium which could mineralize it, relied on the assumption that such an organism had either evolved as a result of long exposure to traces of this recalcitrant compound or on the fact that such degradative traits had been conferred to the bacterial phenotype due to the de-regulation of inherent biochemical pathways (Coco *et al.*, 1989).

MATERIALS AND METHODS

Artificial Seawater (ASW) of pH 6.8 was prepared with modifications according to Gherna & Pienta (1989). A double strength salts solution (gm/liter of D/W) was prepared using stock A: NaCl, 17.52g, $MgCl_2$, 5.0g, $MgSO_4$.7H2O, 3.0g, $CaCl_2$, 0.5g, KCl, 1.0g, $FeSO_4$, 1.0mg, $(NH_4)_2SO_4$, 1.0g and stock B: KH_2PO_4, 0.5g, K_2HPO_4, 1.1g. Both these stocks were autoclaved separately and then mixed to get required single

strength working volumes.

Dibenzothiophene (E Merck) at a concentration of 0.01% to 0.1% was used as the sole source of carbon and energy either directly as crystals (w/v) or from a 10% stock in Dimethyl Formamide (DMF) (v/v). Sodium benzoate, was used at a concentration of 0.08% (v/v) from a 1M filter sterilized stock. All chemicals used were of Analytical grade.

METHODS

Coastal sampling

Major devastation occurred along the Goan coast as a result of a cyclone in 1995. A shipping vessel, "*M. V. Sea Transporter*" ran aground off the bay of Aguada, Sinquerim, Goa. As a result, large amounts of oil were released from the ballast into the sea thereby contaminating the entire stretch of beaches of Calangute and Candolim with oily residues and tar like clumps. Our attempts at isolation began in November 1995 and continued till January 1996 and were restricted to five different areas relatively close to the shipwreck (Fig. 2.1). 500ml of seawater samples were collected in screw-capped plastic bottles at these different sites, at a depth of around 0-5 meters (m) and a distance of around 10 to 20 feet from the shoreline.

Deep-sea sampling

During a scientific cruise aboard the research vessel "*ORV Sagar Kanya*" in October, 1995 seawater samples (500mls) were collected at different depths ranging from surface to 2000m, using a Niskin sampler mounted on a CTD device from 20 different

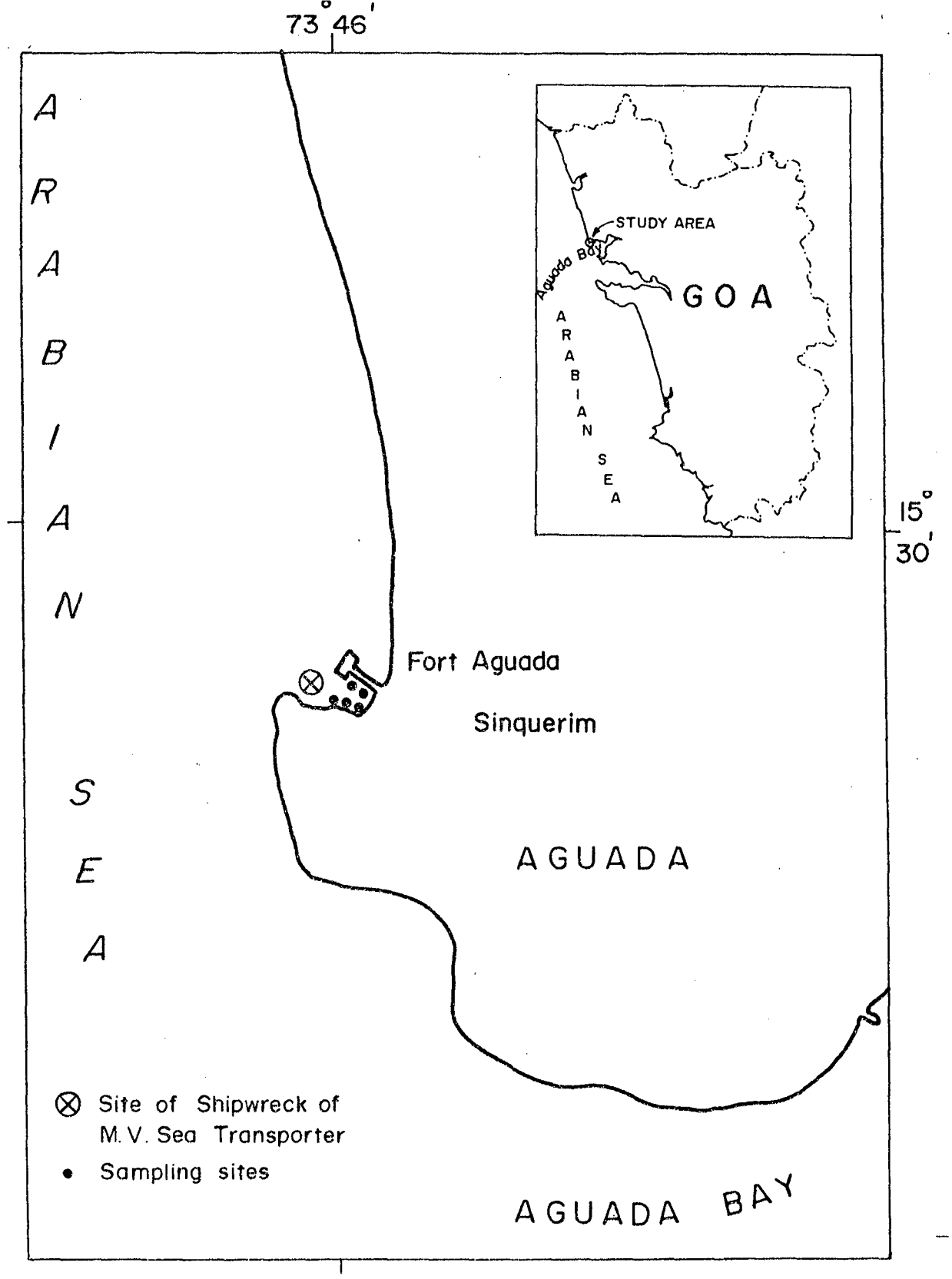


Fig.2.1 Map depicting the location of the sampling sites, off the coast of Sinquerim, Goa, in the vicinity of the ship wreck of *M.V. Sea Transporter*.

stations in the Bay of Bengal. The cruise track is shown in Fig. 2.2.

Processing of the water samples

The water samples were brought to the laboratory and filtered through a 0.22μm sterile membrane filter (Millipore). The filter was then dispensed in sterile artificial seawater medium (5mls) containing dibenzothiophene (0.1%w/v) or sodium benzoate (0.08%v/v), aspirated and aseptically removed. Besides seawater samples were also analyzed for bacterial content, on board the ship itself, by plating onto Zobell's Marine Agar plates and the number of viable cells per ml of seawater was determined.

Direct enrichment technique

Water samples were filtered as above and the filter was dispensed directly into tubes containing DBT (0.1% w/v) and ASW. Sample tubes giving formation of colored metabolites and turbidity were serially diluted and plated on ASW agar incorporated with DBT (0.1% v/v).

Benzoate enrichment technique

Seawater samples were also filtered as above and the filter was dispensed in autoclaved screw capped tubes containing 5ml of ASW with 0.08% (v/v) sodium benzoate and then aseptically removed after aspiration. The tubes were incubated at room temperature on the shaker (100-200 rpm) and visibly checked for turbidity. Serial dilution's of the samples giving turbidity was performed followed by spread plating on ASW agar with 0.08% (v/v) sodium benzoate.

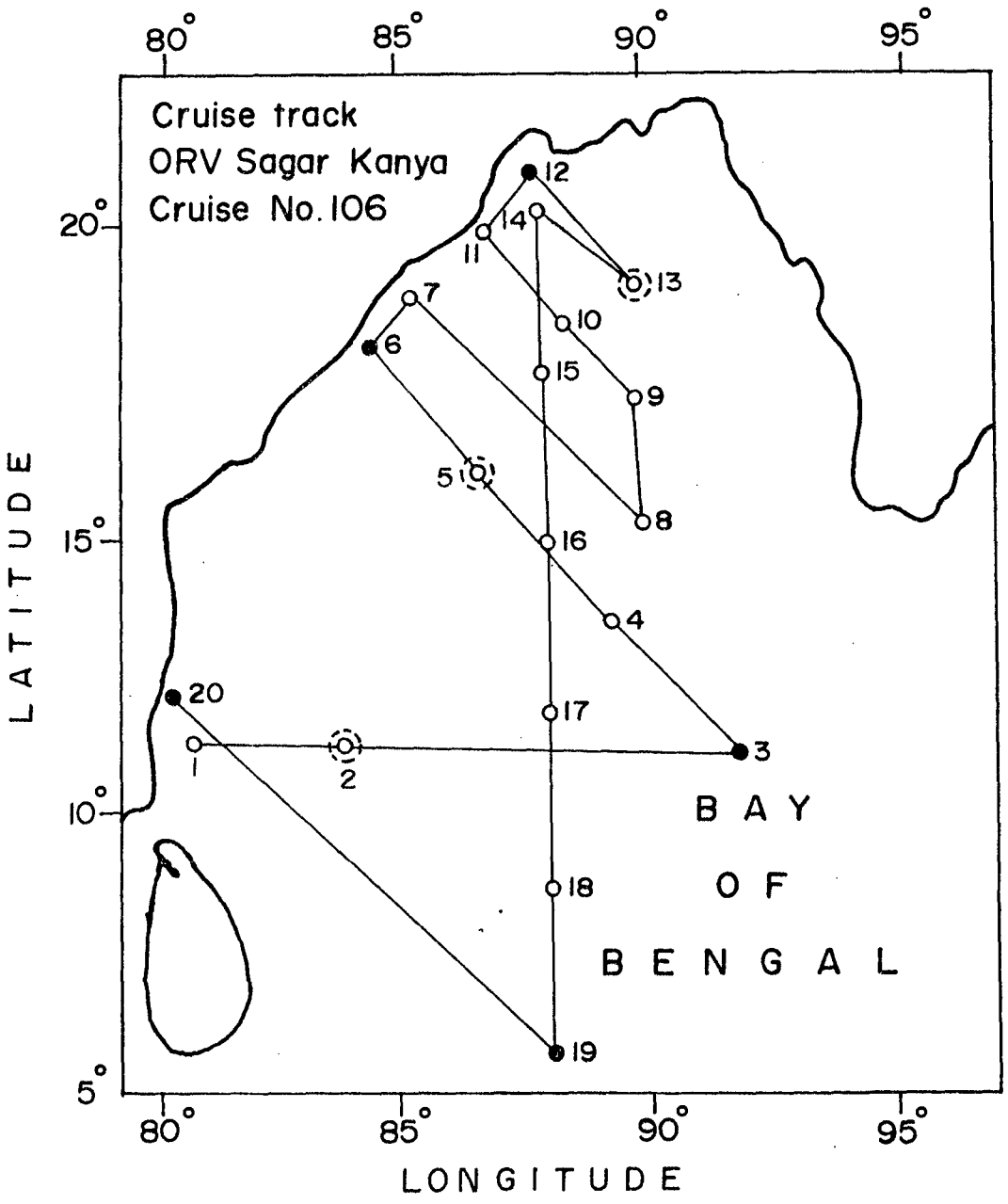


Fig. 2.2 Route map showing sampling stations (o) in the Bay of Bengal aboard research vessel ORV Sagar Kanya Cruise No. 106 in October, 1995. Sampling stations showing presence of DBT degraders in surface waters (●) and at depths (⊙)

Detection of DBT degrading bacterial strains

A rapid procedure devised by Kiyohara *et al.*, (1982) for the detection on solid media of bacteria able to degrade water insoluble solid hydrocarbons was used. Once colonies appeared on the ASW benzoate plates, the plate was sprayed with solution of DBT (0.1%w/v) in ether and incubated at room temperature and checked visibly for the formation of colored metabolites around the colony. The various colonies showing DBT positive phenotype were streaked on ASW agar plates with 0.1% DBT (v/v). The colony showing quickest formation of red colored metabolite was selected for further physiological, morphological and biochemical investigations. On ASW agar plates incorporated with DBT, colonies, which were red or maroon in color, were selected.

Strain identification

All tests for identification of this isolate was done according to the Bergey's Manual of Determinative Bacteriology (Kreig & Holt, 1984). Tests for *ortho* and *meta* cleavage was done according to Stanier *et al.*, (1966). Test for pigment formation (King *et al.*, 1954) were also performed. The culture was also sent to Institute of Microbial Technology (IMTECH), Chandigarh, for identification and deposition in their culture collection.

Estimation of growth

The growth of the culture in tubes was observed visibly as turbidity, in comparison with the appropriate blanks. Turbidity was measured using a UV-visible spectrophotometer (Milton Roy Spectronic 1201) at an optical density of 560nm. The counts of

hydrocarbon degrading marine bacteria in the deep-sea water samples were determined using MPN (Most Probable Number) method (Atlas, 1978; Colwell, 1978).

RESULTS

Dibenzothiophene is a model thiophene compound and it represents the sulfur-containing component of petroleum. In order to isolate marine microorganisms capable of degrading the sulfur heterocycle, this representative molecule was used as sole source of carbon and energy.

Enrichment and Selection of DBT degrading marine bacterium from Coastal waters

Using the Enrichment Technique described in the Materials & Methods, the water samples collected off the Sinquerim-Candolim coast yielded both benzoate and DBT degrading marine microbes.

Detection of DBT degrading bacteria after benzoate enrichment

On ASW benzoate agar plates, colonies with three different morphologies were obtained. When these agar plates were sprayed with a 0.1% (v/v) solution of DBT in ether, they revealed the formation of orange colored metabolite around some of the colonies. The numbers of colonies with this DBT positive phenotype was very low. There were between 1 to 10 organisms with DBT positive phenotype for every 100 to 1000 benzoate-degrading organisms. The colonies having white nucleated morphology on benzoate containing agar possessed the ability to produce the colored metabolite

from DBT, in a period of approximately two to six days.

Detection of DBT degrading bacteria after direct enrichment on DBT

Enrichment of the marine bacteria from the seawater samples directly on DBT resulted in the production of an orange to red coloration in the sample testubes in within 2 weeks. Samples from these tubes when plated onto ASW-DBT (0.1%) agar, revealed that the number of DBT degraders after enrichment on DBT, varied from between 10 to 100 organisms per ml of sea water depending upon the site of sample collection.

Selection and characterization of DBT degrading marine bacterium

One of the red colored colonies growing on ASW-DBT agar was picked and the strain further purified on the same medium. On DBT containing agar plates this colony was pinpoint dark red and this pure culture was used for all our further studies (Fig.2.3A). On Zobell's Marine Agar the colonies were cream in color, slimy, circular, with a convex elevation. In an ASW liquid medium with DBT as the sole carbon source, the entire medium turns transiently vermilion or orange in color and during this period the medium is almost transparent. Gradually in a period of 2 to 3 days the color of the medium changes to a dark maroon with a corresponding increase in the turbidity (Fig.2.3B). If the flask was kept under static conditions the cells settled to the bottom almost immediately and the supernatant medium was clear and pale yellow in color. Phase contrast micrographs of culture broth revealed the presence of bacterial cells occurring as clumps.

On medium with carbon source other than DBT, this marine strain showed

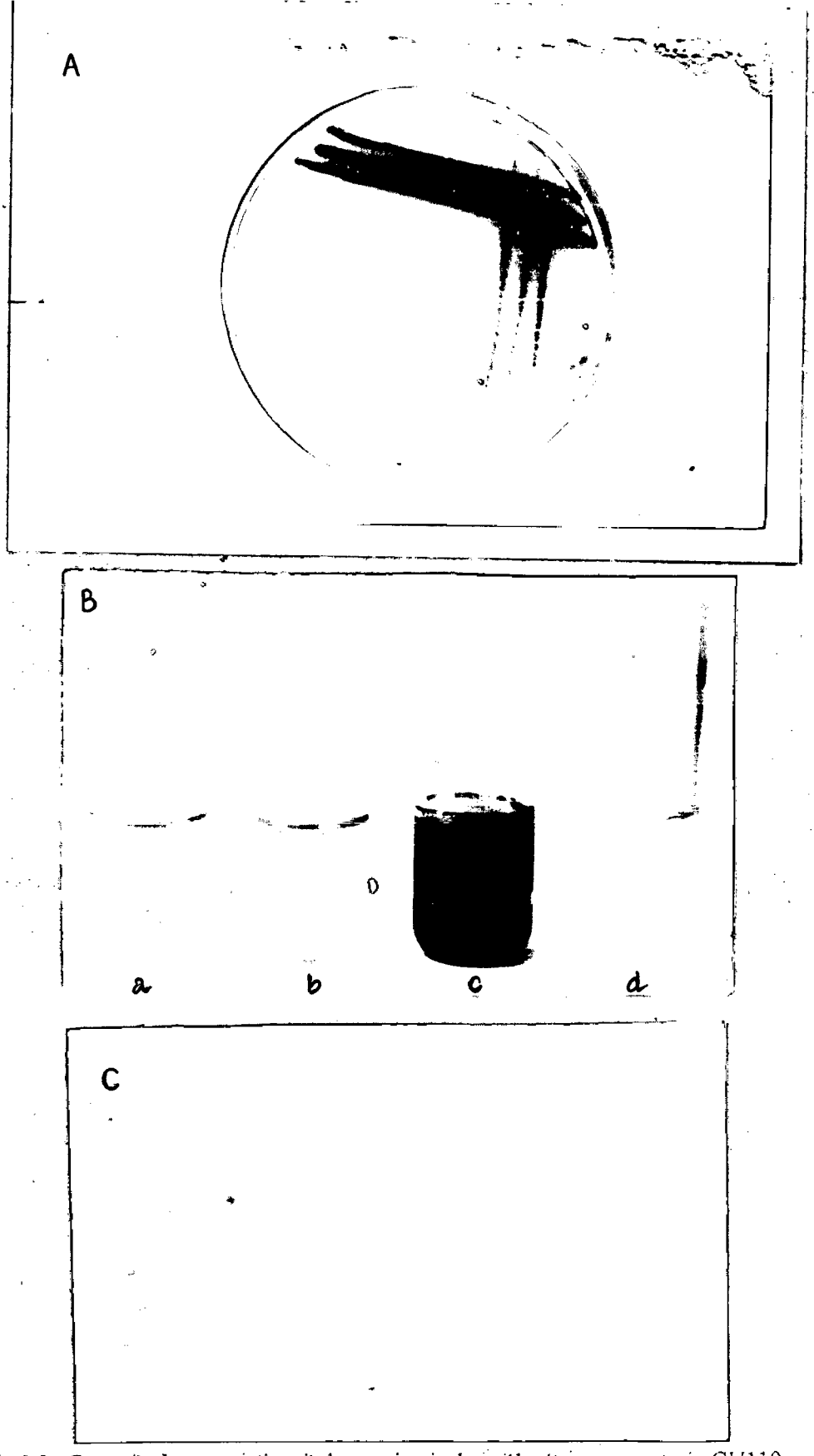


Fig.2.3 Cultural characteristics of the marine isolate *Alcaligenes* sp. strain GU110
(A) On ASW-agar with DBT.
(B) In ASW media containing DBT (in testtubes) inoculated with strain GU110, at different time intervals of growth. Note the transient vermilion color produced in the medium in the testube (a). Un-inoculated control in testube (d).
(C) Gram negative character of strain GU 110 as observed under the microscope (100X magnification under oil immersion objective).

characteristic exopigment formation. On King's medium B, the strain produced a yellow visible pigment, which fluoresced green under ultraviolet light. The strain GU110 also produced a greenish blue exopigment in ASW liquid and agar medium containing sodium lactate, mannitol or sodium benzoate as the carbon and energy source. This phenomenon was also observed in rich medium like Luria Bertanii and Zobell's Marine agar and broth. Pigment formation increased with increasing substrate concentrations of yeast extract and tryptone in the medium but increasing benzoate (>0.32%) and lactate (>0.4%) concentrations inhibited the formation of pigment. On peptone medium with different concentrations of sodium chloride GU110 grew optimally at 3%NaCl.

The strain was an aerobic, motile and gram-negative cocco bacilli (Fig.2.3C), and we temporarily termed it GU110 till further identification. A battery of tests was conducted in accordance with the Bergey's manual and the strain showed a similarity with that of the *Pseudomonas* sp. (Table 2.1). These results corroborated with those carried out at the Institute of Microbial Technology (IMTECH), Chandigarh (Table 2.2). The culture was identified as an Alcaligenes sp. and given the deposition number MTCC 3317. The strain was designated as *Alcaligenes* sp. strain GU110 (MTCC 3317).

Physical and chemical factors affecting DBT degradative ability of strain GU110

GU110 could oxidize DBT even at a 7.5% concentration of NaCl but maximal degradation in terms of turbidity and product formation was observed at 1.75% concentration (Fig.2.4a) but no oxidation of DBT was observed at 0% NaCl

TABLE NO. 2.1 : MORPHOLOGICAL PHYSIOLOGICAL AND BIOCHEMICAL TESTS PERFORMED ON THE MARINE BACTERIAL ISOLATE, STRAIN GU110

Tests	Results
Colony Morphology	
Margin	Entire
Elevation	Convex
Color	Cream
Size	1-2mm
Consistency	Butyrous
Shape	Circular
Grams Reaction	Gram negative
Motility	Motile
Biochemical Tests	
Indole	-
Methyl red	-
Voges Proskauer	-
Citrate Utilization	+
Urea Hydrolysis	+
Casein Hydrolysis	+
Starch Hydrolysis	+
Tween 80 Hydrolysis	+
Catalase Test	+
Gelatin Liquefaction	+
Arginine Dihydrolase	+
Oxidase Test	+

TABLE NO.2.1

Acid Production from carbohydrates		Results
Glucose		-
Lactose		-
Maltose		+
Mannitol		-
Sucrose		-
Fructose		-
Sorbitol		-
Screen for Antibiotic Sensitivity		
Antibiotic	Concentration (μg/disc)	Sensitivity
Streptomycin	25	S
Erythromycin	10	S
Amikacin	30	S
Chloramphenicol	10	S
Gentamycin	10	S
Tetracycline	10	S
Neomycin	10	S
Penicillin	10	R
Amoxycyillin	25	S
Kanamycin	30	R

S = Sensitive R = Resistant

TABLE NO. 2.2: MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS PERFORMED ON THE MARINE BACTERIAL ISOLATE, STRAIN GU110 AT IMTECH, CHANDIGARH

MORPHOLOGICAL TESTS

TESTS	RESULTS
Grams Reaction	Negative
Shape	SR
Motility	+
Fluorescence(UV)	Negative

PHYSIOLOGICAL TESTS

GROWTH AT TEMPERATURES	**RESULT**
4°C	--
10°C	ND
15°C	+
22°C	+
26°C	+
30°C	+
37°C	+
42°C	+
55°C	--
65°C	--
GROWTH AT pH	
pH 5.0	Weak
pH 5.7	+
pH 6.8	+
pH 8.0	+
pH 9.0	+
pH 11.0	+
GROWTH ON NaCl (%)	
2.5	+
5.0	–
7.0	–
8.5	–
9.0	–
10.0	–
GROWTH UNDER ANAEROBIC CONDITION	–

TABLE NO. 2.2: BIOCHEMICAL TESTS

TESTS	RESULT
Growth on MacConkey agar: a) Lac fermentor	+
Indole Test	–
Methyl Red Test	–
Voges Proskauer Test	–
Citrate Utilization	–
Casein Hydrolysis	–
Starch Hydrolysis	+
Urea Hydrolysis	–
Nitrate Reduction	–
Nitrite Reduction	–
H_2S Production	+
Cytochrome Oxidase Test	+
Catalase Test	+
Oxidation/Fermentation(O/F)	–
Gelatin Liquefaction	+

ACID PRODUCTION FROM CARBOHYDRATES	RESULT
Adonitol	–
Arabinose	–
Cellobiose	–
Dextrose	–
Dulcitol	–
Fructose	–
Galactose	–
Inositol	–
Inulin	+
Lactose	–
Maltose	+
Mannitol	–
Mannose	+
Melobiose	–
Raffinose	–
Rhamnose	–
Salicin	–
Sorbitol	–
Sucrose	–
Trehalose	–
Xylose	–

(ND = not detected)

Strain GU 110 is an *Alcaligenes* sp. and was given the deposition no. MTCC 3317.

concentration. DBT could be degraded at both 37°C and at room temperature, but could not be oxidized at higher temperatures as seen in the Fig.2.4b.

Metabolism of hydrocarbon compounds by strain GU110

Ring fission mechanisms employed by GU110, after growth on a simple aromatic compound like benzoate or protocatechuate, were of an ortho cleavage as indicated by the formation of a deep purple colored ring by the Rothera's test.

GU110 was capable of growing on a range of hydrocarbon moieties comprising, simple aromatics like benzoate, polycyclic aromatic compounds like naphthalene and phenanthrene, nitrogen containing heterocyclic compounds like indole, n-alkanes like hexadecane and complex hydrocarbon mixtures like crude oil and tar balls (Table 2.3).

Distribution of DBT degrading bacteria in the open seas

In order to understand the distribution of bacterial sulfur heterocycle degraders in the deep seas, water samples were collected from 20 different stations (Fig.2.2) in the Bay of Bengal during a scientific cruise aboard *ORV Sagar Kanya* (October 1995) at different latitudinal and longitudinal positions and at various depths (Table 2.4). The temperature, pH, salinity and depth profile in the Bay of Bengal was also determined since the ambient environmental characters prevailing at the time of sampling could affect the distribution of the hydrocarbon degraders in the water column (Fig. 2.5). Although the pH, salinity remained relatively constant with the increasing depth, the temperature drop was quite steep from surface up to a depth of 500 meters and

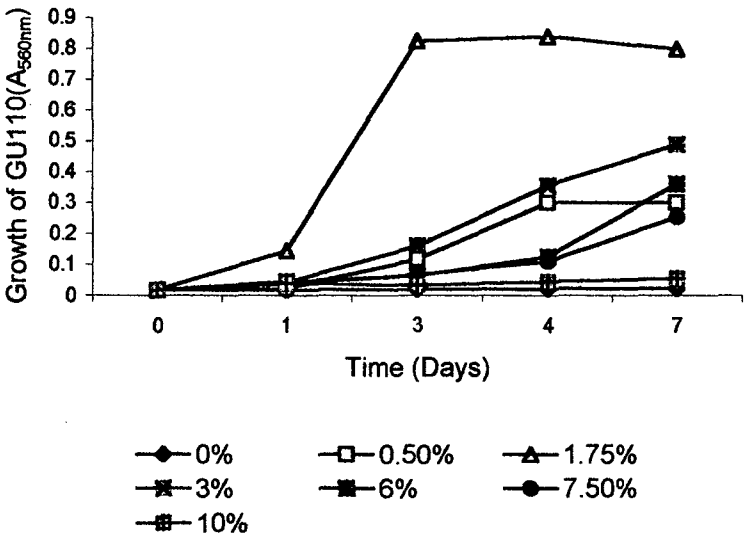


Fig. 2.4a Growth of strain GU 110 on DBT as sole carbon source at different NaCl concentrations.

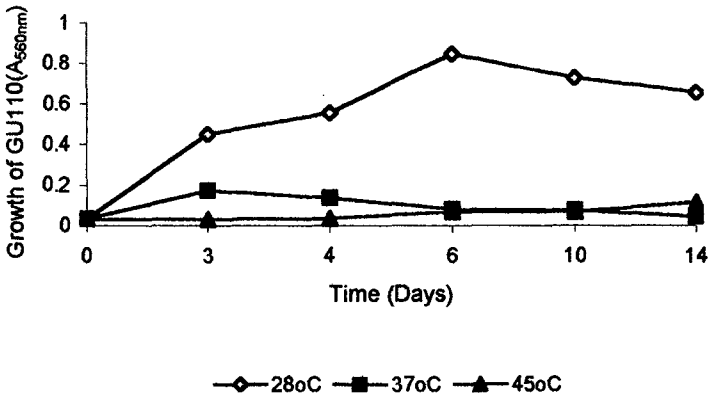


Fig.2.4b Growth of strain GU 110 on DBT as sole carbon source at different temperatures.

Table No. 2.3 HYDROCARBON DEGRADATIVE CAPACITY OF STRAIN GU110

SUBSTRATE	GROWTH (TURBIDITY AT 560nm)
SIMPLE AROMATICS	
Sodium benzoate	+
Catechol	+
Protocatechuate	+
Gentisate	+
Phenol	-
Pthalate	-
Carboxybenzaldehyde	-
Salicylate	+
Salicylaldehyde	+
m-toluic acid	+
Xylene	-
POLYCYCLIC AROMATICS	
Naphthalene	+
1-hydroxy-2-naphthoic acid	+
Naphthoresorcinol	+
Phenanthrene	+
Fluorene	+
Pyrene	-
Anthracene	-
NITROGEN CONTAINING HETEROCYCLICS	
Pyrridine	-
Indole	+
Quinoline	-
SULFUR CONTAINING HETEROCYCLICS	
Thianaphthene	
Dibenzothiophene(DBT)	+
DBT sulfone	+
OILS	
Crude oil	+
Tar balls	+
Engine oil	+
Parrafin oil	+
PARRAFINS	
Hexadecane	+
Heneicosane	+

TABLE NO. 2.4 : GENERAL DESCRIPTION OF SAMPLING STATIONS

DATE	STATION NO	LOCATION		DEPTH(mts) AT WHICH SAMPLES WERE COLLECTED
		LATITUDE N	LONGITUDE E	
95.10.4	1	$11^0$29.98'	$81^0$00.23'	0,500
95.10.5	2	$11^0$30.33'	$83^0$59.86'	0,500,1000,1500,2000
95.10.8	3	$11^0$30.31'	$92^0$00.15'	0,500
95.10.9	4	$14^0$00.17'	$89^0$00.068'	0,500,1000,1500,2000
95.10.11	5	$16^0$48.99'	$85^0$29.622'	0,500,1000,1500,2000
95.10.12	6	$17^0$58.72'	$83^0$58.53'	0
95.10.12	7	$18^0$37.609'1	$84^0$47.60'	0
95.10.14	8	$15^0$00.006'1	$89^0$59.87'	0,500
95.10.15	9	$16^0$59.97'	$89^0$59.54'	0,500,1000,1500,2000
95.10.15	10	$18^0$35.789'	$87^0$59.99'	0,500,1000,1500,2000
95.10.16	11	$19^0$47.90'	$86^0$29.75'	0
95.10.17	12	$20^0$43.71	$87^0$59.58'	0
95.10.18	13	$19^0$00.045'	$89^0$59.712'	0,500
95.10.18	14	$20^0$00'	$87^0$59.367'	0,500
95.10.19	15	$17^0$00.39'	$88^0$0049'	0,500
95.10.21	16	$13^0$59.94'	$87^0$59.938'	0,500
95.10.22	17	$11^0$02.2426'	$87^0$59.1055'	0,500
95.10.22	18	$7^0$59.6512'	$87^0$58.558'	0,500
95.10.26	19	$4^0$03.4490'	$87^0$57.7371'	0,500
95.10.30	20	$13^0$05.7317	$80^0$25.2835'	0

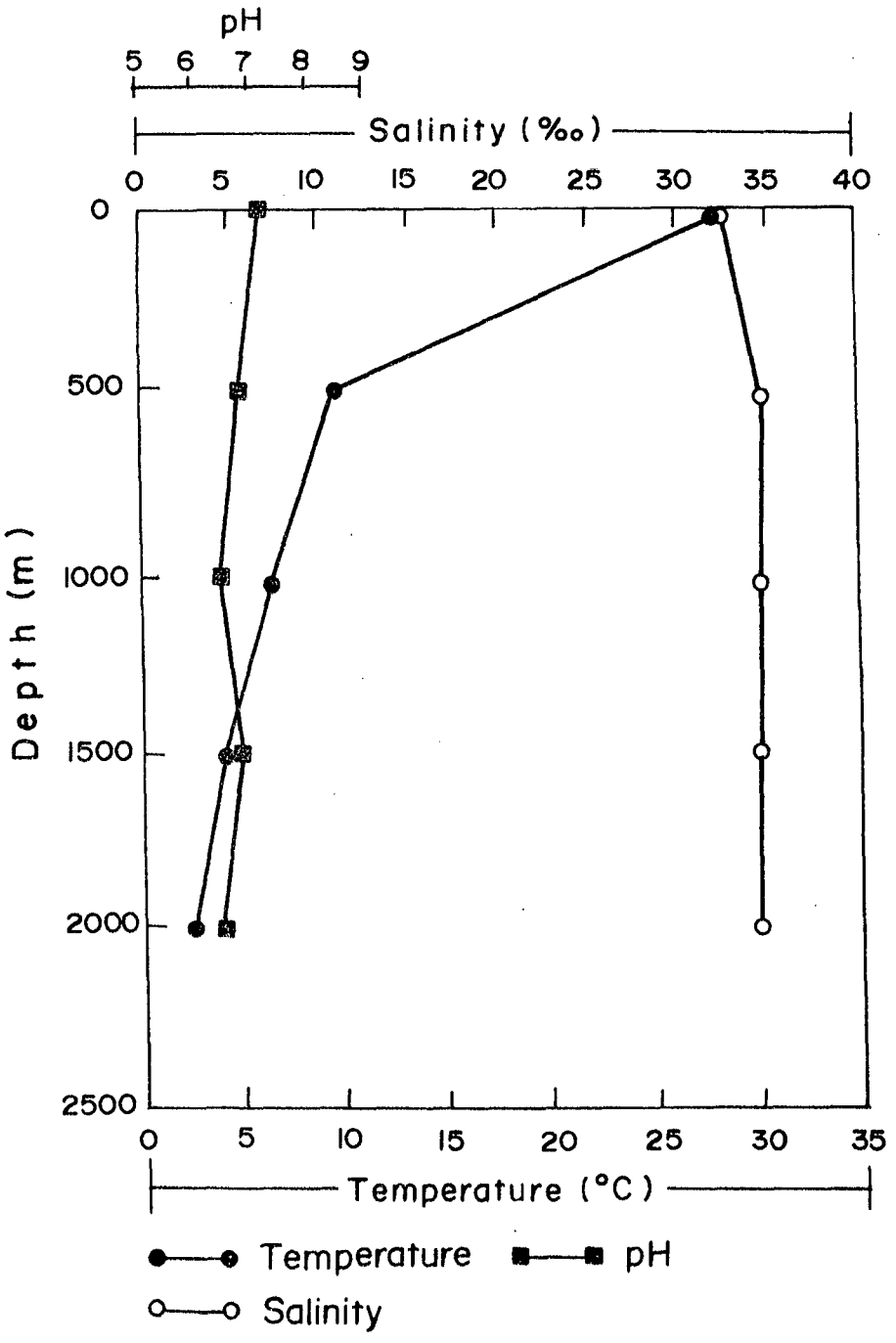


Fig.2.5 Temperature, pH and salinity profiles in the water column of the Bay of Bengal.

thereafter remained relatively constant up to 2000 m. Total viable counts performed from the sea water on Zobell's Marine agar revealed that a milliliter of sea water possessed between 100 and 1000 numbers of marine bacteria and these decreased with an increase in depth (Table 2.5).

Samples from all 20 stations and at all depths provided us with bacteria that could degrade benzoate. When these benzoate enriched bacterial samples were subcultured on DBT, after the culmination of the cruise, in our laboratory, few could yield bacteria with DBT degradative ability. But these results also tallied with those obtained by directly enriching the bacteria in the water samples on DBT itself (Fig.2.2). Coastal waters, where the depth of the ocean bed did not exceed more than 500 m showed a high precedence of DBT oxidizers but these could also be observed at depths of 500 m or more in the open waters. These results indicated that benzoate enrichment could target DBT degraders. By the MPN technique for determination of cell density, we found that marine bacteria when selectively enriched on benzoate and screened for the presence of DBT degraders revealed between 10 to 100 DBT degrading microorganisms for every 10,000 benzoate degraders, with a slightly higher count obtained for surface dwellers but these were present even at depths of 2000 meters (Table 2.6). These results therefore indicate the efficacy of the benzoate enrichment method for selection of DBT degrading marine bacteria.

DISCUSSION

Although bacterial numbers vary by several orders of magnitude from place to place

TABLE NO. 2.5 : DISTRIBUTION OF BACTERIA IN THE BAY OF BENGAL

STATION NO.	TOTAL VIABLE COUNTS(CFU/ML OF SEAWATER)	
	SURFACE	DEPTH(500mts)
1	1.47×10^4	1.52×10^2
2	2.72×10^3	7.50×10^2
3	4.20×10^2	1.15×10^3
4	3.20×10^1	1.60×10^2
5	1.52×10^3	5.80×10^2
6	1.24×10^3	NA
7	1.38×10^3	NA
8	0.81×10^1	7.0×10^1
9	1.0×10^2	5.0×10^1
10	2.0×10^1	1.50×10^2
11	2.30×10^2	NA
12	7.20×10^2	NA
13	5.0×10^3	7.50×10^2
14	4.70×10^2	3.40×10^2
15	4.32×10^3	1.0×10^2
16	9.70×10^2	3.30×10^2
17	5.10×10^2	9.0×10^2
18	1.0×10^2	1.0×10^2
19	1.0×10^2	NA
20	NA	NA

NA = Not Applicable

TABLE NO. 2.6: THE RATIO OF DBT TO BENZOATE DEGRADING MARINE MICROORGANISMS IN THE BAY OF BENGAL

STATION NUMBER	DEPTH(METERS)	RATIO OF DBT TO BENZOATE DEGRADERS IN 100ml OF SEA WATER
2	Surface	-
	500	-
	1000	$1:10^4$
	1500	$1:10^5$
	2000	-
3	Surface	$1:10^4$
4	Surface	-
	500	-
	1000	-
	1500	-
	2000	-
5	Surface	-
	500	-
	1000	$1:10^5$
	1500	$1:10^5$
	2000	$1:10^5$
9	Surface	-
	500	-
	1000	$1:10^5$
	1500	-
	2000	-
12	Surface	$1:10^5$
13	Surface	-
19	Surface	$1:10^5$

and from time to time, almost all samples of seawater contain bacteria capable of oxidizing some of the different components of petroleum (Floodgate, 1984). Since hydrocarbons are natural products, these hydrcarbonoclastic bacteria comprise almost < 1% of the total bacterial population even in pristine environments (Atlas, 1981). When the marine environment is exposed to petroleum contamination, the indigenous flora responds to the increase in oil loading by increasing the oil- degradative capacity, either by selecting strains that are oil degrading or else by inducing the enzymatic mechanisms within the strains, or both (Floodgate, 1984). This results in an increase in the proportion and number of bacterial populations with hydrocarbon-degrading ability (Atlas, 1995) often exceeding 10% of the total bacterial populations (Atlas, 1981). Asphaltenes are the most persistent components of crude oil and the mere presence of hydrocarbon degrading strains does not imply mineralization of these components unless a specific degradative pathway has evolved within the strain. Since the sea is such a vast medium, we decided to restrict our search for a dibenzothiophene degrading marine microorganism, to a coastal environment, which had a history of exposure to hydrocarbons. The intertidal regions off the coast of Sinquerim and Candolim, which had been exposed to oil pollution because of a shipping accident, provided us with such an environment.

Efficacy of the method employed for the isolation of hydrocarbon degrading marine microorganisms

Although the sites selected for sampling had known histories of exposure to hydrocarbons and therefore greater degree of hydrocarbon degrading bacteria, collection of representative seawater samples could largely obviate our attempts to

generate selective hydrocarbon degrading biomass, and we could easily miss out on the hydrocarbon degrader of our choice. An ingenious strategy developed in our laboratory by Fernandes *et al.*, (1994), helped alleviate this difficulty by concentrating the bacterial population from a given quantity of seawater sample by filtration through a Millipore filter (0.22μm). Dispensing the bacterial biomass on this filter in a limited amount of sterile ASW with the hydrocarbon of choice helped increase the numbers and proportions of hydrocarbon degraders, thereby selectively enriching the bacteria of choice. Use of sodium benzoate, a relatively non-toxic aromatic moiety, as sole source of carbon and energy, helped in preliminary enrichment and in increasing the numbers and proportions of DBT degrading bacteria. Besides, we attempted using dibenzothiophene directly, as a substrate for selection of S-heterocycle bacterial degraders. Both these methods proved very effective in selecting for dibenzothiophene degraders. Infact, both methods revealed the presence of a DBT degrading marine isolate having similar morphological and cultural characteristics, thereby proving that the benzoate enrichment method was as effective as direct enrichment on DBT itself.

The spray plate method developed by Kiyohara *et al.*, (1982) for selection of bacteria capable of degrading insoluble hydrocarbon substrates, also helped in selecting the microbial strain of our choice from a plate containing different benzoate degraders.

Characterization of the DBT degrading marine bacterial strain

The pure isolate obtained on DBT-ASW agar, indicates clearly that the strain utilizes DBT as sole carbon source and the characteristics in Table 2.1 & 2.2 led us to identify this microorganism as an *Alcaligenes* sp. and it was designated as, strain GU110

(MTCC 3317). As a result of extensive phenotype analysis Baumann *et al.*, (1972) accommodated by a process of elimination 4 new groups of aerobic marine bacteria in the genus *Alcaligenes* as *A.aestus, A.cupidus, A.pacificus, and A.venustus.* These organisms were similar to the genus *Pseudomonas* and were distinguished only on the basis of their flagellation. Most studies on oxidation of dibenzothiophene deal with the genus *Pseudomonas.* Incidentally, the genus *Pseudomonas* has been the subject of much research as regards its prevalence in oil contaminated marine environments (Atlas, 1981; Leahy & Colwell 1990) and in its ability to degrade PAH's (Foght and Westlake, 1988; Kiyohara *et al.*, 1982). Monticello and Finnerty (1985) have shown that the oxidation of DBT is plasmid mediated in two *Pseudomonas* species. Two soil isolates, strains of *P. alcaligenes* and *P. putida* were found to harbor a similar 72kb plasmid coding for DBT oxidation and growth on naphthalene and salicylate. Lal & Khanna (1996) have isolated an *Alcaligenes odorans* P20 from soil, which degrades DBT as well as both alkane and aromatic fractions of Bombay High and Gujarat crude oil. Fedorak and Westlake (1983) reported removal of alkyl benzothiophene's and dibenzothiophene from Prudhoe Bay crude oil by mixed microbes of marine origin and observed that incubation of marine samples from polluted waters with naphthalene increased the yield of DBT oxidizing microorganisms.

Early treatments of the genus *Pseudomonas* included pigmentation as a generic character, amongst which the soluble pigments; the fluorescent pyoverdins and the pyocyanins are predominant (Krieg & Holt, 1984). GU110 produced a pyoverdin pigment on Kings medium B, which fluoresced green under ultraviolet light. On Kings medium A the yellow pigment was also produced but did not fluoresce under UV. It

has been reported that pigment production in the medium may become erratic or cease depending upon prolonged cultivation under laboratory conditions. A green blue pigment is produced by GU110 on rich medium like L.B. or on ASW medium with easily utilizable carbon sources like lactate and mannitol and simple aromatic moiety like benzoate. The fact that it is produced only on aeration and agitation proves that its production is oxygen dependent. Reduction with potassium metabisulfite results in loss of color. This could be due to the fact that the pigment is a quinone like compound and loss of oxygen changes the configuration of the molecule so as to change its physical properties. This characteristic of pigment production is prominent amongst marine bacteria, which are often characterized on this basis.

Versatile nature of the Alcaligenes sp. strain GU110 (MTCC 3317)

A great number of marine bacteria are capable of a wide variety of metabolic reactions and consequently can utilize a wide variety of nutrients (Rheinheimer, 1976). Macleod (1965) believes that the ability to live in the sea is the only characteristic, which clearly distinguishes marine organisms from other bacteria but also assumes that only a few mutational steps are needed to change a marine form into a one, which will survive in a non-marine environment. Therefore marine microbial forms are highly adaptable and can live in a great diversity of habitats. Their genetic plasticity, metabolic diversity and physiological adaptability enable them to carve out a successful existence from cozy nutrient rich niches to starvation conditions at extremes of temperatures, pH and salinity. Most of the research work concerning the oxidation of DBT deals with microorganism of terrestrial origin. To date, few reports have dealt with the oxidation of DBT by a pure culture of marine origin. The strain GU110 was capable of

attacking DBT and utilizing it as sole source of carbon and energy unlike most others, which co-metabolize DBT. It was also capable of growing on a plethora of various hydrocarbon moieties ranging from simple aromatics to polycyclic aromatic hydrocarbons (PAH's) like naphthalene and phenanthrene as seen in Table 2.3. In their studies on the degradation of PAH's in marine sediment slurries, Bauer & Capone (1988) have found that microbial populations acclimated to single PAH's have the enhanced ability to degrade that PAH as well as a broader range of alternate PAH's. The degradative capability of strain GU110 is more extensive than the *Pseudomonas* sp. HL7b (Foght & Westlake, 1990). This *Pseudomonas* strain was capable of growing on a range of mono, di and tri cyclics including DBT but cannot use DBT as sole source of carbon and energy.

MacLeod (1971) states that a majority of the gram-negative bacteria found in sea require seawater or salt mixtures on isolation. It is now generally believed that a true marine microorganism can be distinguished from terrestrial species by this criterion. The total salt content of normal seawater is 35ppt. A large number of cultures isolated from the sea grew almost equally well in freshwater medium. The range of salinity tolerance by marine bacteria varies but in general gram-negative bacteria are rather sensitive osmotically and rarely grow above 10% salt (MacLeod, 1971). The *Alcaligenes* sp. strain GU110 grew optimally at 3% sodium chloride concentration in a peptone rich medium and could oxidize DBT optimally at 1.75% NaCl in ASW medium. As the concentration of salt increased in the medium, the growth of the culture and its ability to oxidize DBT decreased, as seen by the decrease in the turbidity of the medium and in the formation of DBT oxidation products. But on prolonged

incubation the organism was capable of attacking DBT even at concentrations of 7.5% sodium chloride thereby confirming the high degree of adaptability of this strain. Ward and Brock (1978) showed that the rates of hydrocarbon metabolism decreased with increasing salinity in the range 3.3% to 28.4% and attributed this to a general reduction in microbial metabolic rates.

Distribution of DBT degraders in the deep-seas

Areas of chronic pollution are found within the shelf seas close to shipping lanes, ports and estuaries (Floodgate, 1984). Mironov (1970) and Mironov & Lebed (1972) found highly elevated populations of hydrocarbon utilizing microorganisms in the oil tanker shipping channels of the Indian Ocean and the Black sea. In general, the population levels of hydrocarbon utitlizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons. In unpolluted systems HC utilizers generally constitute less than 0.1% of the microbial community; in oil polluted ecosystems they can constitute up to 100% of the viable organisms (Atlas, 1981).

Although strain GU110 was isolated from marine habitats on the West Coast of India, in the Arabian Sea; it was pertinent to understand the distribution of DBT degraders in the waters along the East Coast and in the open seas of the Bay of Bengal. The Bay of Bengal is the northern part of the Indian Ocean bordering on India, Bangladesh and Burma. A number of important seaports on the Bay, which include that of Calcutta, Madras, Rangoon and several others, contribute to the increased degree of shipping activity in its waters. Besides a number of major rivers like that of the Ganges,

Brahmaputra and Irrawady empty their waters into the Bay. The increased shipping activity, in addition to pollution by inland sewer systems, seepage from the industry, households and farmlands bring harmful chemicals and pollutants into this land locked ocean.

The ubiquity, precedence and distribution of hydrocarbon degraders in the water column of the Bay of Bengal up to a depth of 2000 meters serves as an indicator of hydrocarbon contamination in its waters. The hydrocarbonoclastic bacterial populations are substantially greater than in non-polluted waters, presumably because of continuous enrichment (Zobell *et al.*, 1969). The precedence of DBT degrading marine microbes in the Bay of Bengal is low as compared to that of benzoate degrading organisms but these were present even at depths of 2000 meters therefore reflecting the historical exposure of these waters to complex polycyclic aromatic moieties. The prevalence of DBT degraders in the waters of the Arabian Sea, as well as in the Bay of Bengal is therefore evident from our studies.

In Conclusion

DBT degrading microbes could be enriched on a simple aromatic moiety like benzoate as well as on DBT itself. On further purification a marine strain, which could utilize DBT as the sole source of carbon and energy, was identified and designated as *Alcaligenes* sp. strain GU110 (MTCC 3317).

The versatility of *Alcaligenes* sp. strain GU110 can be exploited to combat hydrocarbon contamination and oil pollution in the natural marine or even terrestrial environments. One approach often considered for the bioremediation of petroleum pollutants after an

oil spill is the addition of microorganisms that are capable of degrading hydrocarbons thereby enabling the elimination of these recalcitrant compounds from the environment. The broad enzymatic capacity for hydrocarbon degradation indicated the microbial potential of strain GU110 for removal of DBT, as well as many constituent hydrocarbon components of crude oil.

The ease with which this species and other DBT oxidizers from the Bay of Bengal was isolated suggests the efficacy of the enrichment method used as well as the fact that comparable organisms exist in the vast tropical marine environment surrounding peninsular India. These can be exploited environmentally for their degradative capabilities as discussed in the following chapters. The occurrence of such degraders is also of importance when studying the fate of recalcitrant aromatic compounds in nature.

CHAPTER III

MECHANISM OF UTILIZATION OF DBT BY THE MARINE ISOLATE *ALCALIGENES* SP. STRAIN GU110 (MTCC 3317).

DBT is representative of a group of sulfur heterocyclic compounds which includes thiophene, benzothiophene, the benzonaphthothiophenes and more complex sulfur containing polyaromatic hydrocarbons. There have been numerous biodegradation studies using DBT as a model, condensed thiophene. Reports describing DBT oxidation by *Pseudomonas* sps., *Rhizobium & Acinetobacter* sps., *a Beijerinckia* sp. *and a Sulfolobus acidocaldarius* and other uncharacterized cultures have appeared in literature. Two mechanisms of DBT biodegradation have been proposed. The carbon destructive pathway involves the conversion of DBT to water-soluble forms with the sulfur still attached to the moiety. Other isolates have been reported to use DBT as sole source of sulfur and not carbon, via the 4S pathway. Both of these pathways are of potential use in the Biocatalytic Desulfurization (BDS) process, but organisms which possess the second pathway in which only the organically bound sulfur from petroleum is removed without diminishing the calorific value of the oil, are industrially more promising.

Most of these degradative processes involve terrestrial microbes which co-metabolize DBT and were not able to utilize it as sole source of carbon and energy. In the last chapter the isolation of a marine bacterial strain was described which could grow on DBT as a sole source of carbon and energy in an artificial seawater medium, with a visible change in the color of the medium to a transient vermilion and then a dark maroon. This bacterial isolate was identified as an *Alcaligenes* sp. strain GU110 (MTCC 3317).

In this chapter we verified the ability of this marine isolate to utilize DBT, examined the water-soluble colored oxidation products formed and determined their similarity with those described previously (Kodama *et al.*, 1970 & 1973) and thereby inferred the

biochemical pathway involved in the oxidation of DBT by this marine microbe. We could thus predict the fate of DBT and other sulfur heterocycles in a natural seawater environment when it is encountered by inherent naturally occurring marine microbial populations like *Alcaligenes* sp.

MATERIALS & METHODS

Chemicals

DBT (E Merck), DBT sulfone, (synthesized in the laboratory as described below), Gibbs's reagent (2,6 dichloroquinone- 4 - chlorimide) (Hi Media), Folin Ciocalteau Reagent (SRL chemicals), bovine serum albumin (Hi Media), resorcinol (Hi Media). Silica gel G for TLC from Hi Media Ltd. and for silica gel for column chromatography (60-120 mesh size) from S.D.Fine-Chem Ltd.

All other chemicals were of analytical grade. All organic solvents were of HPLC grade (E Merck).

Inoculum & Culture conditions

The media used to verify the growth of *Alcaligenes* sp. strain GU110 (MTCC 3317) was ASW. This media was modified so that it was free from sulfates, in order to determine the ability of the culture to grow on DBT as sole source of carbon, energy and sulfur. The carbon source used was DBT either as crystals (w/v) or from a 10% DBT stock in dimethylformamide (DMF).

Microorganisms from maintenance flasks were subcultured into fresh ASW medium with DBT. Inoculum was used from these actively growing cultures for growth studies. *Alcaligenes* sp. strain GU110 cells were incubated at normal room temperature (28°C to

32°C) under continuous aeration (100 to 200 rpm) on a rotary shaker for 48 to 72 hours. Volumes from these actively growing cultures were inoculated into 500ml Erlenmeyer flasks containing 50ml media with different concentrations of DBT to obtain an inoculum size of between 2 to 4 % v/v. Control flasks containing ASW medium and DBT without microorganisms were kept to determine the non-biological oxidation of DBT.

Measurement of Growth

1ml volumes of culture media were harvested at different time intervals and the optical density at 560 nm was determined in a Milton Roy (Spectronic1201) spectrophotometer. The cells from this sample were harvested by centrifugation for 10 minutes at 12500 rpm (Beckman Microfuge E^{TM}) and 1ml of 0.1N NaOH was added to the cells. After digestion at 80°C for one hour in a water bath, the biomass was estimated as protein by the method of Lowry *et al.*, (1951) using bovine serum albumin (BSA) as a standard.

Analysis of DBT oxidation products

The concentration of hydroxylated aromatic metabolites from DBT degradation was determined by an adaptation of the method outline by Box (1983) which uses the Folin Ciocalteau reagent. At different time intervals of growth of strain GU110 on DBT, a 0.5ml aliquot of cell free supernatant was taken and diluted with 7ml of distilled water (D/W) with thorough mixing in testubes. 0.5 ml of Folin Ciocalteau reagent was then added to the mixture and the samples were thoroughly mixed again. After standing for three minutes, 1ml of 20% Na_2CO_3 (saturated solution) was added and mixed, and 1ml of D/W was added to make the final volume to 10 ml with continuous mixing. The tubes were allowed to stand for one hour and the absorbance of the colored solution was read at 725nm in a spectrophotometer. A standard curve was prepared with resorcinol

and the concentration of the hydroxylated metabolites was expressed in micrograms of resorcinol equivalents (RE) per ml.

DBT degradation products were also analyzed at different stages of growth by demonstrating changes in the UV-visible spectra of cell free culture supernatants by measurement on a Milton Roy (Spectronic 1201) spectrophotometer or a Shimadzu UV-1601 spectrophotometer, between 220nm to 600nm.

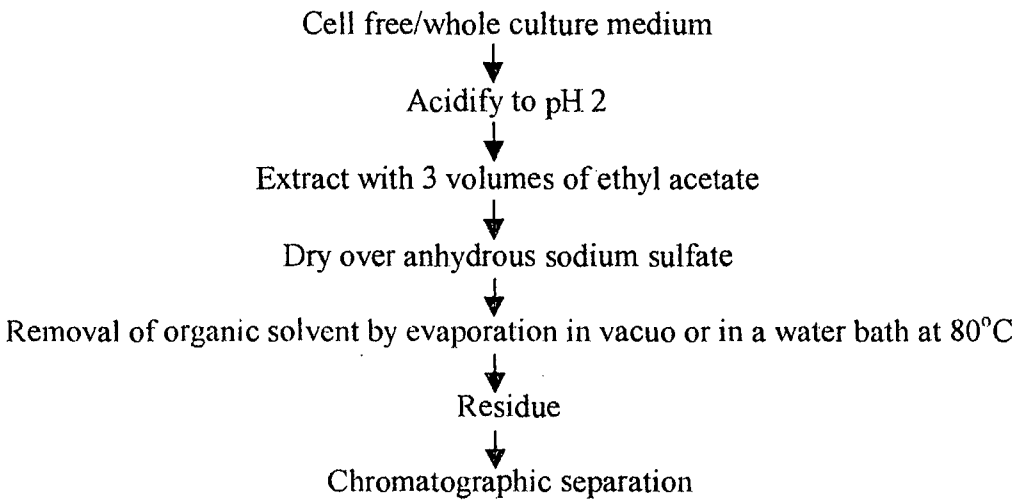
Assessment of sulfate released from DBT

The microbial oxidation of DBT was followed in a modified ASW medium free from sulfates. Sulfate released into the medium was measured turbidimetrically as barium sulfate (kargi & Robinson, 1984). 1ml of 10% barium chloride was added to 1ml of cell free culture supernatant taken at different time intervals of growth and the turbidity of the final mixture was immediately measured spectrophotometrically at 450nm. A standard curve using known quantities of potassium sulfate in distilled water was determined and the concentration of the barium sulfate precipitate was measured in terms of mg/ml.

Solvent extraction of DBT oxidation metabolites

Whole culture broths and cell free supernatants were acidified to pH of 2.5 with 6N HCl and extracted with 3 volumes of ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The residue was then re-dissolved in appropriate organic solvent and subjected to different purification techniques by chromatography.

Flow chart for the extraction and separation of the DBT oxidation products



Separation and purification of oxidation products of DBT

The dried organic residue was subjected to various chromatographic techniques, which included;

(a) Paper Chromatography: The residues were applied to a Whatman Paper No.1 in a solvent system consisting of ethanol, water and ammonium hydroxide (20:15:1). Spots were detected by observing for fluorescence under UV or by spraying with an alcoholic solution of Gibb's reagent (2% w/v) (2,6-dichloroquinone-4-chlorimide). Gibb's reagent reacts with aromatic hydroxyl groups to form chromogenic complexes. Hence the hydroxylated nature of the compound could be determined.

(b) Thin layer Chromatography (TLC): The residues were also applied onto preparative silica gel G TLC plates and then developed in a solvent system of chloroform and methanol (100:5). After locating the fluorescent spots under UV light, the silica gel was carefully scraped out and mixed with methanol or chloroform to elute out the compounds of interest. Further these purified products were analyzed to determine the

maximum absorbancies (λ_{max}) by scanning in the UV-visible spectrum. They were also subjected to gas chromatographic analyses to determine their purity.

(c) Column Chromatography: The dried organic extract obtained above was also separated by chromatography on silica gel (Mesh size 60 - 120) packed in glass columns (1.5 cm by 30 cm) using sequential elution by 4 column volumes of chloroform, chloroform-methanol (2:1), chloroform-methanol (1:2), and methanol.

(d) Gas chromatography (GC): The residues were also dissolved in ethyl acetate and 0.5µl was injected into a GC (Shimadzu 14-B) equipped with a flame ionization detector (FID) and a capillary column packed (DB-1). The injector temperature was 320°C, detector temperature was 320°C, carrier gas, nitrogen and the program rate was set to rise from 60°C to 300°C @ 6°C/min.

Product depletion studies

Alcaligenes sp. strain GU110 was grown in ASW with DBT (0.1% w/v) as the sole source of carbon, incubated at room temperature with continuous shaking at 100 rpm. After 7 days to allow for product accumulation the cells and particulates were removed by centrifugation at 10,000 rpm by using a Sorvall RC5C centrifuge. Supernatants were filter sterilized by passage through 0.22µm pore size Millipore filters. 50ml portions of this cell free, product containing medium were dispensed into sterile 500ml flasks and inoculated with log phase cells from a benzoate enriched (4% v/v) and DBT enriched culture (4% v/v) GU110 culture. The depletion of product at a particular wavelength and the UV-visible spectral changes in the medium were studied using a Shimadzu-1601 UV-visible spectrophotometer.

Synthesis of Dibenzothiophene oxide or DBT sulfone

Dibenzothiophene-5-oxide and DBT-5-dioxide (DBT sulfone) was synthesized in laboratory by the oxidation of DBT with hydrogen peroxide according to the method of Gilman & Esmay (1952). Re-crystallization from benzene gave a compound, which melted at approximately 180°C. GC data revealed a single peak at 26 minutes and no peak at 21 minutes, which is the retention time for DBT. Infra Red spectroscopy (Shimadzu FTIR-8101A) of this compound revealed absorbancies at $1175cm^{-1}$ & $1300cm^{-1}$ which was typical of sulfones (Fig. 3.1).

***Utilization of DBT sulfone by Alcaligenes* sp.**

DBT sulfone, from a 5% stock in DMF was supplied to 50mls of ASW medium in 500ml flasks, as sole source of carbon and energy, at a concentration of 0.01%(v/v). Late log phase cells of *Alcaligenes* sp. strain GU110 (MTCC 3317) (4% v/v) growing on DBT-ASW was used as inoculum. Growth was estimated as protein content and cell free supernatants were screened for the presence of polar metabolites by methods described earlier.

RESULTS

Growth and utilization of DBT by *Alcaligenes* sp. strain GU110 was evident; visual examination of the culture flasks revealed turbid maroon coloration and production of water-soluble colored products in the medium.

Utilization of DBT as sole source of carbon and energy

Growth of *Alcaligenes* sp. strain GU110 was observed, at the expense of DBT, which was supplied in the seawater medium as the only carbon source. The increase in the turbidity of the medium at 560nm with time was concomitant with an increase in the

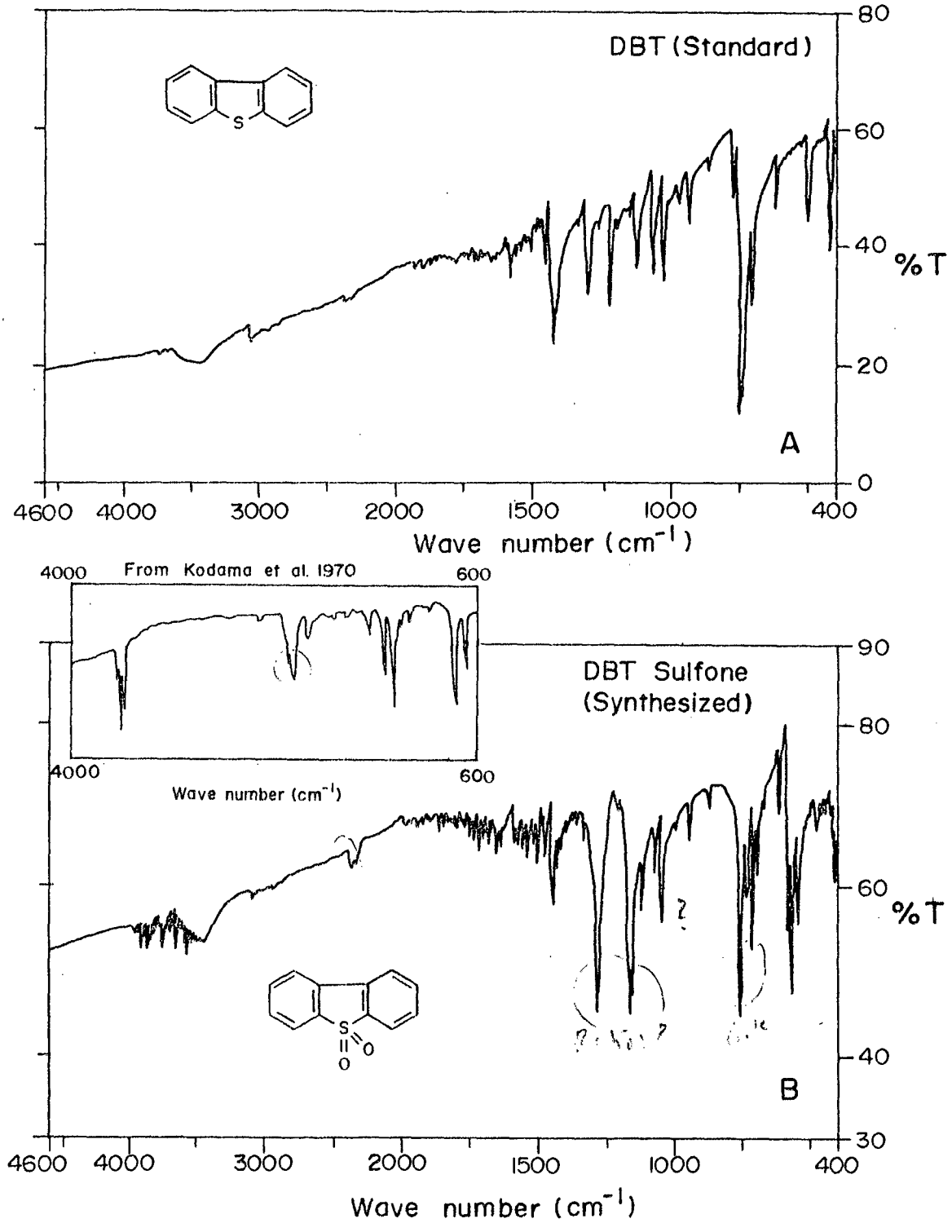


Fig.**3.1** Infrared spectrum of DBT(A) and (B) DBT sulfone (synthesized in the laboratory). Inset shows IR spectrum of DBT sulfone as reported by Kodama *et al.*, (1970).

protein concentration (Fig 3.2), thereby confirming the release of carbon from the aromatic ring of the DBT molecule and its assimilation as cell carbon or cell protein. Increasing concentrations of DBT, were not toxic or inhibitory to the growth of GU110, and the generation times achieved by GU110 cells was similar at these different concentrations (Fig. 3.3). Whole cell culture broths when observed under the microscope revealed the presence of reddish brown clumps of cells adhering to DBT needles (Fig. 3.4).

Formation of DBT oxidation products

Growth of GU110 on DBT was accompanied by the formation of metabolites and the concomitant depletion of DBT in the medium which could be distinctly observed in GC profiles of ethyl acetate extracts of cell free culture supernatants (Fig. 3.5). UV-visible scans of these colored cell free supernatants, performed at different time intervals of growth on DBT, revealed the presence of peaks at 485nm, 390nm, 309nm, 281nm, & 260nm (Fig. 3.6). The absorbance at 390nm and 480nm represent the characteristic absorption maxima's (λ_{max}) of 3-hydroxy-2-formyl benzothiophene (HFBT) and *trans* 4-[2-(3 hydroxy)-benzothiophene]-2-oxo butenoic acid (*trans* HTOB) as described by kodama *et al.*, (1970 & 1973).

The optical densities at these two particular wavelengths in cell free supernatants were recorded and these intermediates were termed as **metabolite II** (A_{485nm}) and **metabolite III** (A_{390nm}) respectively. The growth of GU110 on DBT was accompanied by an increase in the formation of these two metabolites and in a corresponding increase in the polar phenolic metabolic intermediates in the medium (Fig. 3.7). Increasing concentrations of DBT in the medium resulted in an initial increase in the amounts of

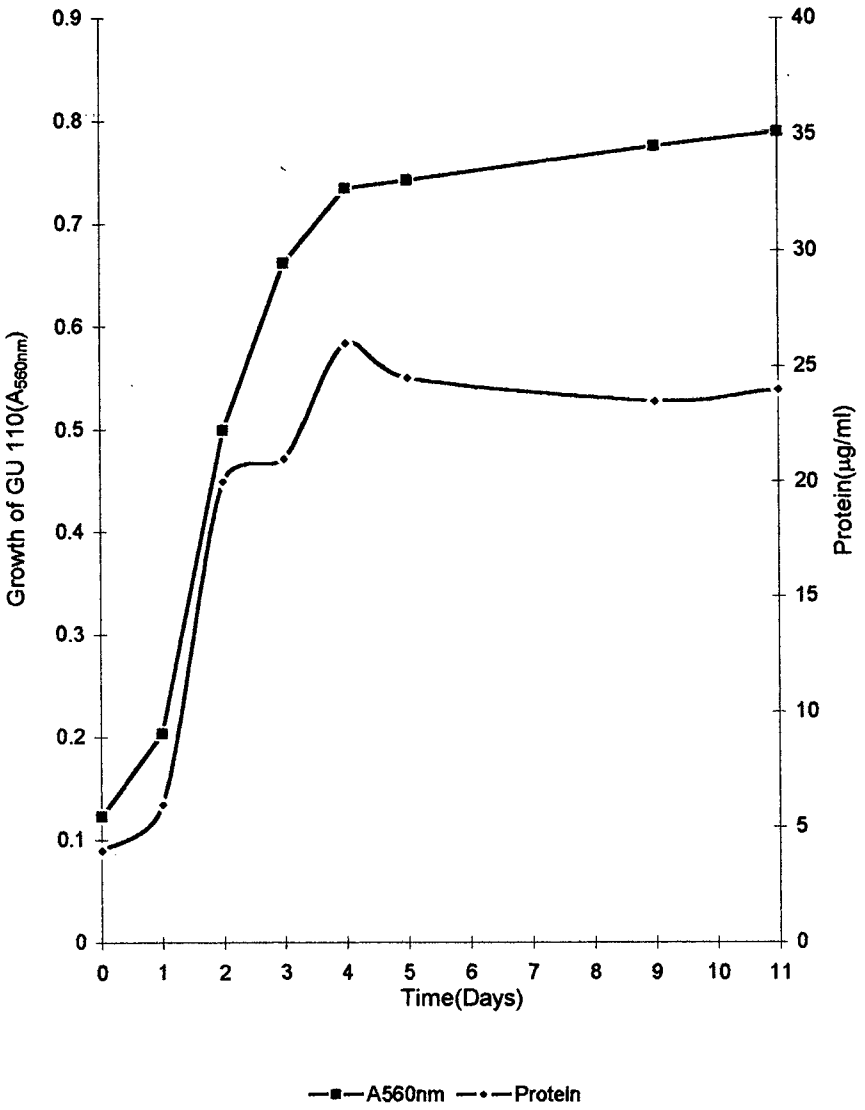


Fig.3.2 Growth of strain GU110 on DBT(0.01%) as sole carbon source recorded as an increase in turbidity(A_{560nm}) and protein concentration(µg/ml).

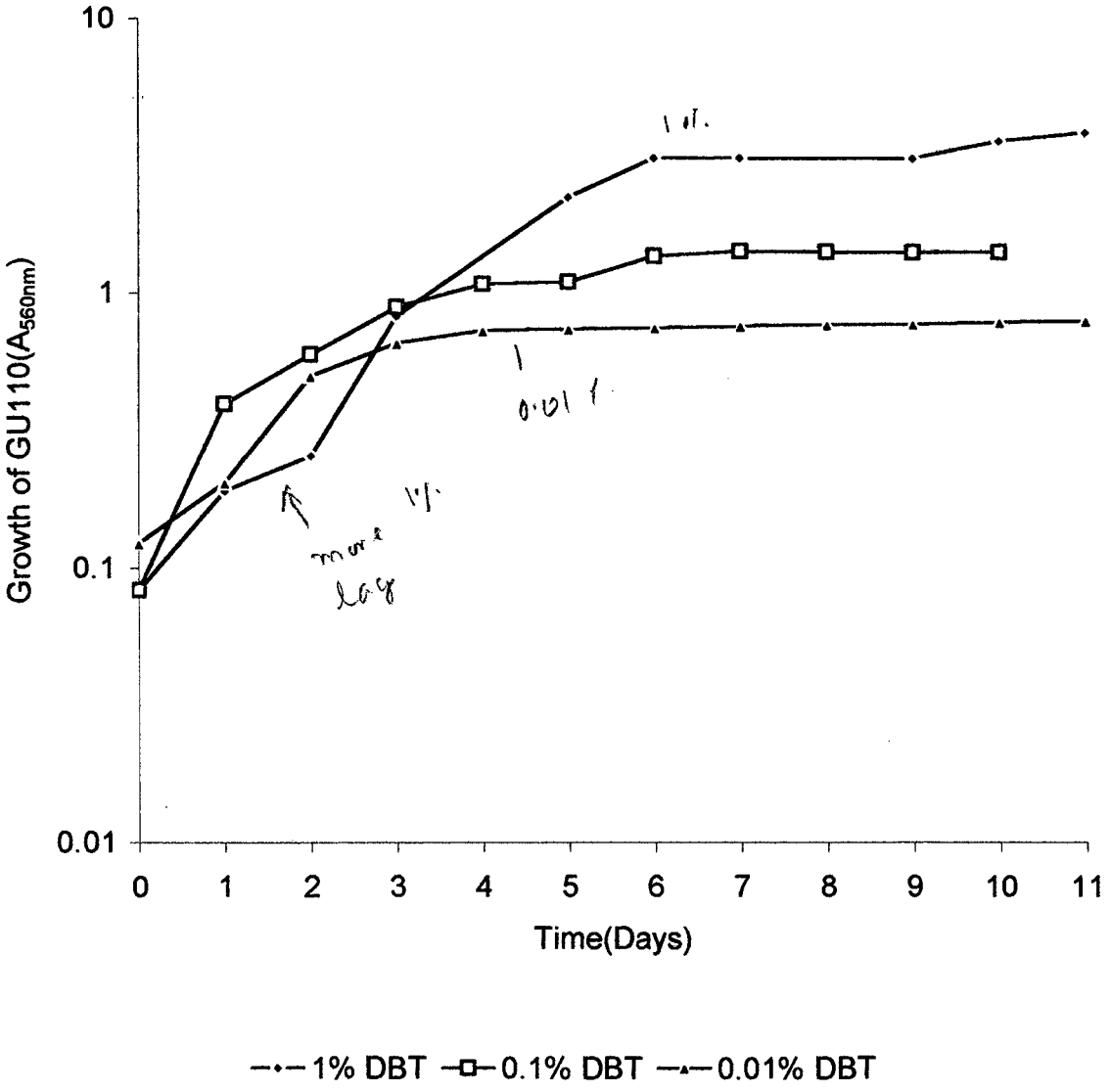


Fig.3.3 Effect of increasing concentrations of DBT on the growth of strain GU110.

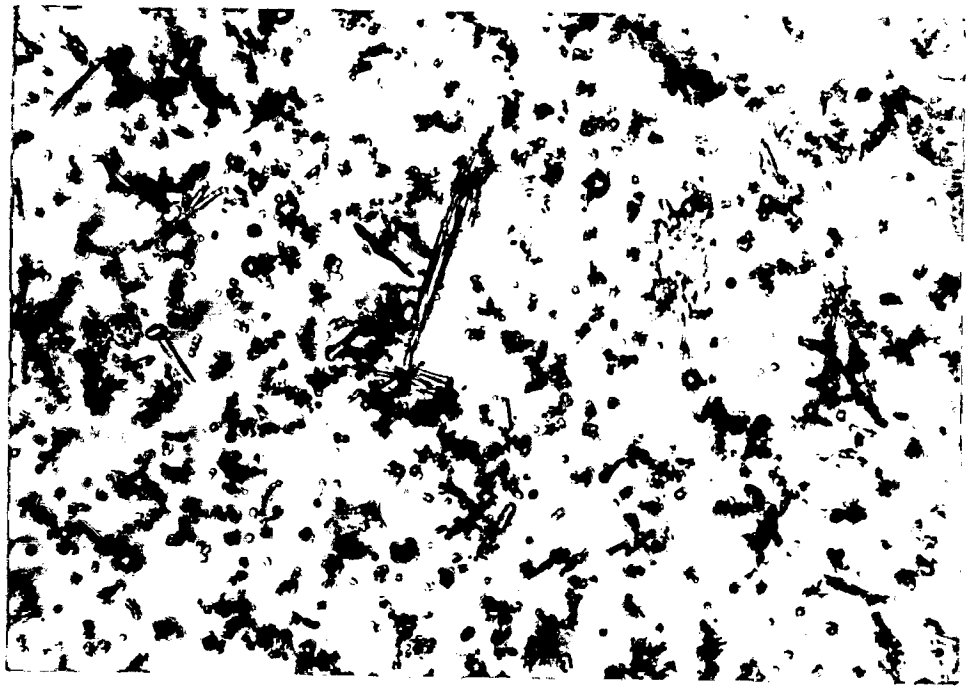


Fig. 3.4 Photomicrograph portrays the presence of strain GU110 cells in clumps (40X magnification) adhering to the needle like crystals of DBT.

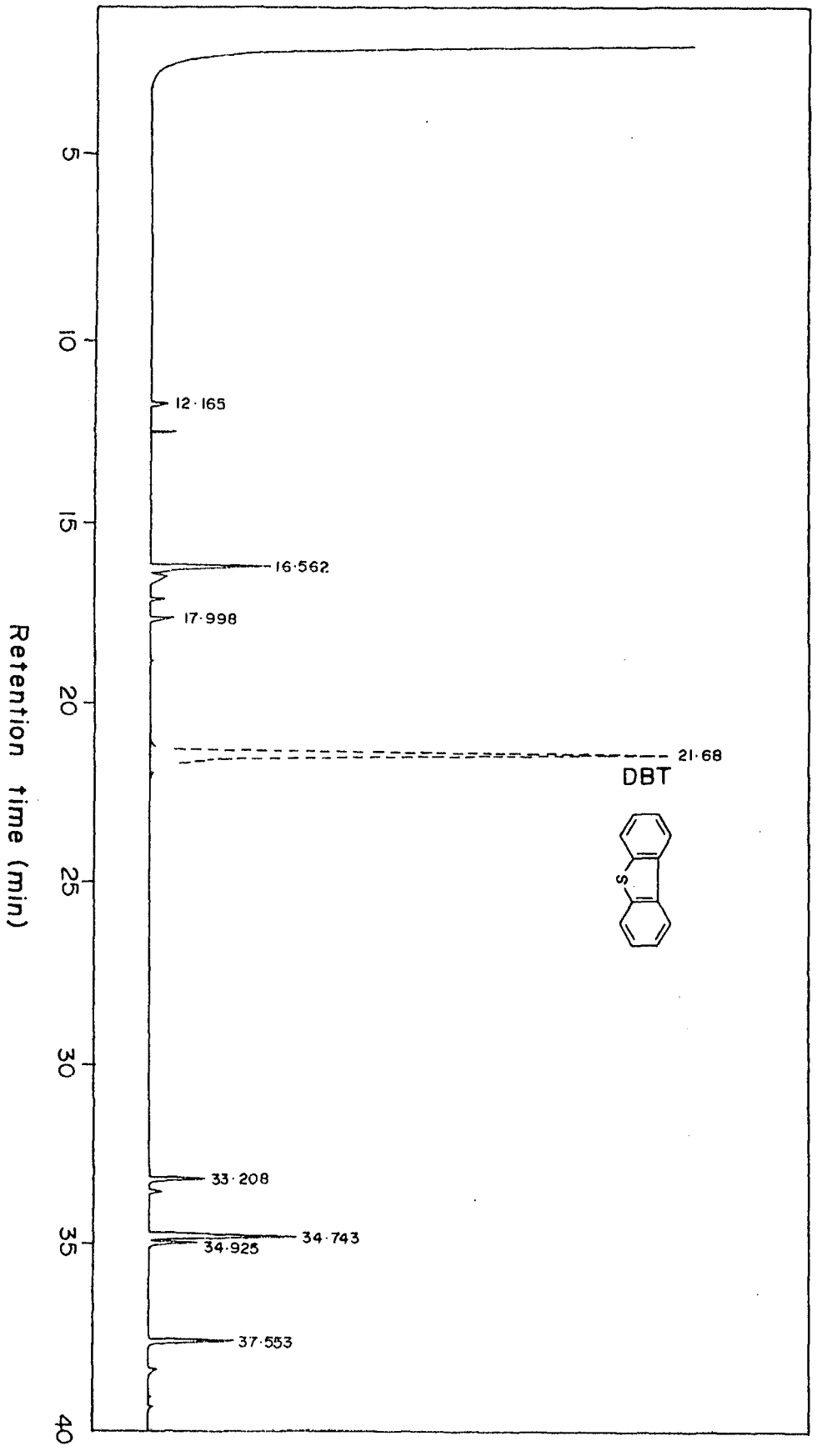


Fig.3.5 GC profile of acidic extracts of GU110 cells grown on DBT as sole source of carbon. Formation of metabolite peaks at different retention times is concomitant with the depletion of the DBT peak (retention time = 21.0 minutes) which is represented by the dotted line.

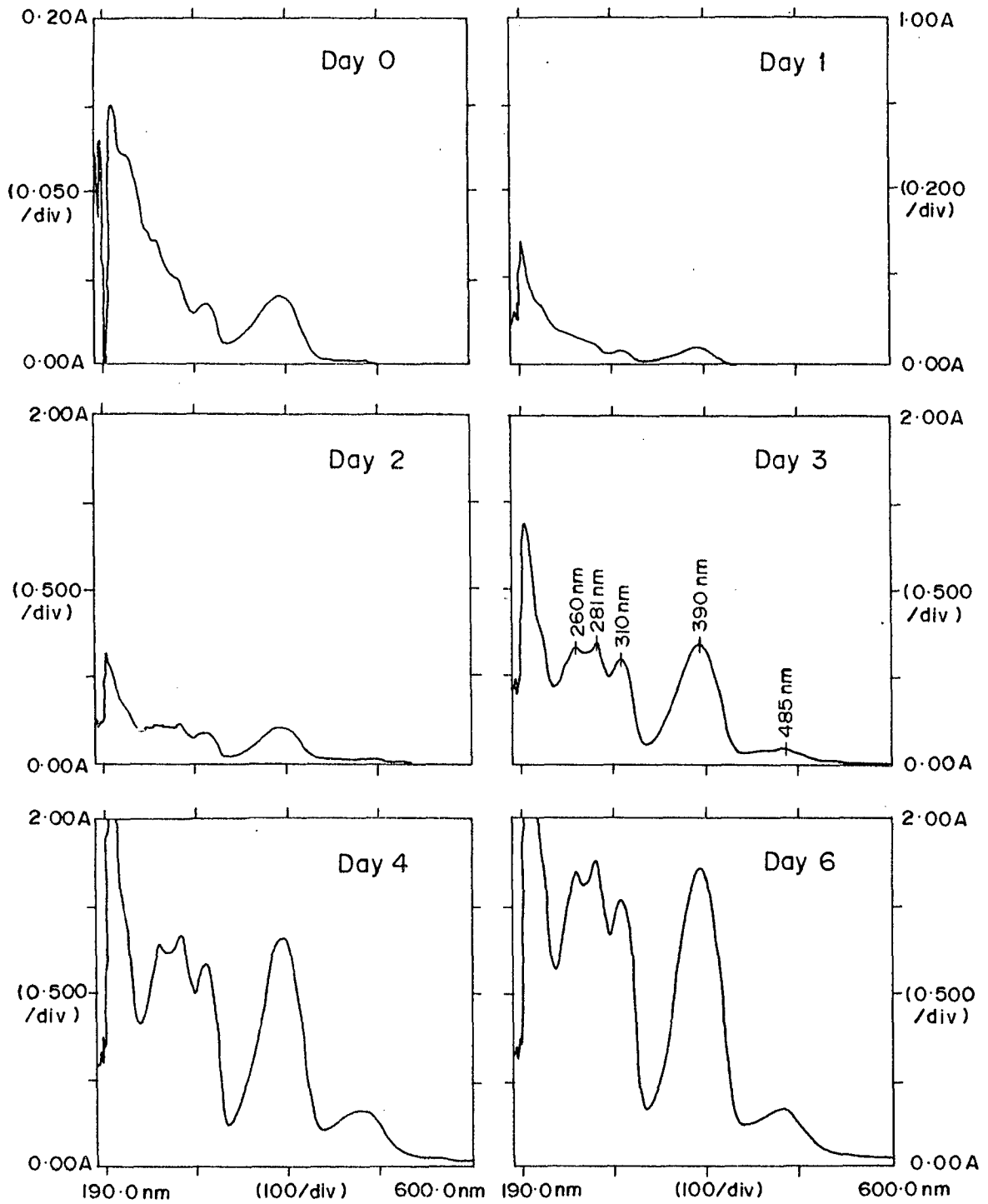


Fig.3.6 UV-visible scans of strain GU110 cell free supernatants performed at different time intervals (day-wise) of growth on DBT as sole carbon source in ASW medium.

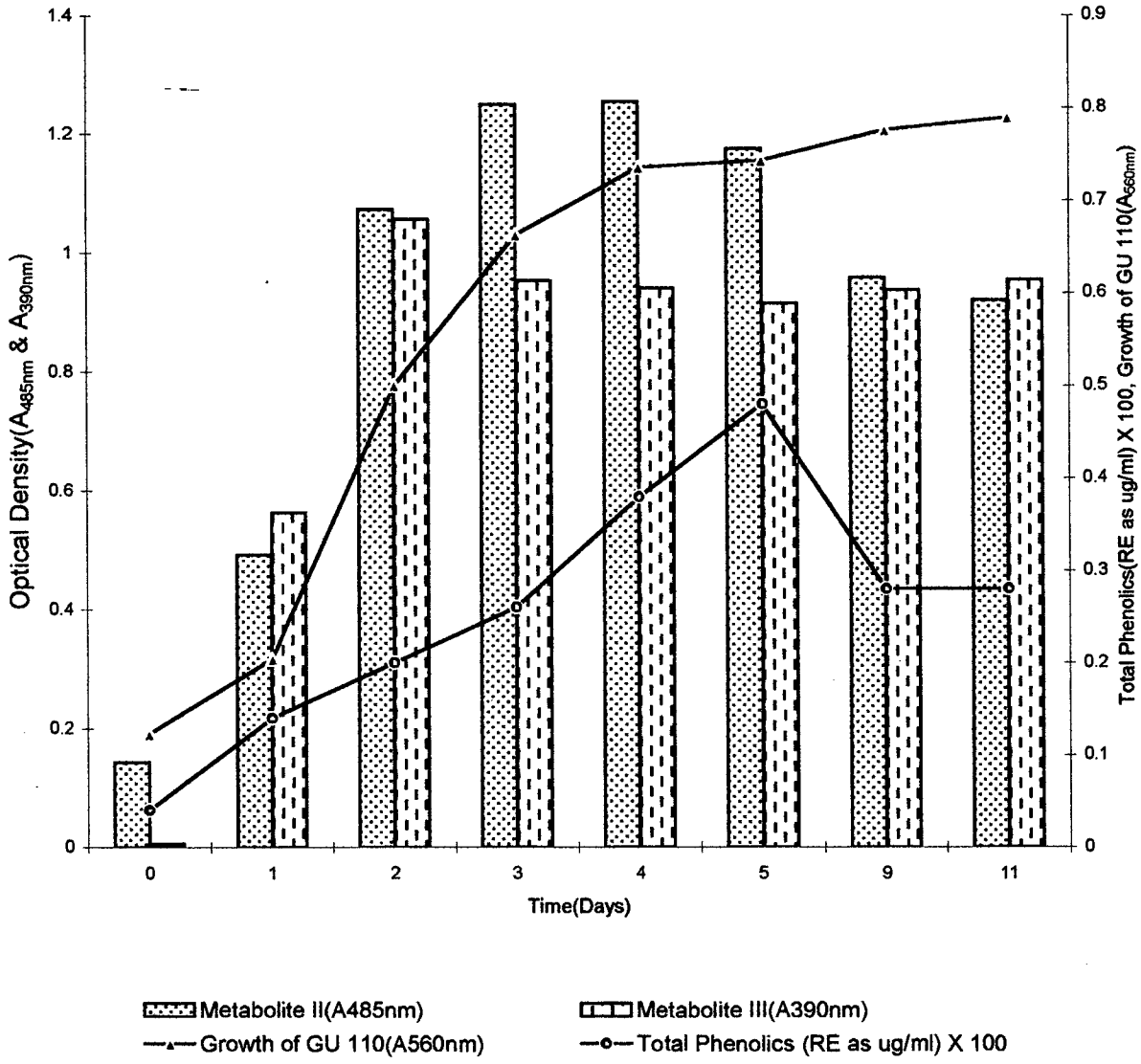


Fig.3.7 Formation of water soluble metabolites (A_{485nm} & A_{390nm}) in cell free supernatants quantified as Total Phenolics(Resorcinol Equivalents or RE) during growth of strain GU110 on DBT.

these intermediates in the medium but at higher concentrations of DBT these amounts remained almost constant (Fig. 3.8).

Utilization of DBT as source of sulfur by strain GU 110

GU110 was capable of growing at the expense of DBT as a source of sulfur, and this was observed by following its growth as increase in turbidity, in a modified ASW medium, free from any sulfur. The culture was maintained for three sequential 5ml transfers in 500ml Erlenmeyer flasks containing 50ml of this medium, in-order to dilute the sulfate in the maintenance ASW medium to negligible background levels. Control flasks containing the same medium and DBT, without any microorganisms were used to determine the non-biological oxidation of DBT. The cell densities achieved by GU110 on DBT, in such a medium were relatively lower than those achieved in a normal sulfates containing seawater medium (Fig. 3.9). Absence of sulfates in the medium, limits the utilization of DBT by GU110, but the oxidation of DBT in such a medium results in the production of metabolites with characteristic absorbancies at 485nm and 390nm, which were the same as those obtained in normal ASW. Utilization of DBT by GU110 in such a medium results in the release of sulfur as sulfate, and this was detected as very low levels of barium sulfate precipitates in the cell free growth medium (Fig. 3.10).

Purification of metabolites formed by strain GU 110 from DBT

Acidification of cell free supernatants with HCl (6N) prior to extraction with organic solvent (ethyl acetate) resulted in a shift in the absorption maxima's at 485nm and 390nm to peaks at 360nm and 305nm with a shoulder at 440nm characteristic of keto-enolic tautomerism of aromatic ring systems (Fig. 3.11).

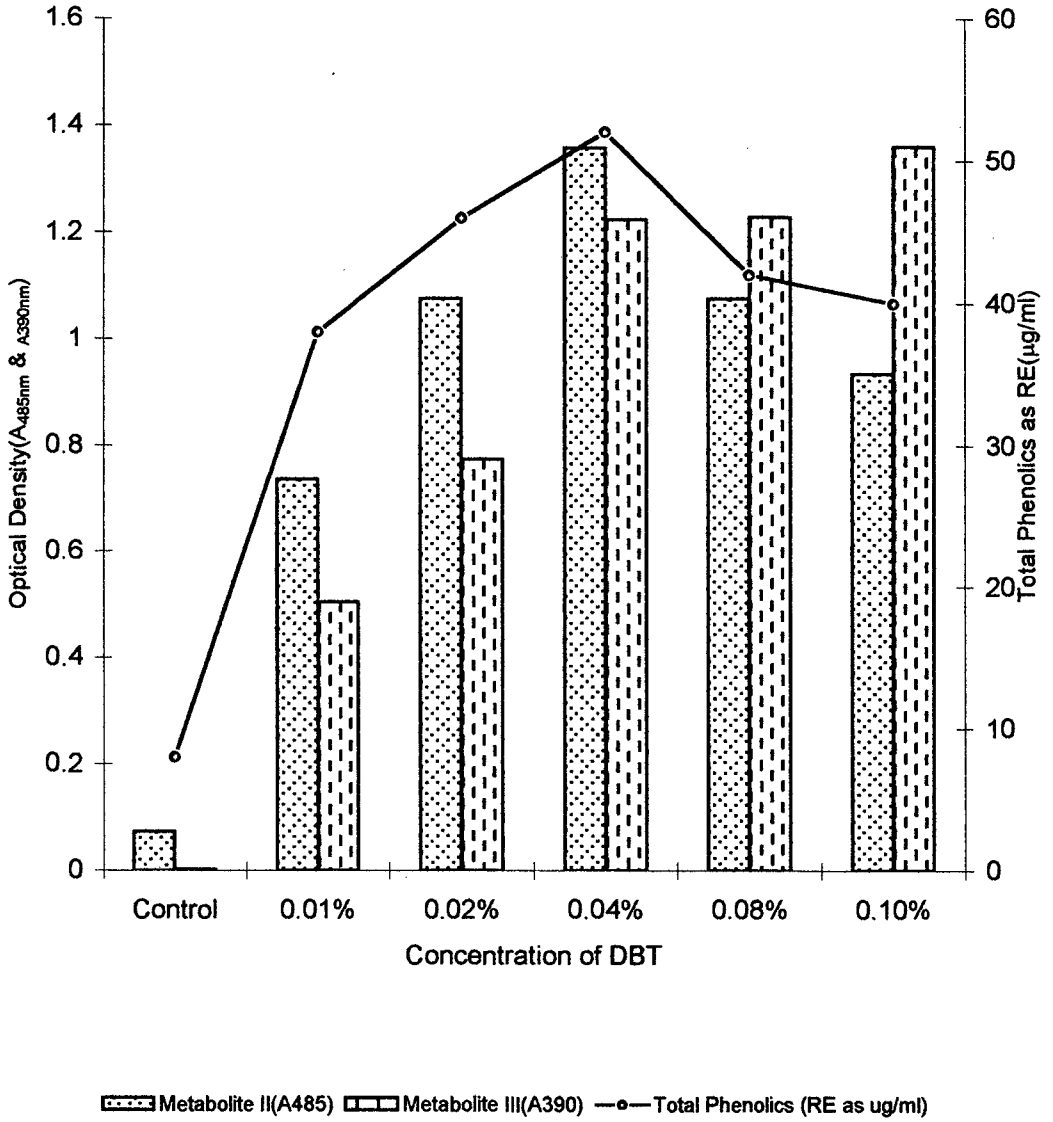


Fig.3.8 Total Phenolic intermediates (Resorcinol equivalents,RE) and water soluble metabolites (A_{485nm} & A_{390nm}) formed by growth of strain GU110 on increasing concentrations of DBT.

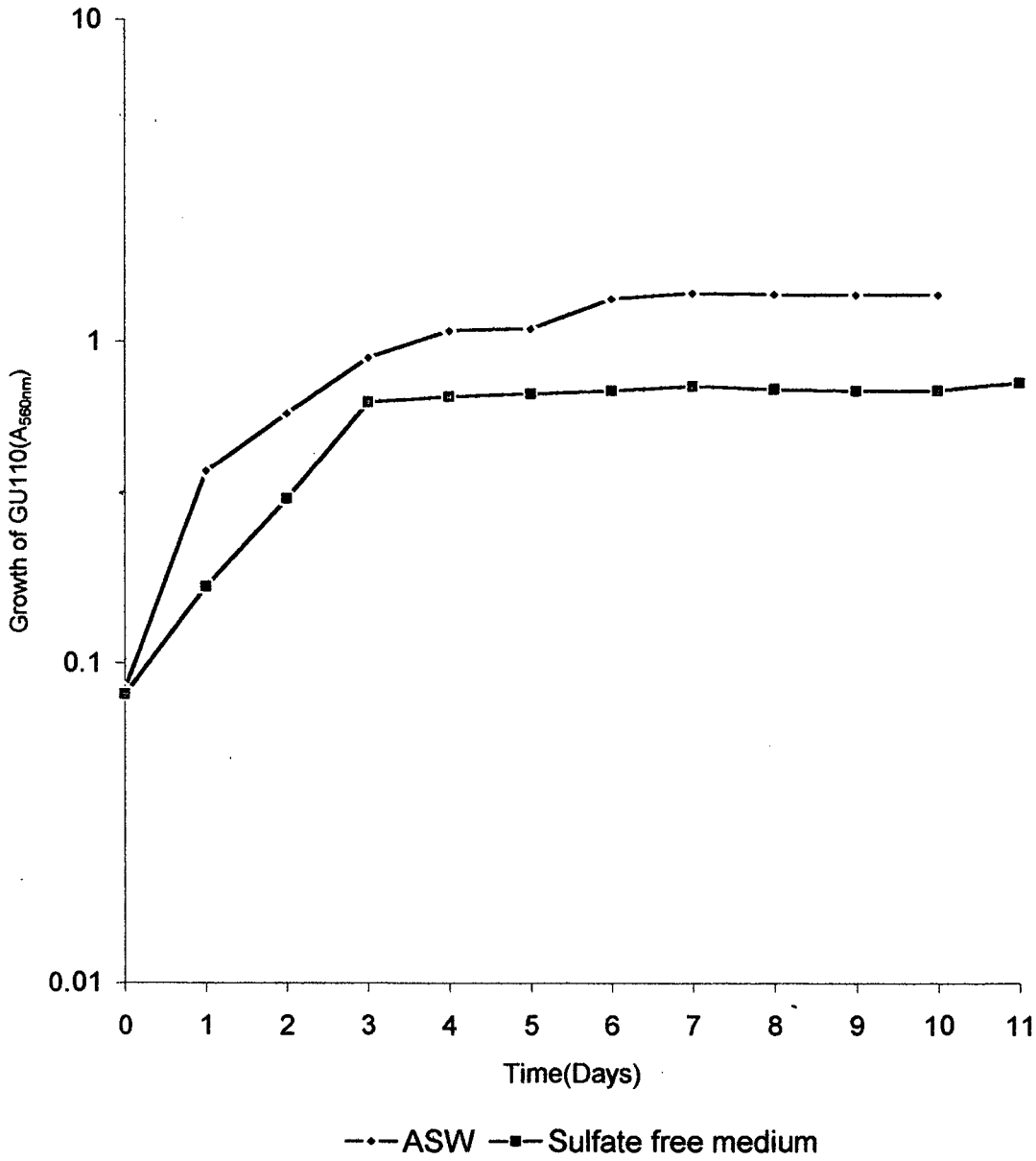


Fig.3.9 A comparison of the growth of strain GU110 on DBT(0.1%) in ASW and in a modified ASW medium free from sulfates.

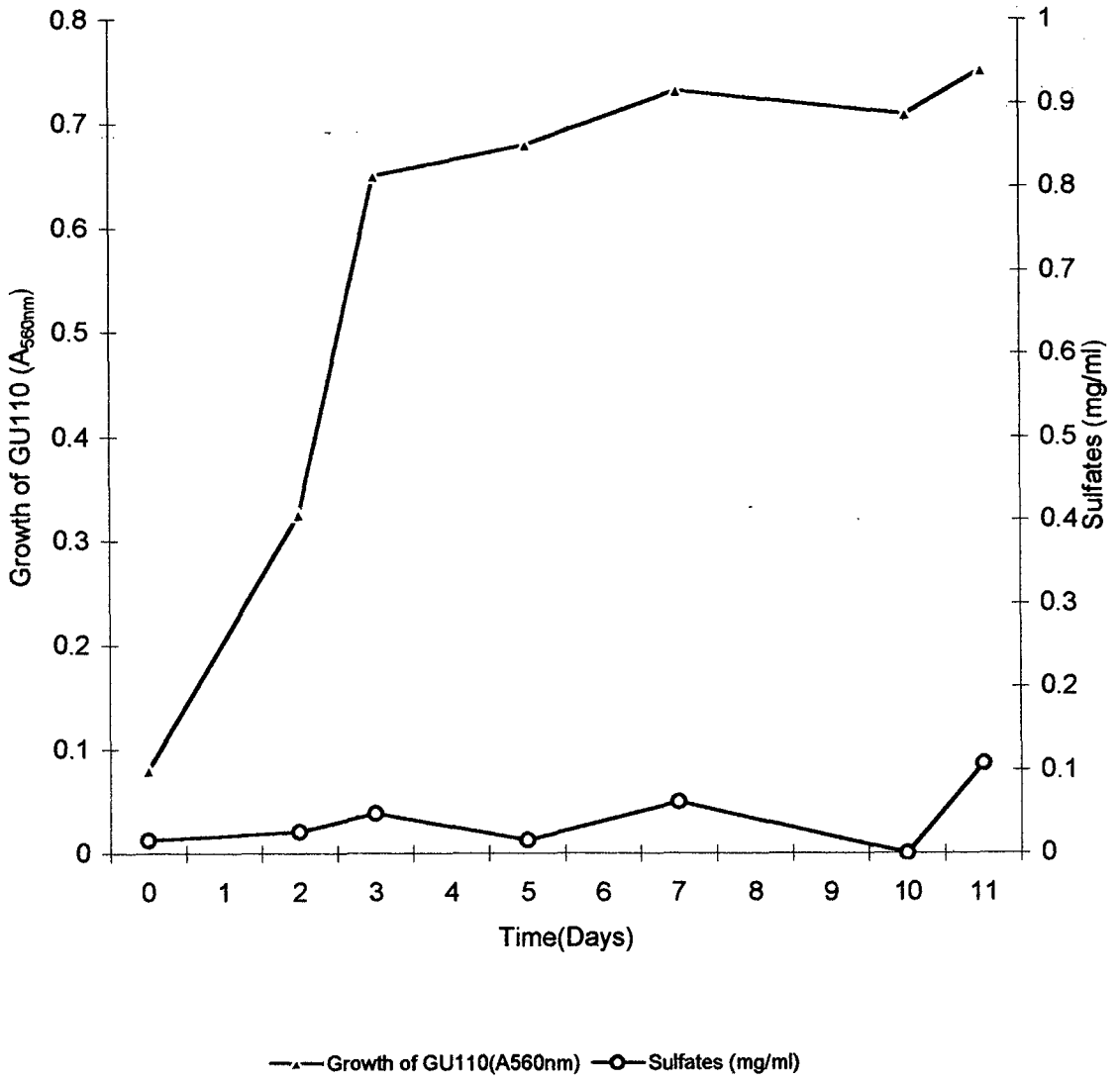


Fig.3.10 Release of sulfates in a modified ASW medium free from sulfates, during growth of strain GU110 on DBT(0.1%).

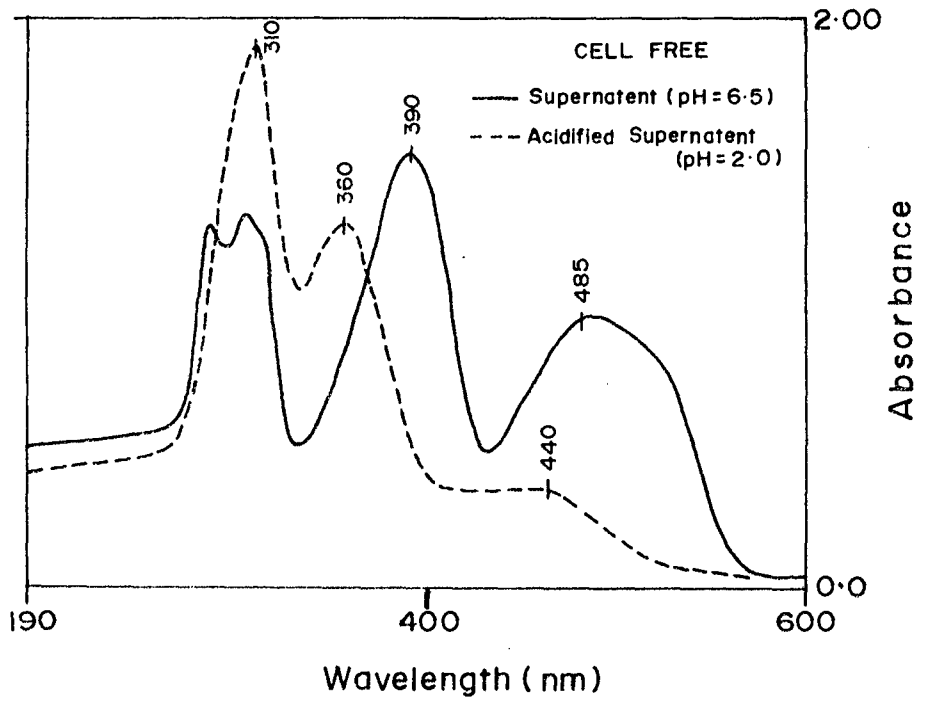


Fig.3.11 UV-visible spectra of cell free supernatant of strain GU110 grown on ASW medium containing DBT at pH's of 7.0 and 2.0.

DBT oxidation products were separated by chromatography on silica gel columns by sequentially eluting with varying proportions of non-polar solvent, chloroform and polar solvent, methanol as described in Materials and Methods. A dark fluorescent pink compound eluted in the chloroform fraction and was designated as **metabolite I**. The next product eluted out in the chloroform-methanol fraction and was a visible bright yellow in color and termed as **metabolite II**. The extremely polar metabolites eluted out in the final washing of the column with methanol and were dark brown in color. Other prominent compounds, were separated by preparative TLC using a chloroform-methanol solvent system (100:5), as they were not successfully separated by column chromatography. These included a visible yellow compound, which fluoresced blue under UV (**metabolite III**) and a purple compound (**metabolite IV**).

Characteristics of the purified metabolites

Metabolite I: TLC's of metabolite I, in a solvent system comprising chloroform: methanol (100:5) revealed a dark fluorescent pink, homologous spot which migrated close to the solvent front (Rf=0.9). It fluoresced purple pink under UV and reacted with Gibb's reagent to form a purple compound. UV-visible spectral analysis revealed no single characteristic peak at any specific wavelength but maximum absorbance was observed in UV regions of less than 250nm.

Metabolite II: This yellow colored compound fluoresced yellow under UV and had a Rf of 0.34 on TLC's. Gas chromatographic analyses revealed a single peak at a retention time of 18 minutes. A compound which had a maximum absorbance at 485nm was identified as the *trans* form of 4[2- (3-hydroxy)-thianaphthenyl]-2-oxo3-butenoic acid or *trans* HTOB by Kodama *et al.*, (1973), and a compound having similar absorbance maxima was clearly observed in the cell free supernatants prior to extraction (Fig. 3.11).

But metabolite II exhibited a maximum absorbance at 445nm (Fig. 3.12) and could possibly have been formed from the *trans* form of HTOB, by the acidification and extraction procedures employed.

Metabolite III: was purified from preparative TLC's and was yellow in color but fluoresced blue under UV and reacted with Gibb's reagent to form an orange pink compound. These characteristics, plus the absorption spectrum of the purified product (λ_{max}=390nm) (Fig. 3.13) are identical to the characteristics reported for 3 hydroxy -2-formyl benzothiophene (HFBT) by Kodama *et al.*, (1970).

Metabolite IV: was purple on TLC plates and fluoresced pink under UV. It exhibited an absorption maxima at 540nm which was similar to that of the DBT oxidation product identified by Kodama *et al.*, (1970) as 3-oxo-[3-hydroxy-thionaphthenyl-(2)-methylene]-dihydrothionaphthene (Fig. 3.14), the non-enzymatic dimerization product of 3-hydroxy-2-formyl-benzothiophene.

Transient nature of DBT oxidation products

Thin layer chromatographic analysis of supernatants from the log phase, using a chloroform methanol solvent system (100:5), revealed the presence of metabolites I, II, III and IV. TLC's of supernatant from late stationary phase showed absence of metabolites II, III and IV and the appearance of a brown spot having an Rf of 0.34, which fluoresced pink under UV-visible spectral analysis (Fig. 3.15). A similar absence of metabolites II and III was seen in scans of supernatants from the late stationary phase (Fig. 3.16) when compared to the scans of supernatant from the mid log phase of growth. We studied the depletion of HFBT or metabolite III (A_{390nm}) by *Alcaligenes* sp. strain GU110 cells enriched on DBT and benzoate. UV-visible scans performed at different time intervals showed the decrease in the peak at 390nm with the concomitant

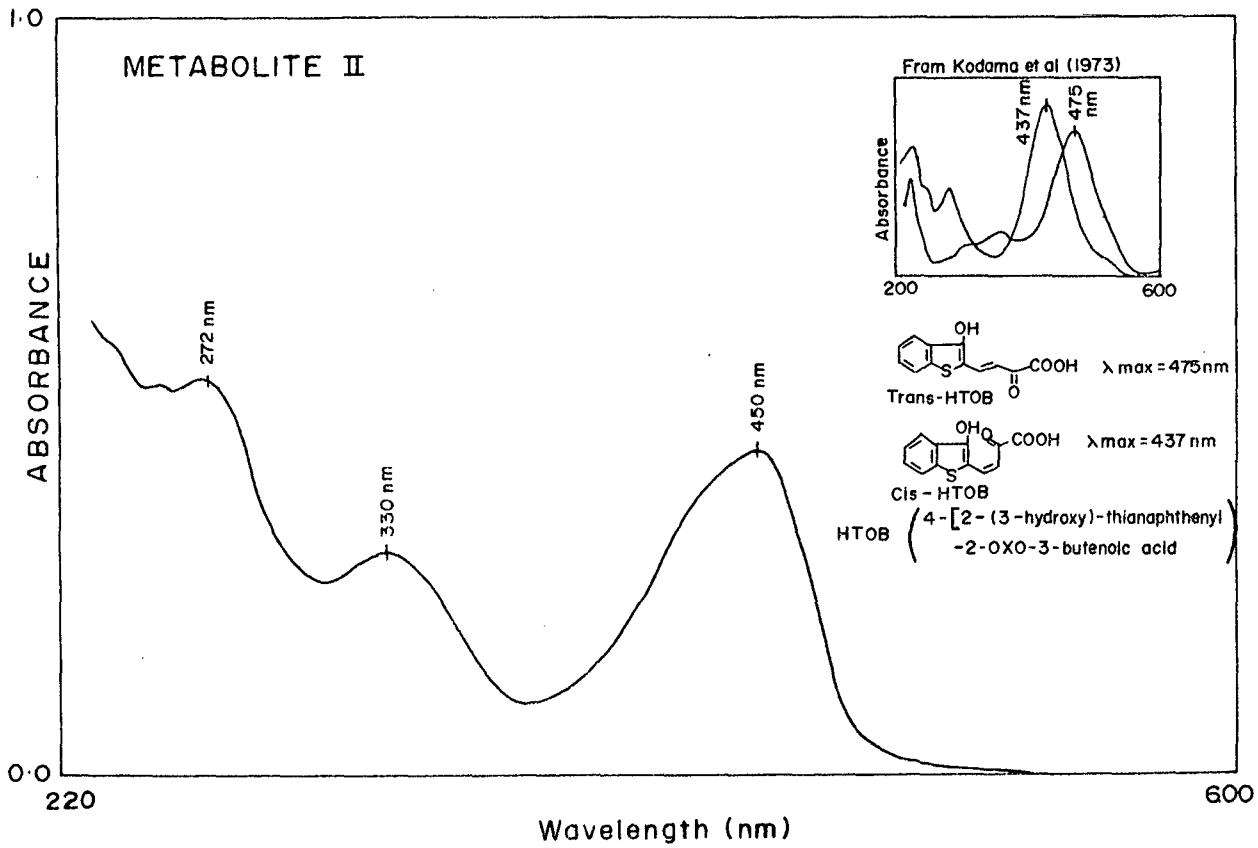


Fig.3.12 UV-visible spectra of **metabolite II** in chloroform. Metabolite II was separated by column chromatography and displayed spectral characteristics which were similar to that of HTOB or 4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid, a DBT oxidation product purified by Kodama *et al.*, (1973) [inset].

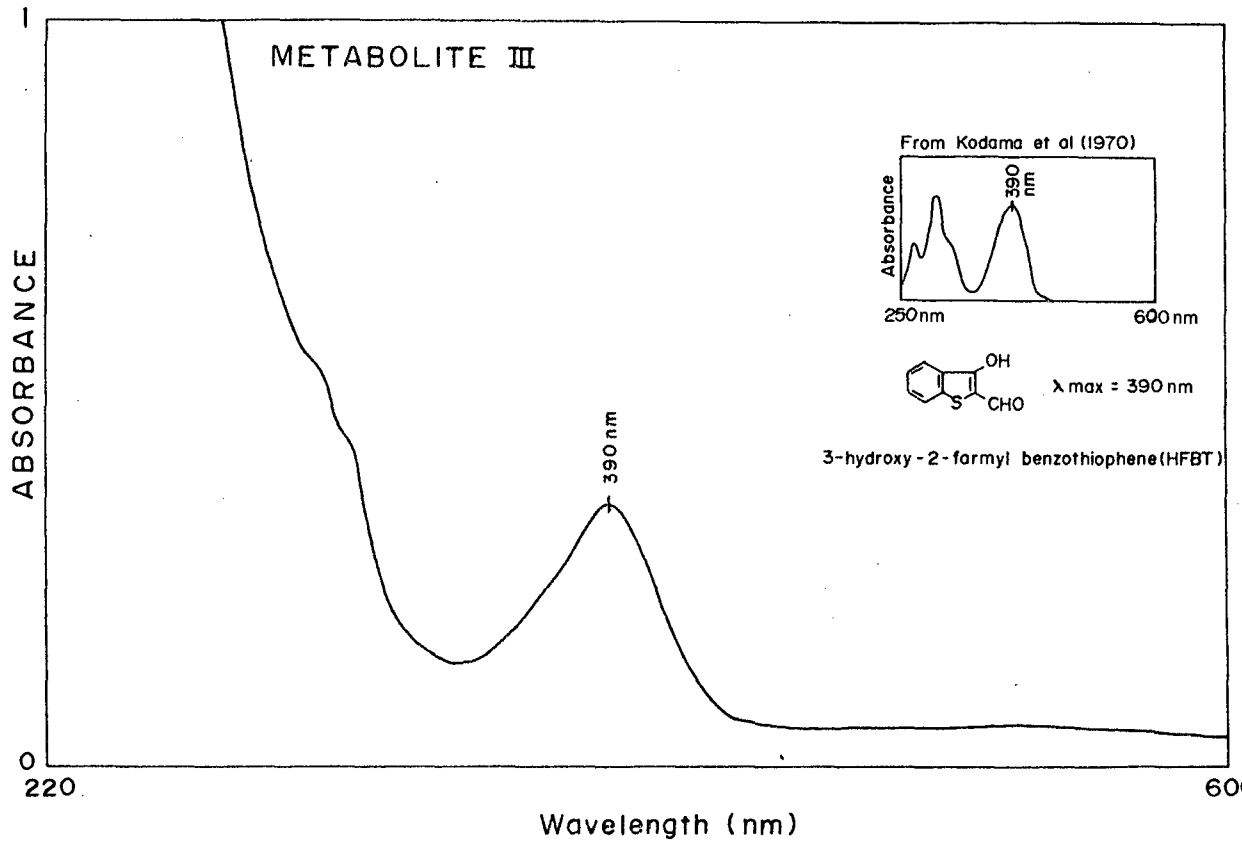


Fig.**3.13** UV-visible scan of **metabolite III** (in chloroform) depicting a compound with a maximum absorbance (λ_{max}) at 390nm which is similar to that obtained by Kodama *et al.*, (1970) for 3-hydroxy-2-formyl benzothiophene (HFBT) [inset].

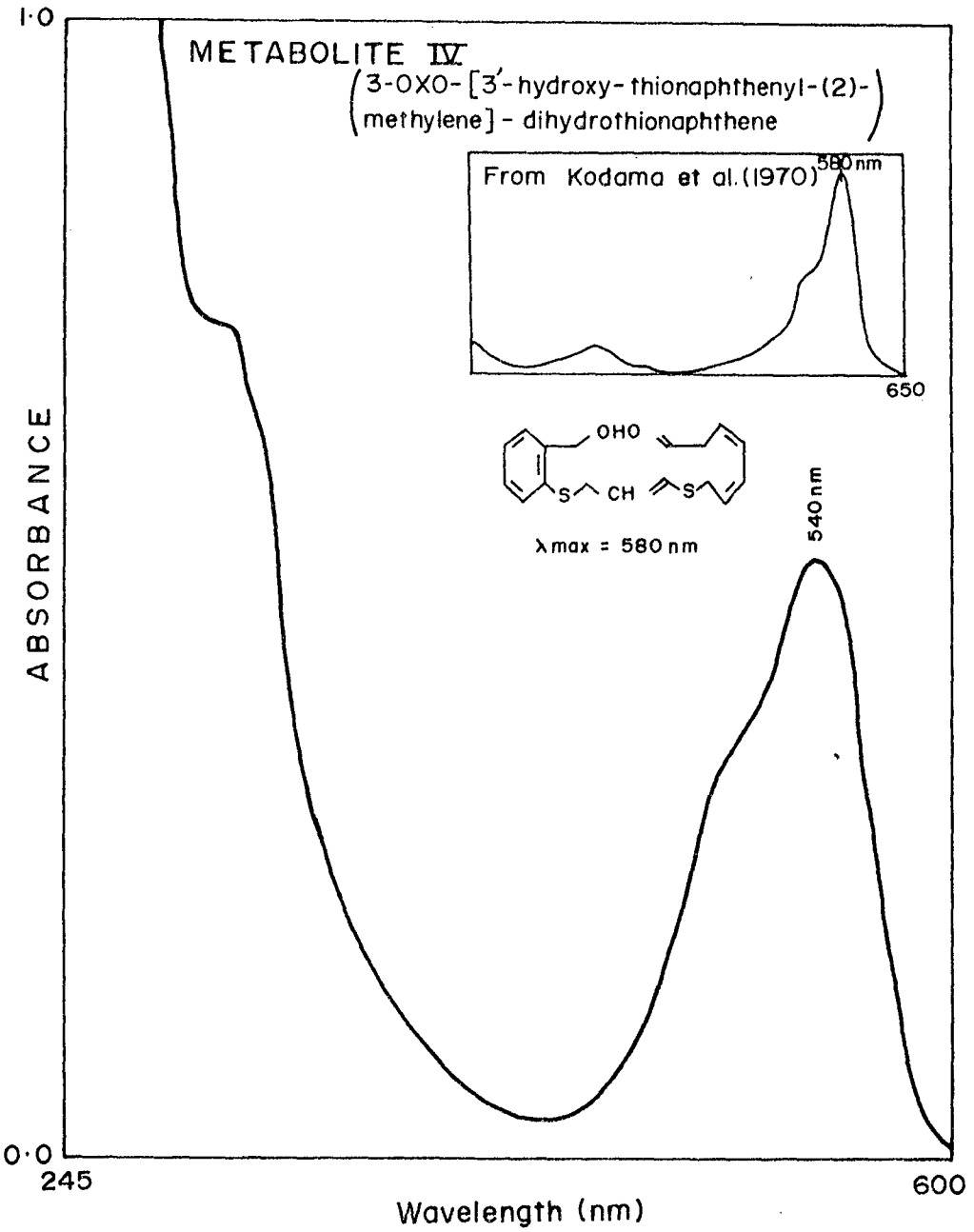
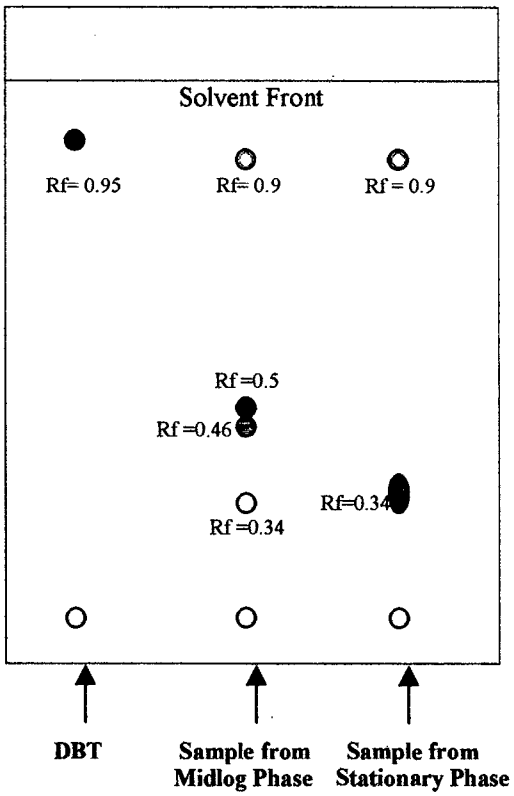


Fig.**3.14** UV-visible scan of **metabolite IV** (in chloroform) displayed a compound with a maximum absorbance (λ_{max}) at 540nm. 3-oxo-[3-hydroxy - thionaphthenyl-(2)-methylene]-dihydrothionaphthene revealed a similar λ_{max} as reported by Kodama *et al.*, (1970) [inset].

Fig. 3.15 Line drawing of a Thin layer chromatogram (TLC) depicting the separation of components from acidic extracts of cell free supernatants taken at different stages of growth of strain GU110 on DBT. Spots were detected under UV and exhibited different colors.



Solvent system : Chloroform – Methanol (100 : 5)		
Rf	Metabolite	λ_{max}
0.9	I	
0.5	IV	580nm
0.46	III	390nm
0.34	II	445nm

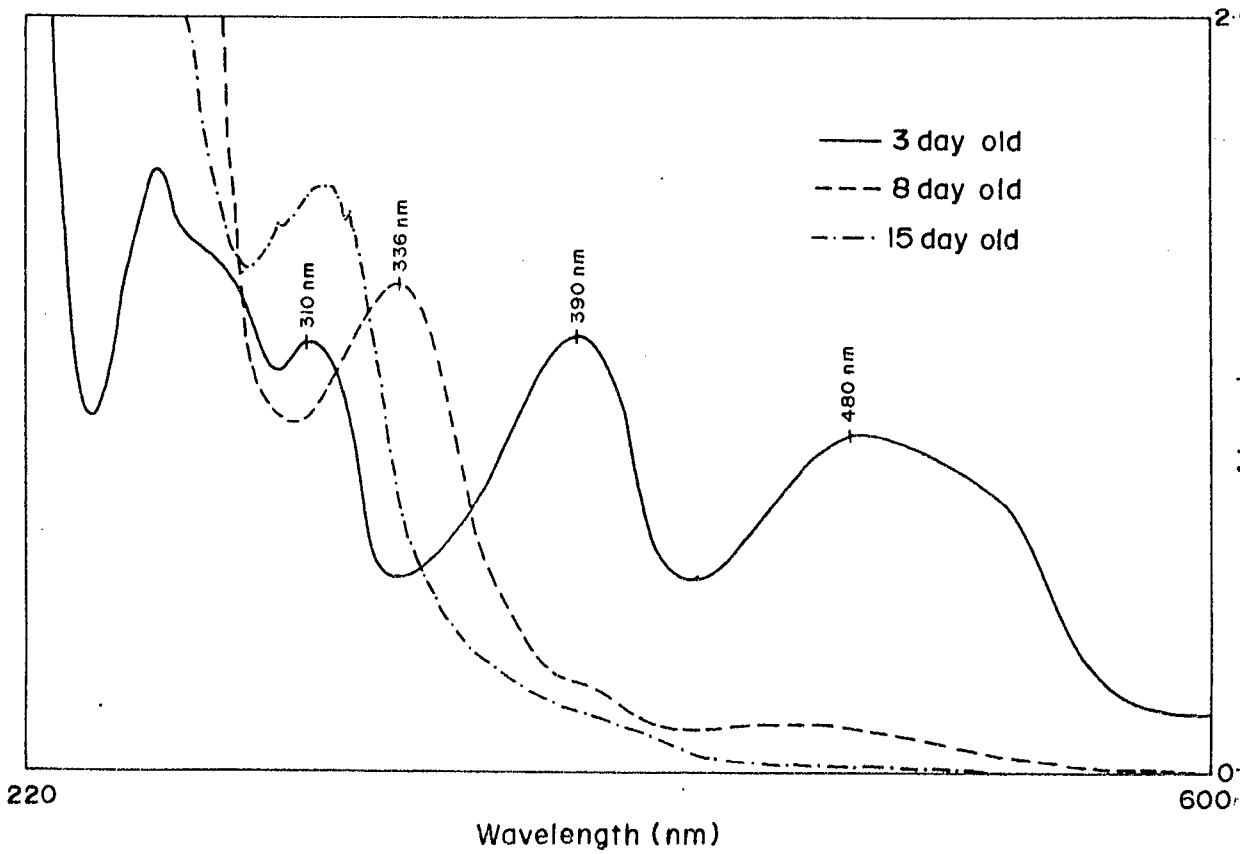


Fig.**3.16** UV-visible scans of cell free supernatants of strain GU110 cells grown on DBT (ASW medium), taken at different time intervals, revealed that metabolite III (A_{390nm}) and metabolite II (A_{485nm}) did not accumulate in the medium but were produced only transiently.

formation of a small peak at 336nm when compared to sterile control flasks (Fig. 3.17). These results confirm that HFBT does not accumulate in the medium but is only produced transiently.

Utilization of DBT sulfone as sole source of Carbon and Energy

DBT sulfone, which was synthesized in the laboratory, supported a very faint growth of culture GU110 when supplied to the media in the form of crystals (w/v). Increase in cell density of GU110 at the expense of this aromatic moiety could be confirmed only on the basis of an increase in the protein concentration. UV-visible scans of the cell free supernatants revealed no formation of new products or intermediates. Thin layer chromatography of supernatant extracts on silica gel G using a chloroform-methanol solvent system (100:5) revealed the presence of 2 faint spots having an Rf of 0.318 and 0.09 respectively and these fluoresced blue under ultraviolet light (Fig. 3.18). These results confirm the utilization of DBT sulfone by strain GU110.

DISCUSSION

DBT has been widely used as a model compound representing the organic sulfur component of fossil fuels. Numerous studies have been reported on transformation, degradation and desulfurization of DBT. Two major types of pathways for DBT metabolism are now recognized.

Kodama *et al.*, (1970, 1973) identified intermediates that led them to conclude that DBT is metabolized by a series of oxidation's analogous to those by which naphthalene is degraded. In this pathway, dioxygenation of one of the aromatic rings of DBT leads to the degradation of the ring without releasing sulfur, to yield the final product, 3-hydroxy-2-formyl-benzothiophene. Several bacteria that partially degrade DBT by this

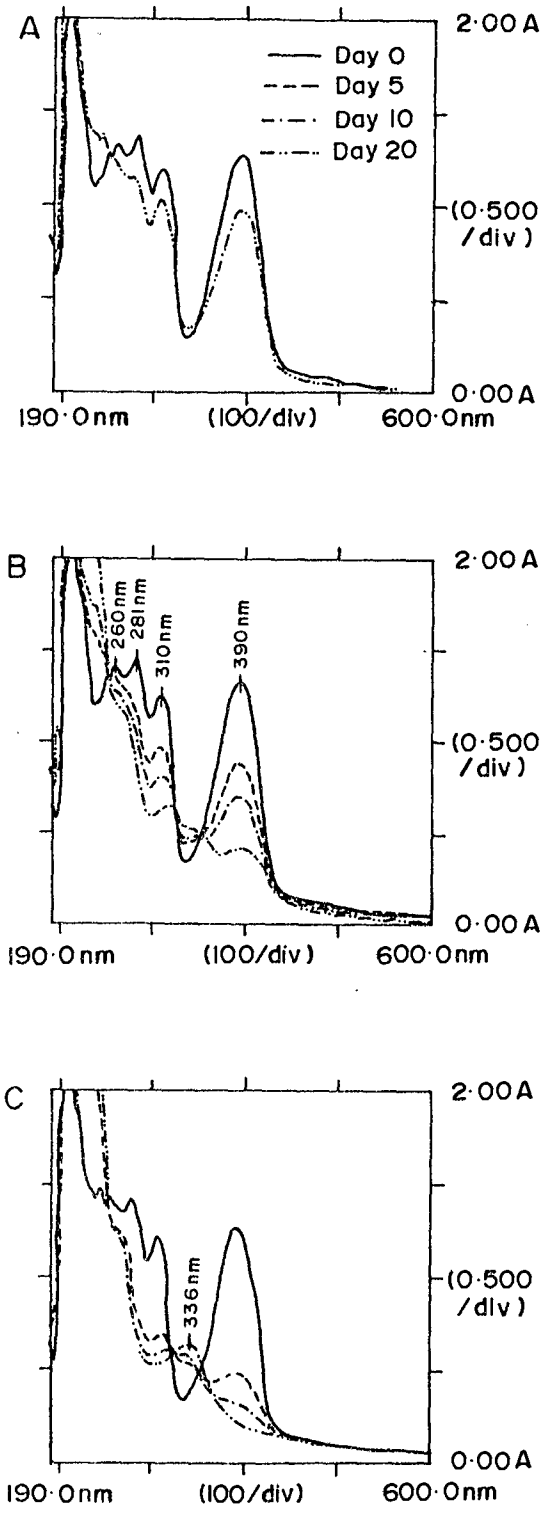


Fig.3.17 UV-visible scans reveal the transient nature of the product, HFBT or **metabolite III** having a λ_{max} at 390nm. Day-wise depletion of this product was studied.

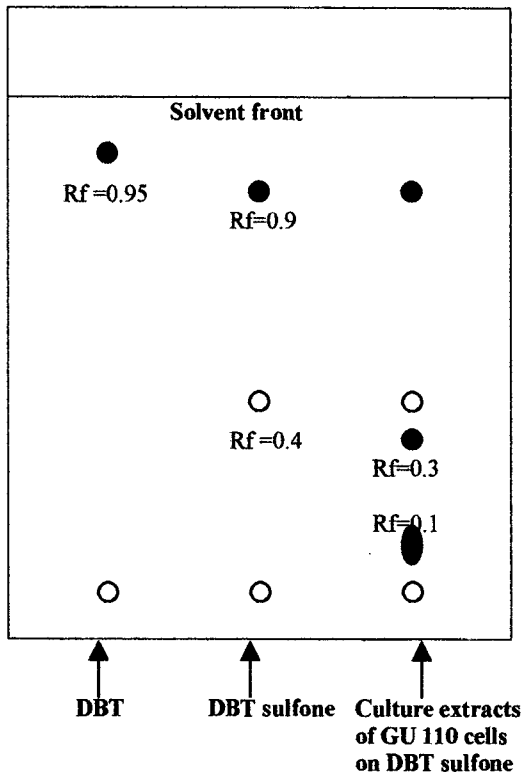
(**A**) Control : Cell free supernatant showing presence of HFBT.

(**B**) Test: Cell free supernatant with HFBT inoculated with benzoate enriched cells of strain GU110.

(**C**) Test: Cell free supernatant with HFBT inoculated with DBT enriched cells of strain GU110.

The supernatant in the un-inocualted control did not reveal any change in the UV visible spectra. Depletion of the peak at 390nm was seen in both the test samples with the corresponding formation of a new peak at 336nm.

Fig. 3.18 Line drawing of a Thin layer chromatogram (TLC) depicting differential migration of spots of authentic standard DBT and synthesized DBT sulfone. All spots were detected under UV and exhibited different colors.



(Solvent system: Chloroform – Methanol, 100:5)

Note : The presence of spots at an Rf of 0.3 and 0.1 respectively were obtained from culture extracts of GU110 cells grown on DBT sulfone.

route have been isolated. Cultures of *Pseudomonas* (Hou and Laskin, 1976; Kodama *et al.*, 1970; Kropp *et al.*, 1997a), *Acineobacter* (Malik, 1978) and *Beijerinckia* (Laborde and Gibson, 1977) have been reported to aerobically degrade DBT to 3-hydroxy-2-formyl benzothiophene.

The second pathway is one in which the sulfur is oxidized first and then removed from DBT with or without subsequent degradation of its two aromatic rings. Holland *et al.*, (1986) and Crawford & Gupta (1990) showed than *Cunninghamella elegans* oxidizes the sulfur moiety to DBT-5-oxide (DBT-sulfoxide) and DBT-5-dioxide (DBT-sulfone). No additional degradation products were observed. Van Afferden *et al.*, (1990) isolated a *Brevibacterium* sp., that oxidizes the sulfur in DBT but continues to degrade these intermediates to sulfite and benzoate, which itself is degraded.

A very specific version of this second pathway has been described by Kilbane *et al.*, (1989, 1990a) in the gram positive isolate *Rhodococcus* sp. strain IGTS8. In this case sulfur is extracted from DBT without further degradation of the aromatic carbon to yield 2-hydroxy biphenyl (2-HBP) as final product (Kilbane, 1990; Kilbane and Bielaga, 1990a; Kilbane *et al.*, 1989; Olson *et al.*, 1993).

Most of studies involving, DBT degradation by these two pathways deal with microbial cultures that cannot grow on DBT as sole source of carbon and energy and can only co-metabolize DBT in the presence of an alternate more easily utilizable carbon source. Saftic *et al.*, (1993) have reported a 1-methyl-naphthalene degrading *Pseudomonas* strain BT1 which can grow on DBT as its sole carbon and energy source. Grifoll *et al.*, (1995) have also isolated a fluorene degrading bacterial isolate *Pseudomonas cepacia* F297 that could also grow on and transform DBT.

Here, a marine bacterial strain, *Alcaligenes* sp. strain GU110 is reported which could utilize DBT as sole source of carbon, energy and even sulfur. Products formed in a sulfur free media were similar to those obtained in normal ASW with DBT, but their proportions were smaller, establishing the sulfate requirement of this isolate. Microgram quantities of sulfate were found to be released in the medium during growth but this release seemed quite erratic suggesting that since sulfate is absent in this media, these small quantities could support the sulfate requirement of the culture. The sulfate that is released could have arisen from the enzymatic oxidation and subsequent release of the sulfur, which is present in the thiophenic moiety of DBT but we could not confirm this observation. It could generally be concluded that DBT was degraded to compounds, which are very polar, even in such a sulfate-limiting medium.

Utilization of the S-heterocycle probably occurs by adherence of GU110 cells to this hydrophobic, water insoluble aromatic moiety. Phase-contrast micrographs of culture broths revealed the presence of clumps of bacterial cells on the fine needle like crystals of DBT. Since GU110 could grow on the crystal of DBT itself, we could conclude that this polyaromatic compound does not exhibit a toxic growth limiting effect on the GU110 strain. When increasing amounts of DBT was supplied to GU110, in ASW medium, the cell densities reached were similar at all concentrations of DBT above 0.01% (w/v) further supporting the observation that DBT is not inhibitory to growth of GU110. We therefore assumed that because of its insoluble nature, only fixed trace amounts of sparingly solubilized DBT could be scavenged by GU110, so that complete removal of DBT is achieved only at low concentrations of 0.01% (w/v) and below. Concentrations above these will not affect the utilization of DBT by GU110, but the excess of the un-utilized DBT will remain in the medium. The inherent nature of

GU110 cells to form clumps, and to attach to the DBT crystals, obviated our attempts to enumerate these cells by the plate count method. Therefore biomass was estimated as protein by the method of Lowry *et al.*, (1951).

Attack or uptake of DBT by GU110 cells is immediately followed by the formation of water-soluble polar, colored metabolites in the media. Metabolite accumulation is a common characteristic of PAH catabolism by bacteria (Abbot & Gledhill, 1971). The most common intermediates produced from PAH are the *ortho*-hydroxy compounds and carboxy compounds, which result from the cleavage of the terminal ring. Conversion of DBT to such water-soluble non-toxic metabolites in natural environments aids in their utilization by natural populations of bacteria since oxidized forms of DBT have been reported to be less toxic than the parent molecule itself.

Separation and purification of these polar products from cell free supernatants, revealed their similarity to those isolated and identified by Kodama *et al.*, (1970 and 1973) during the co-metabolism and utilization of DBT by *Pseudomonas jianii.*

Metabolite I, a fluorescent pink colored compound, was not reported to have been formed by the Kodama pathway but Mormille and Atlas (1989) have reported the formation of a red product in DBT grown cells of *Pseudomonas putida.* They could not identify this compound but presumed it to be a thermally unstable form of DBT possessing a charge sulfonium ion. A compound having an absorption maxima of 480nm similar to that of *trans* HTOB was present in cell free supernatants of GU110 grown on DBT. But when these supernatants were acidified with HCl, for extraction purposes, this peak was converted to a shoulder at 440nm. Metabolite II, which was purified by column chromatography, was bright yellow in color and had an absorption maxima at 445nm. This could probably be an isomeric form of HTOB, formed because

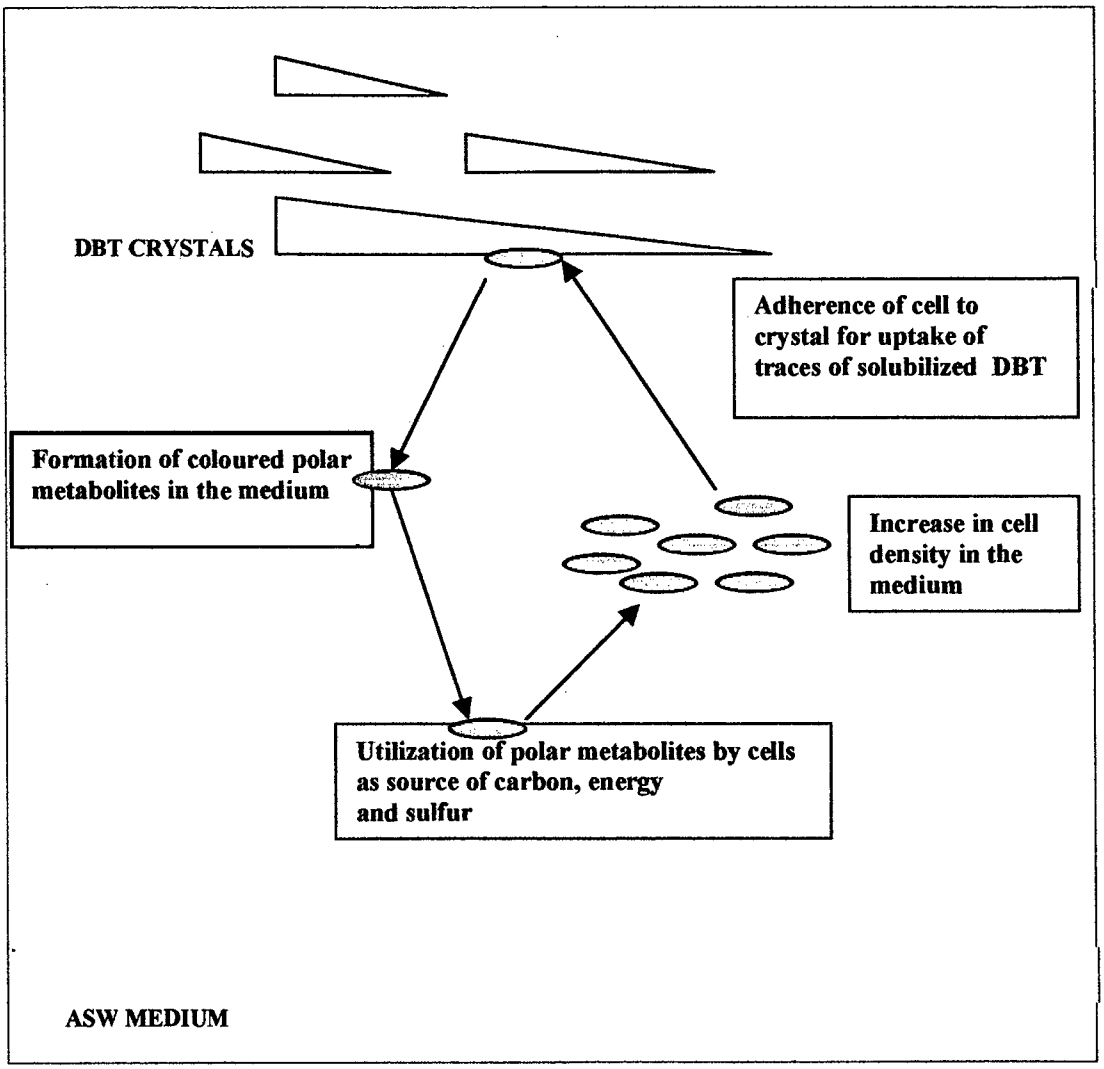
of the extraction procedures that were employed. HFBT (metabolite III) and the dimerization product of HFBT (metabolite IV) were also detected in GU110 cell free supernatants.

It can therefore be concluded, that the marine bacterial culture, *Alcaligenes* sp. strain GU110 attacks DBT by a pathway, analogous to that reported by Kodama *et al.*, (1970 and 1973) in the terrestrial microorganism, *Pseudomonas jianii*. But, DBT degradation by *P. jianii* lead to the accumulation of HFBT and DBT sulfone as dead end products. *Alcaligenes* sp. strain GU110 accumulated HFBT only transiently and could degrade it further to more polar metabolites. A similar phenomenon was reported in DBT oxidizing culture of Bohonos *et al.*, (1977) and Kropp *et al.*, (1997a) which transformed DBT via the HFBT by use of the Kodama pathway with further transformation presumably yielding benzothiophene-2,3-dione. The formation of such a product by strain GU110 could not be confirmed.

DBT-sulfone, which was also a reported dead end metabolite in the Kodama pathway, was not observed in cell free supernatants of GU110 grown on DBT. But the carbon from this aromatic moiety was assimilated into the cell as cell protein, when it was supplied to GU110 as a growth substrate, thereby suggesting the breakdown of the carbon ring structure. Mormille and Atlas (1989) demonstrated carbon dioxide evolution from DBT-5-oxide and DBT sulfone when mixed enrichment cultures were incubated with these substrates.

On the basis of all of the above observations we have proposed a model representation for the utilization of DBT by *Alcaligenes* sp. strain GU110 (Fig. 3.19). When confronted with the DBT molecule in a seawater medium, the GU110 cells attach to the DBT particles and scavenge traces of this sparingly soluble aromatic hydrocarbon. Such

Fig. 3.19 MODEL REPRESENTATION FOR UTILIZATION OF DBT BY *ALCALIGENES SP.* STRAIN GU 110.



an attack results in the formation of water-soluble and colored polar metabolites, which are released into the surrounding medium. Products released by this process serve as substrates for growth of cells. Utilization of these soluble forms by cells leads to their proliferation and thereby an increase in cell density, which will enable further attack and utilization of DBT by strain GU110 cells.

IN CONCLUSION

Alcaligenes sp. strain GU110 (MTCC 3317) uses DBT as source of carbon and energy and even sulfur and metabolizes this moiety by a pathway similar to that described by Kodama *et al.*, (1970 & 1973). But it differed from this pathway in 2 ways;

1) HFBT does not accumulate in cell free supernatants as the end product and is degraded to more polar compounds.

2) It also possessed the capacity to utilize DBT sulfone, a dead end product formed via an alternate pathway reported by Kodama *et al.*, (1973), as sole source of carbon and energy.

Such biodegradation studies that use pure cultures and pure compounds, are useful for the determination of metabolic pathways for the destruction of organic contaminants and are a step towards identification of metabolites whose possible formation in contaminated environments may influence the ability of bioremediation to reduce the toxicity of a contaminated site. The capacity of this *Alcaligenes* sp. to attack the toxic sulfur heterocycle, DBT in stringent nutrient deficient medium, to form non-toxic water-soluble polar metabolites indicates its potential use in bioremediation of oil contaminated marine environments. The pathway described for DBT degradation in this marine strain (GU110) is analogous to that described by Kodama *et al.*, in a terrestrial

strain. Whether this degradative pathway could have evolved independently in the marine environment or had been extended from terrestrial organisms is subject to speculation.

CHAPTER IV

UTILIZATION AND TRANSFORMATION OF INDIVIDUAL PAH COMPONENTS AND PAH MIXTURES BY STRAIN GU110.

Crude oil contains numerous aromatic hydrocarbons and heterocyclic compounds, which range in size from monocyclic to large fused ring structures. The larger polycyclic aromatic hydrocarbons (PAH's) and heterocycles are recalcitrant and their ultimate environmental fate may be determined by microbial degradation.

PAH with three, or more rings, heterocycles and alkyl-substituted homologues are environmentally important, owing to their inferred recalcitrance to microbial degradation and potential toxicity to higher life forms (Gundlach *et al.*, 1983; Vandermeulen, 1981). The ability of bacterial species to metabolize small aromatic hydrocarbons such as toluene, xylenes and naphthalene is widespread and has been reviewed by Gibson and Subramanian (1984). Fewer species have been described which degrade PAH such as phenanthrene and DBT. Those species capable of degrading mono-aromatics have not been reported to metabolize larger aromatics, or have not been tested rigorously for this capability.

Biological technologies are considered to have potential in the remediation of contaminated sites. Their successful application, however, demands a broader understanding of the biochemical pathways by which PAH's are degraded, both individually and in mixtures.

In the last chapter, the degradation of DBT by a marine bacteria, *Alcaligenes* sp. strain GU110 was investigated. A preliminary characterization revealed that this strain was able to utilize, as carbon source, a variety of aromatic moieties ranging from simple to polycyclic aromatic compounds. A study of the biochemical mechanisms involved could provide useful information about the biotransformation processes thereby leading to an understanding of how PAH mixtures are degraded.

In this chapter evidence is presented to show that the utilization of DBT by *Alcaligenes* sp. strain GU110 is based on a set of enzymes analogous to those used in naphthalene degradation.

The inducible or constitutive nature of the DBT degrading enzymes in this marine organism was also determined. The actions of this strain on a variety of aromatic compounds, individually and in mixtures are described, accounting for the potential ability of this organism to remove PAH's with 1, 2 or 3 aromatic rings when incubated with crude oil.

MATERIALS AND METHODS

Chemicals

The aromatic compounds used included, DBT (E Merck), DBT sulfone (synthesized), naphthalene and sodium benzoate from Hi Media Ltd., phenanthrene, naphthoresorcinol, 1-hydroxy-2-naphthoic acid and protocatechuate from Sigma Chemical Co. Glucose, sodium lactate, chloramphenicol, agarose, Tris buffer, EDTA, ethidium bromide were from Hi Media Ltd. All other chemicals used were of analytical grade. All organic solvents were of HPLC grade (E Merck).

PAH mixtures were formulated as follows;

Composition of tertiary PAH mixture

Nature of PAH	Type of aromatic compounds	Concentration used in medium (w/v)
Di-aromatic	Naphthalene	0.1%
Tri-aromatic	Phenanthrene	0.1%
Heterocyclic	Dibenzothiophene	0.1%

Composition of binary PAH mixture

Nature of PAH	Type of aromatic compounds	Concentration used in medium (w/v)
Tri-aromatic	Phenanthrene	0.1%
Heterocyclic	Dibenzothiophene	0.1%

For oxygraph studies, a 2mg/ml stock of all the solid water insoluble aromatics was made in methanol. Filter sterilized sodium benzoate was used from a 1 M stock. Sodium lactate and glucose were used from 20% autoclaved stock solutions.

Media used and supply of growth substrates

ASW medium with pH 6.8 (described earlier) was used for all the growth studies. The aromatic compounds, DBT, phenanthrene and naphthalene were supplied to the ASW medium (50mls) individually or as mixtures either as crystals (w/v), or from a 10% stock in dimethyl formamide (DMF), as sole source of carbon and energy. Benzoate, protocatechuate, lactate and glucose were also used as growth substrates. Actively growing cultures of *Alcaligenes* sp. strain GU110 (4% v/v) from maintenance flasks were inoculated into these Erlenmeyer flasks and incubated at room temperature on the shaker at 100 rpm.

Measurement of Growth

Growth and utilization of aromatic compounds was observed as increase in the optical density at 560nm on a Milton Roy spectrophotometer (Spectronic 1201) and also by observing for visible changes in the color of the medium due to the formation of polar metabolites and by recording the changes in the UV-visible spectral characteristics of cell free culture supernatants.

Flasks of *Alcaligenes sp.* strain GU110 cultures growing on 0.01% DBT (v/v) were supplied with phenanthrene (0.01% w/v) crystals and naphthalene (0.01%w/v) crystals at mid-exponential phase. The effect of these aromatic compounds on the growth of strain GU110 on DBT was determined by comparison with the growth of strain GU110 on DBT alone.

Gas Chromatographic (GC) analyses

Cell free culture supernatants of strain GU110 cells growing on the individual hydrocarbon substrates were acidified and extracted with three volumes of ethyl acetate. The organic layer was then dried over anhydrous sodium sulfate and the solvent was removed by evaporation in a water bath at 60-80°C. The residue was dissolved in ethyl acetate or chloroform and 0.5µl was injected into a GC (Shimadzu GC-14B) equipped with a capillary column and flame ionization detector. Utilization of aromatic substrates was ascertained by removal or depletion of substrate peak and corresponding formation of metabolites at different retention times.

Preparation of washed cell suspensions

Alcaligenes sp. strain GU110 cultures from maintenance flasks were inoculated into Erlenmeyer flasks containing 50 mls of ASW medium and required substrate as sole source of carbon and energy. These GU110 cells were harvested at late exponential phase by centrifuging at 8000 rpm at 4°C in a Sorvall RC5C. The cell pellets were washed twice with sterile ASW to remove any traces of the growth substrate and then resuspended in 2mls of ASW and stored at 4°C in fridge. These washed cell suspensions were utilized within a period of one week.

Measurement of oxygen uptake rates of washed GU110 cells

The oxidation of DBT and other compounds was measured by an initial rate assay with resting cells of *Alcaligenes* sp. strain GU110 grown on a host of compounds like; sodium lactate and glucose, simple aromatics, like sodium benzoate and protocatechuate as well as PAH's like naphthalene, phenanthrene and DBT. A respirometer cell (Gilson 5/6 Oxygraph) equipped with a Clarke-type oxygen electrode and maintained at constant

room temperature (28°C) was used to measure oxygen uptake. Fixed volumes of washed cells were introduced into the respirometer cell which contained 50mM aerated phosphate buffer (1.6ml) and an initial endogenous uptake rate was measured. 10-20µl of stock solutions containing the substrate of interest was injected into the respirometric cell and the oxygen uptake rate was measured again. Oxygen consumption in the presence of substrate was corrected for endogenous respiration. Results were expressed as nanomoles of oxygen consumed per minute per mg dry weight of cells. The dry weight of the cells was determined by weighing the dried cell pellets of a known optical density (560nm) in pre-weighed vials on a Mettler AE 240 balance.

Detection of plasmid DNA

The *Alcaligenes* sp. strain GU110 was subcultured repeatedly on Zobell's Marine Agar, ASW with DBT and with naphthalene respectively as sole carbon source and screened for the presence of plasmid by the alkaline lysis method of Sambrook *et al.*, (1989) and also by 2% SDS lysis in a 10mM Tris EDTA (TE) buffer of pH 8.0, using a 0.8% agarose gel. The gel was stained with ethidum bromide and visualized under UV for plasmid bands, after using an appropriate marker.

RESULTS

Alcaligenes sp. strain GU110 utilizes an array of compounds besides DBT as the sole source of carbon. Amongst these are a set of polycyclic aromatic compounds that are normally present as components of crude oil.

Utilization of individual polycyclic aromatic hydrocarbons (PAH's)

GU110 utilizes both phenanthrene and naphthalene as sole source of carbon and energy.

The biomass obtained on DBT is higher than that obtained on the other two aromatic molecules, when used at the same concentrations. At high concentrations (1% w/v) phenanthrene, like DBT was not toxic to the culture and did not inhibit the growth of *Alcaligenes* sp. strain GU110 although naphthalene did display its toxicity at such high concentrations by inhibiting the growth of GU110 (Fig. 4.1).

When grown on phenanthrene increase in biomass was accompanied by an increase in the formation of water-soluble colored metabolites into the medium. These were pale yellow or brownish in color. When grown on naphthalene at concentrations above 0.05%(w/v) a bright orange compound accumulates in the medium with no corresponding increase in the biomass. UV-visible spectral analysis of these cell free supernatants reveal a peak at 437nm. When a reducing agent like sodium dithionite was added to this colored medium, it became colorless and regained its color on re-oxidation in air. This suggests the accumulation of a quinone type of compound which gets reduced on addition of reducing agent. At low concentrations of naphthalene this compound is produced only transiently. GC analysis of naphthalene and phenanthrene grown GU110 cell free supernatants revealed depletion of these substrates from the medium with the concomitant formation of peaks representative of the breakdown products, at retention times which were similar in both naphthalene and phenanthrene grown cells (Fig. 4.2).

Strain GU110 was also capable of growing on and utilizing naphthalene breakdown products such as naphthoresorcinol, salicylaldehyde and catechol as well as the phenanthrene pathway intermediate which is 1-hydroxy-2-naphthoic acid (1H2NA) (Table 4.1). Utilization of these substrates was determined by an increase in GU110 cell

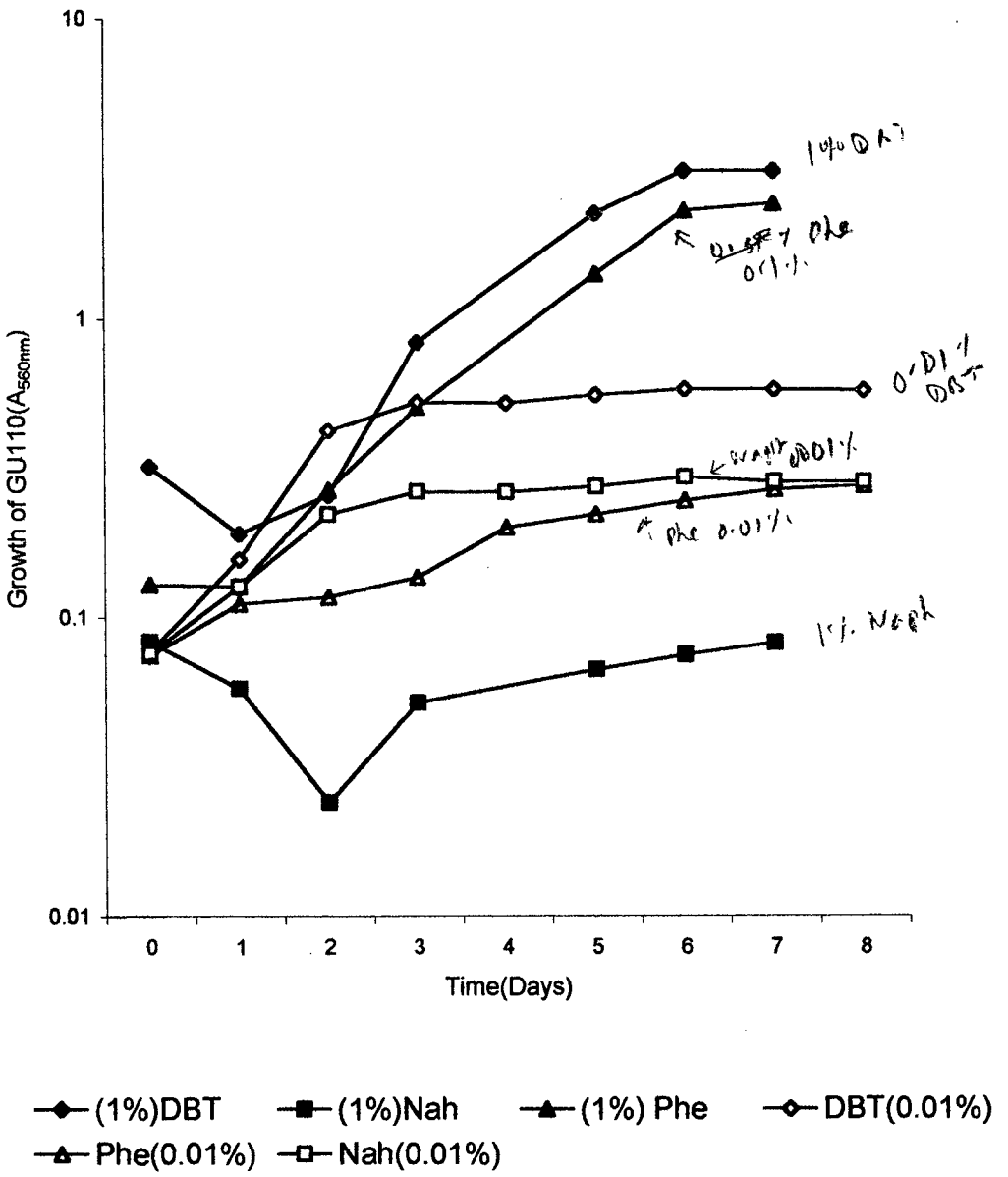


Fig.4.1 Comparison of Growth of strain GU110 on PAH's at different concentrations.

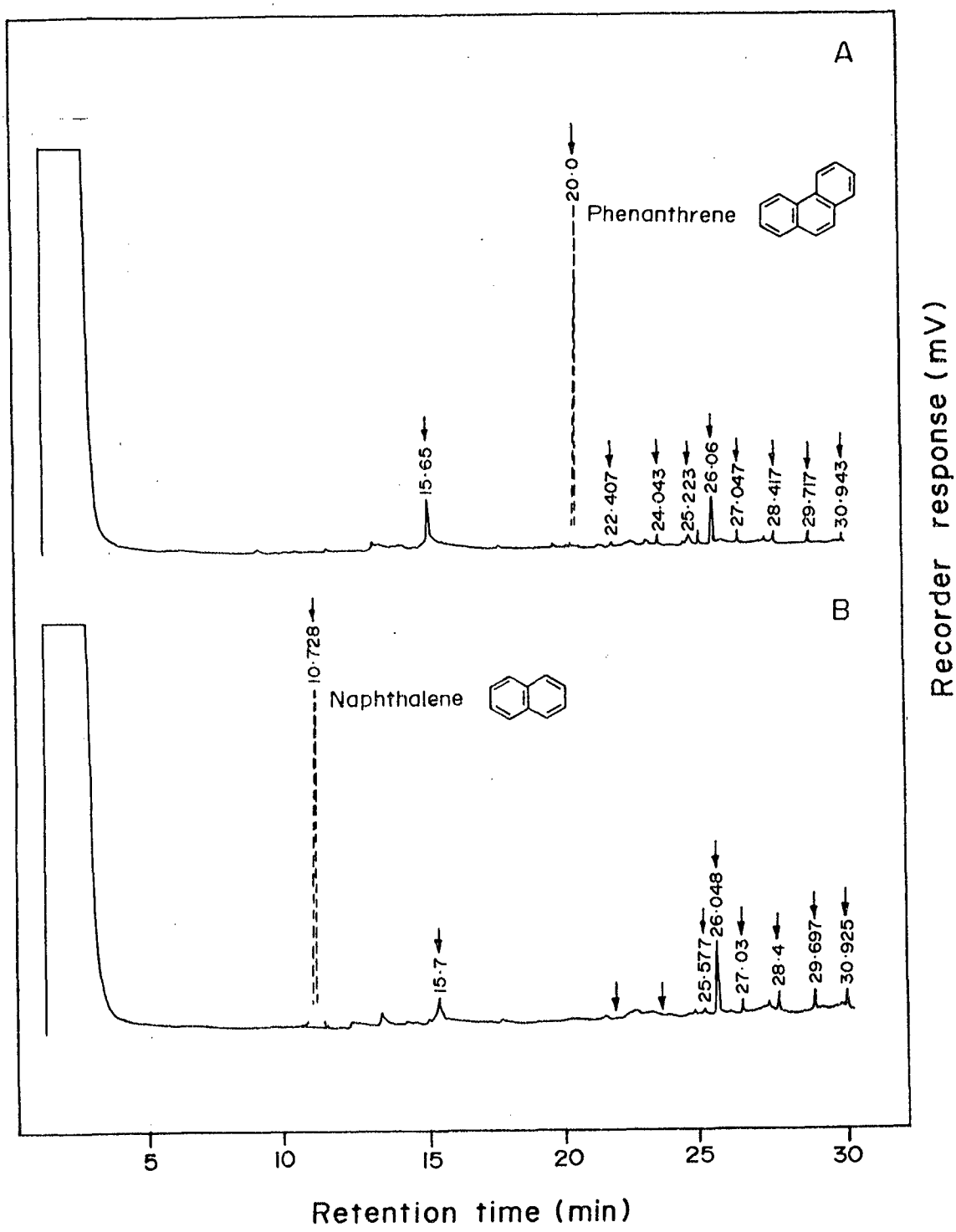


Fig.**4.2** GC profiles of cell free acidic extracts after growth of strain GU110 cells on (**A**) phenanthrene and (**B**) naphthalene .The phenanthrene (retention time = 20.0) and naphthalene (retention time =10.72) peaks were absent after growth and are represented as dotted lines. Arrows depict the presence of metaboiltes at similar retention times in both (**A**) & (**B**).

density at 560nm and by the depletion of these substrate peaks when acidic extracts of these cell free supernatants were subjected to GC analyses (Fig. 4.3).

Effect of phenanthrene and naphthalene on utilization of DBT by GU110

The toxic effect of the aromatic moieties, phenanthrene (a tri-cyclic aromatic) and naphthalene (a di-cyclic aromatic) on the growth of *Alcaligenes* sp. strain GU110 on DBT was determined. Strain GU110 was grown on DBT to mid log phase which is achieved within a period of 2 days after inoculation. At this stage, weighed amounts of aromatic compounds, either phenanthrene (0.01% w/v) or naphthalene (0.01% w/v) were added to these flasks. Addition of the second substrate did not result in a decrease in the optical density at 560nm of GU110 cells growing on DBT when compared to control flask, where-in no second substrate was added (Fig. 4.4).

Utilization of PAH mixtures

Aromatic mixtures were formulated as follows: (a) Naphthalene (0.1% w/v): Phenanthrene (0.1% w/v): DBT (0.1% w/v) and (b) Phenanthrene (0.1% w/v): DBT (0.1% w/v) in 50mls of ASW.

The utilization of these mixtures by GU110 was confirmed by the increase in the optical density at 560nm when compared to growth on DBT alone at the same concentration (Fig. 4.5A). Utilization of the aromatic mixtures was further confirmed by UV-visible spectral analysis of GU110 cell free supernatants grown on these mixtures (Fig. 4.5B).

Kinetics of DBT conversion by naphthalene grown washed cells of GU110

Cultures of *Alcaligenes* sp. strain GU110 were maintained in flasks containing 50mls of ASW medium with naphthalene (0.01% w/v) crystals as sole source of carbon. 2ml volumes of this culture was transferred to fresh medium every 5 days, so that DBT and

TABLE NO. 4.1: Growth of strain GU110 on pathway intermediates.

Growth of strain GU110 on Phenanthrene Pathway Intermediates and Naphthalene Pathway Intermediates	
Intermediate	Growth(OD 560nm)
1-Hydroxy-2-naphthoic acid Salicylaldehyde Catechol	+ + +
Growth of strain GU110 on Phenanthrene Pathway Intermediates	
Intermediates	Growth
2-Carboxybenzaldehyde o-Phthalic acid Protocatechuic acid	- - +

TABLE NO. 4.2 : Oxygen uptake rates of strain GU110 cells grown on PAH's.

	Growth substrates(Oxygen Uptake in nanomoles/min/mg Dry Weight)		
Substrates Oxidized	DBT	Phenanthrene	Naphthalene
Benzoate	15.46	14.99	42.58
DBT	38	22.9	42.55
Phenanthrene	30	59.85	33.40
Naphthalene	36	34.52	63.78
DBT-Sulfone	32.72	36.6	50.55
1-Hydroxy-2-naphthoic acid	22.90	54.94	56.77
Naphthoresorcinol	19.5	39.2	16.949

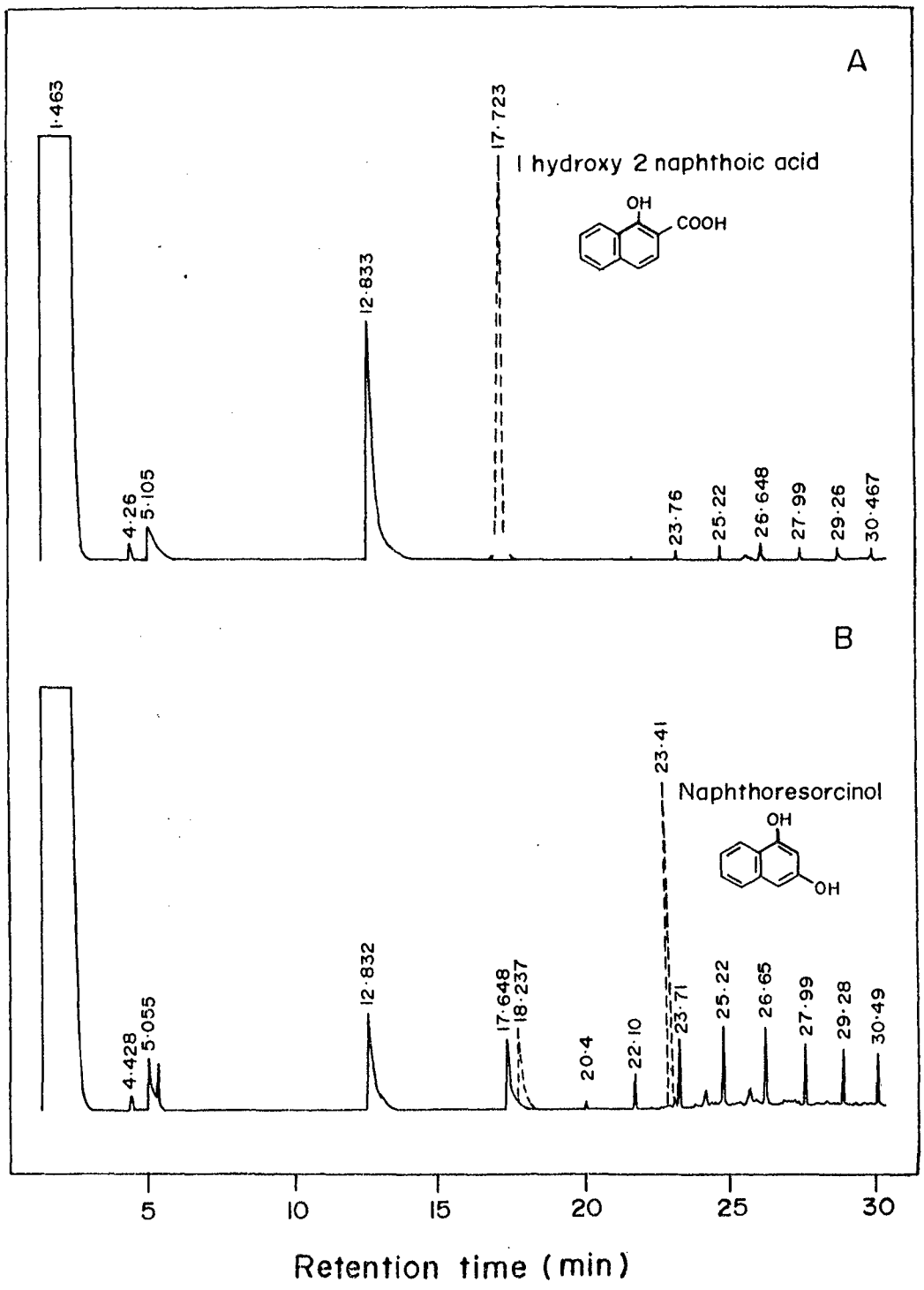


Fig.**4.3** GC profiles of cell free acidic extracts after growth of strain GU110 on (**A**)1-hydroxy-2-naphthoic acid (retention time=17.72), a phenanthrene pathway intermediate and (**B**) naphthoresorcinol (retention time=23.41) a naphthalene breakdown product. Both these substrate peaks were absent after growth of GU110 and are therefore represented as dotted lines. The additional peak at 18.237 minutes in (**B**) was present in the standard naphthoresorcinol sample.

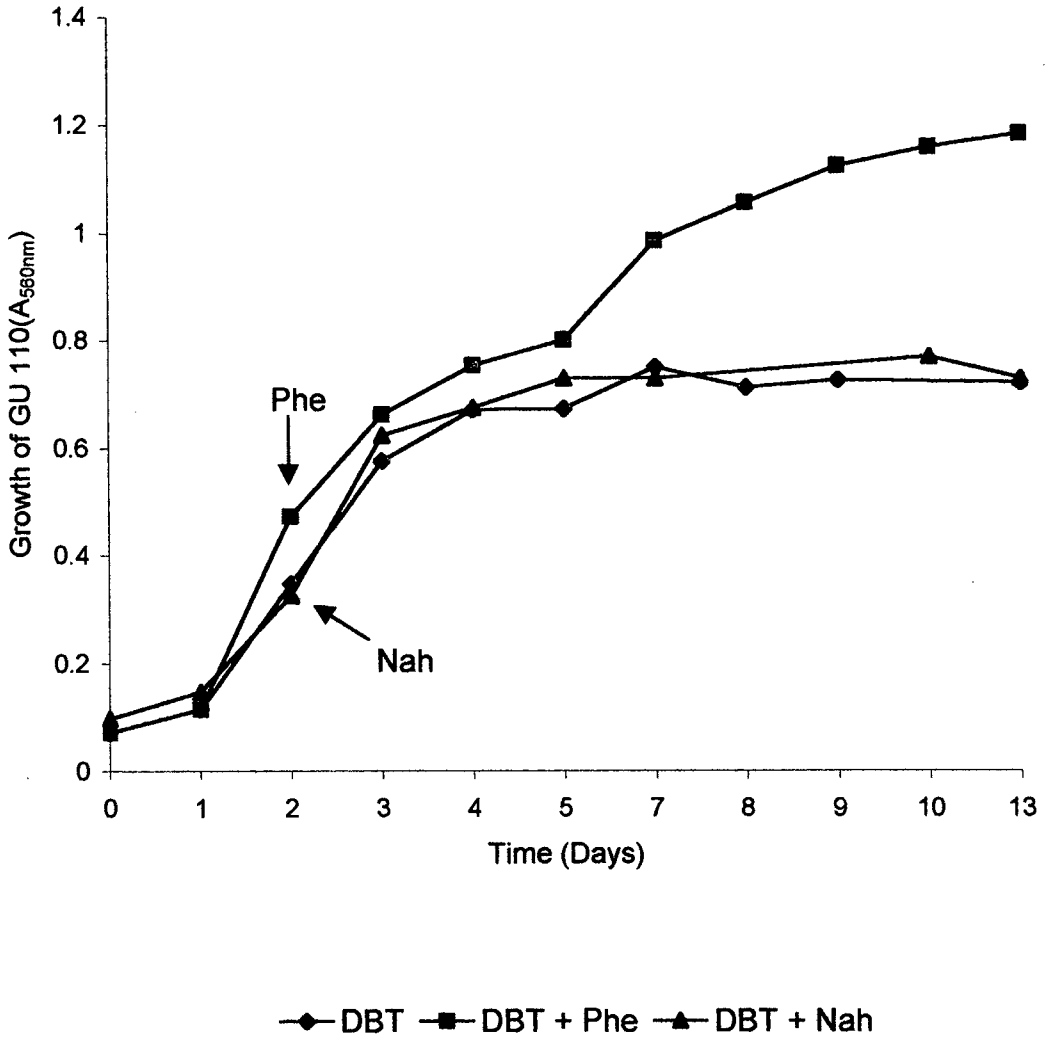


Fig.4.4 Effect of phenanthrene and naphthalene on growth of strain GU110 on DBT. Arrows depict the addition of Phe and Nah at mid log phase of growth of GU110 on DBT (0.1%w/v).

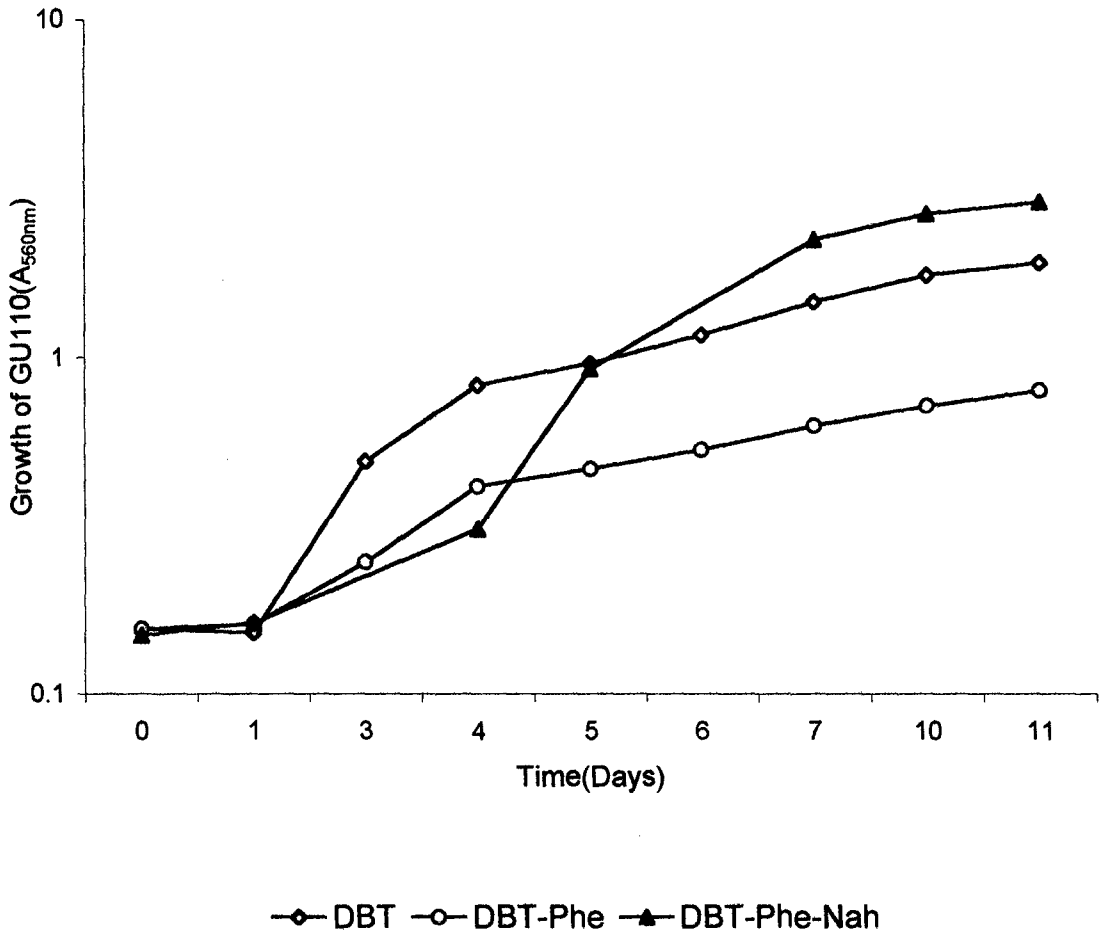


Fig 4.5A Comparison of growth of strain GU110 on tertiary PAH mixture (DBT:phenanthrene:naphthalene), on binary mixture (DBT:phenanthrene) and on single hydrocarbon component, DBT.

Conc → Present?

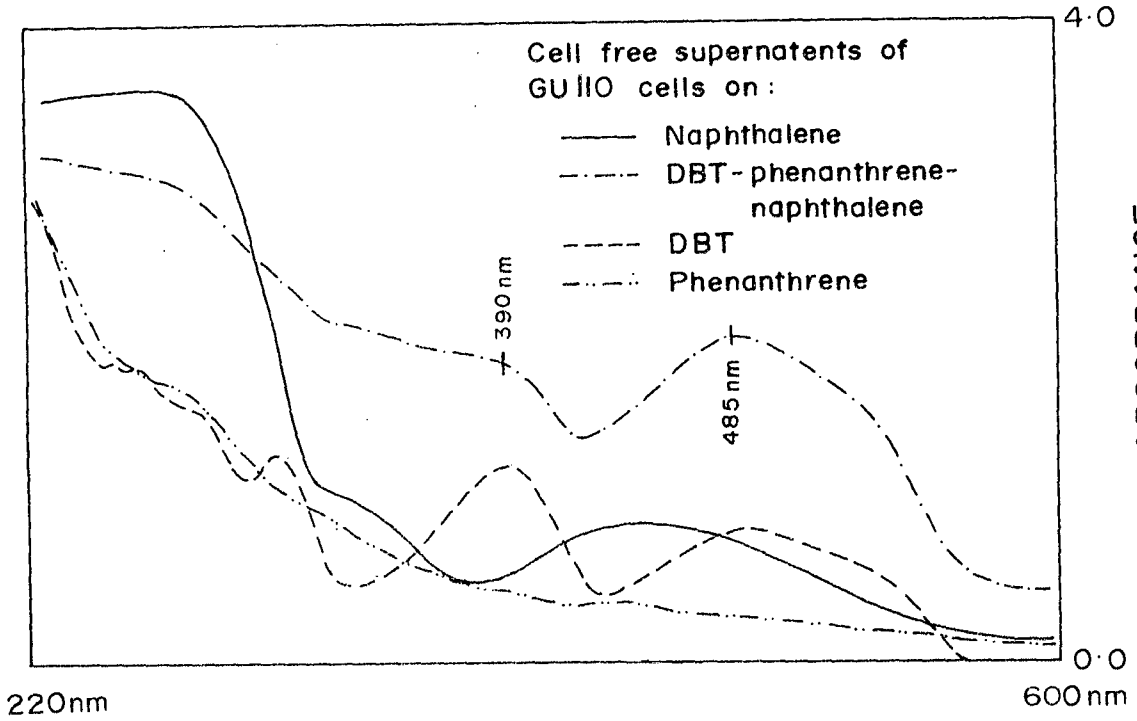


Fig. **4.5B** UV-visible scans of cell free supernatants of strain GU110 grown on PAH mixture and on individual PAH's → Cont'd.

its oxidation products, were diluted to negligible background levels. At the time of the third transfer naphthalene grown cells were harvested at the late exponential phase by centrifuging and the washed cell pellet so obtained was dispensed in ASW stored at 4°C for further use.

(i) Effect of increasing cell density on DBT oxidation by naphthalene grown washed cells: Naphthalene grown resting cell suspensions were diluted in ASW to an optical density of approximately 0.2, 0.6 and 1.0 OD units at 560nm which correspond to a dry weight of 0.04 mg/ml, 0.12 mg/ml and 0.2 mg/ml respectively. 2ml of volumes of these suspensions was introduced into a 3ml quartz cuvette in a UV-visible spectrophotometer (Milton Roy 1201 Spectronic). Baseline was set to zero and DBT was then added (from a 10% stock in DMF) to all these samples to a final concentration of 0.005%v/v or 270 μM. Cuvette contents were mixed regularly. Oxidation of DBT to water-soluble colored metabolites was visually observed in the cuvette, with the corresponding increase in the optical densities at 390nm and 485nm which are the characteristic absorbancies of HFBT and *trans* HTOB respectively (Fig. 4.6). Fig. 4.7A & Fig. 4.7B shows the increase in the formation of these two metabolites over a period of two hours by increasing cell densities of strain GU110.

Because molar extinction coefficients of the DBT metabolites are not known, rates could not be expressed as moles of product formed per unit time but instead were calculated as increase in absorbance units per minute. The linear increase in the absorbance per minute at 485nm and 390nm was used to calculate the rates of formation of *trans* HTOB and HFBT respectively (Fig. 4.8). The rate of product formation and thereby DBT conversion was in direct proportion to the existing cell density.

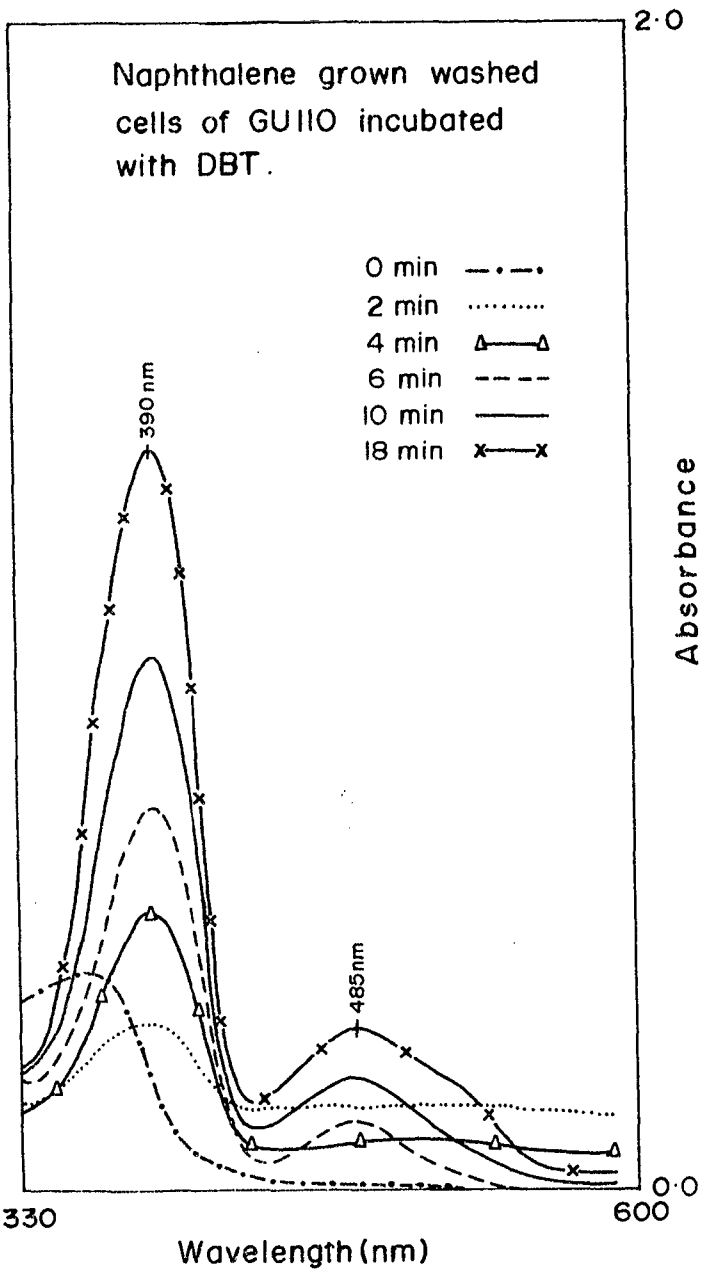


Fig.4.6 UV-visible scans showing time bound formation of metabolite II (A_{485nm}) and metabolite III (A_{390nm}) by naphthalene grown washed cells of strain GU110 incubated with DBT.

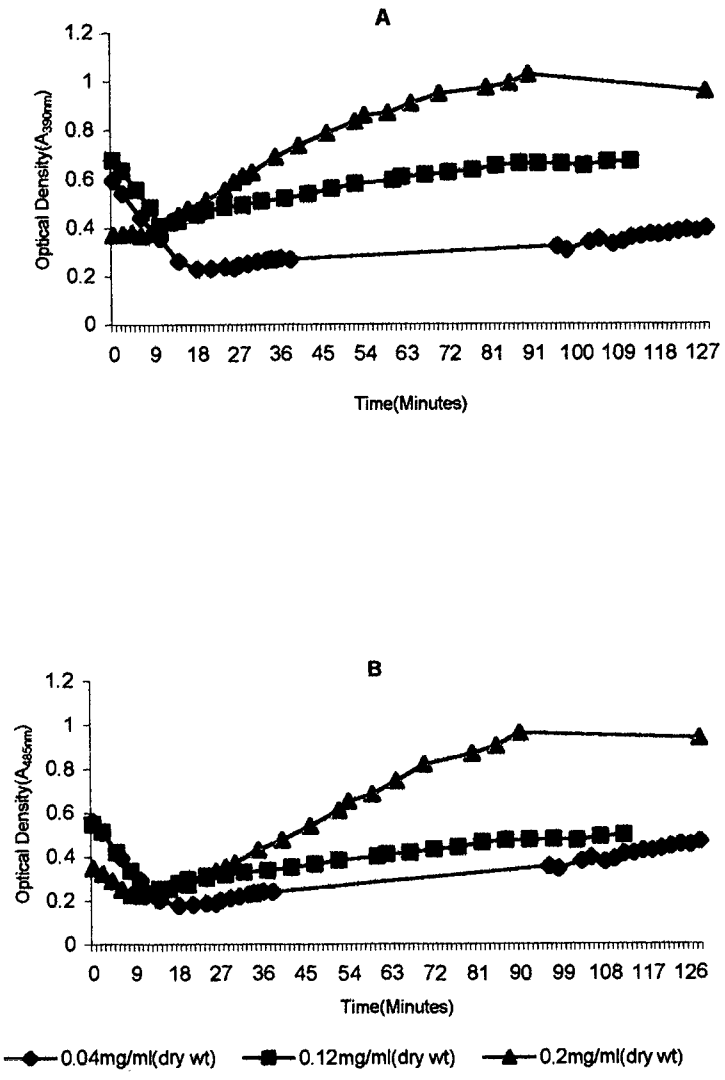


Fig.4.7 Formation of HFBT(A) & trans HTOB(B) by increasing cell densities of Naphthalene grown washed cells of strain GU110 incubated with fixed concentration of DBT(0.005%).

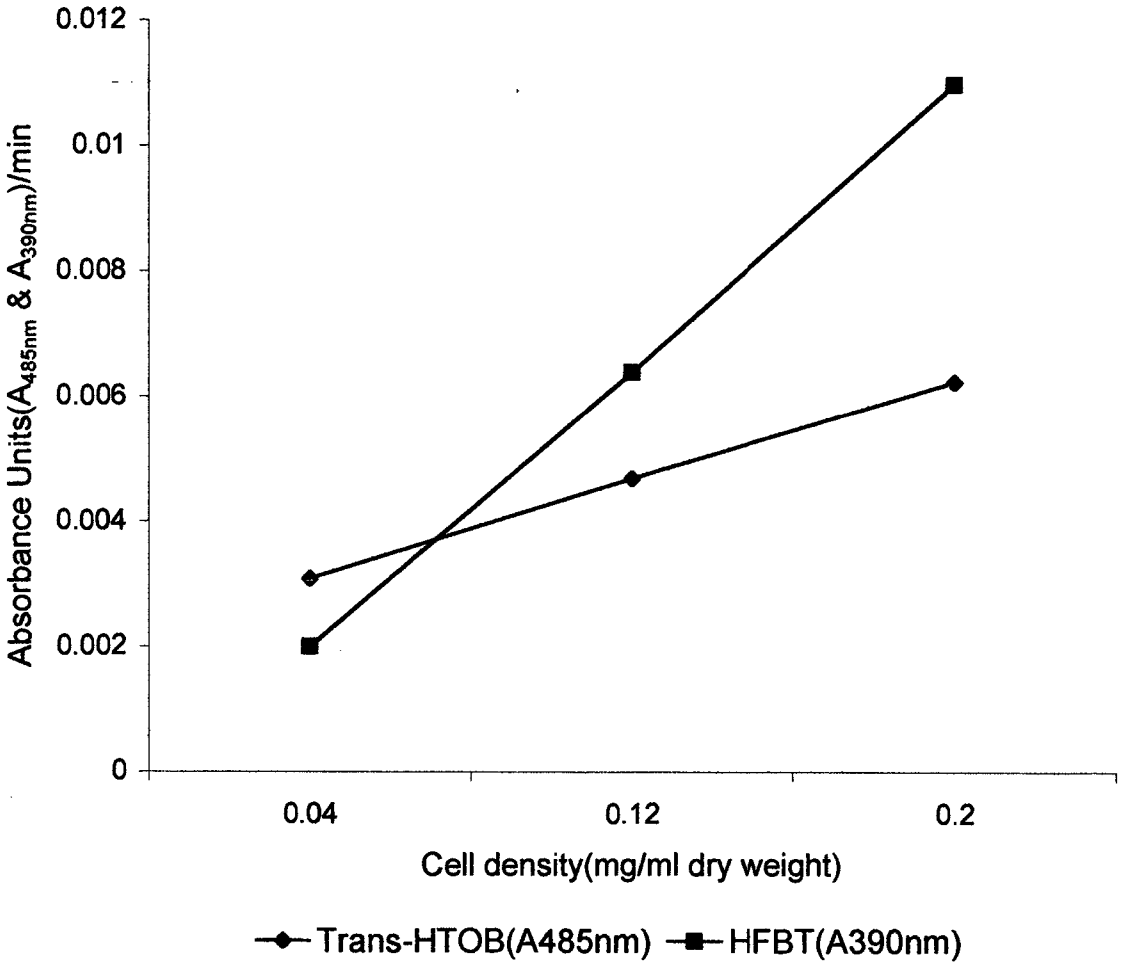


Fig.4.8 Rates of formation of metabolite II (A_{485nm}) and metabolite III (A_{390nm}) by increasing cell densities of naphthalene grown washed cells of strain GU110 incubated with DBT.

(ii) Effect of increasing DBT concentrations on oxidation of DBT by naphthalene grown washed cells: Naphthalene grown, washed resting cell suspensions were made to an optical density of 1.0 at 560nm and 2ml volumes of this suspension was placed in a 3ml quartz cuvette in a Milton Roy spectrophotometer. The baseline was set to zero and DBT from a 10% stock in DMF was added to the cuvette contents to a final concentration of 0.0005% (27μM), 0.001% (54μM), 0.002% (108μM), 0.005% (271μM), 0.01% (540μM) & 0.02% (1080μM) respectively. Addition of increasing concentrations of DBT to these washed cell suspensions resulted in an increase in the formation of HFBT and *trans* HTOB over a period of 2 hours but at lower concentrations of 0.0005% (27μM) and 0.001% (54μM) DBT, a depletion of these two products was observed after 30 minutes of incubation of washed GU110 cells with DBT (Fig. 4.9A & Fig. 4.9B). As the concentration of DBT is increased, the rates of conversion of DBT to HFBT decreased and then remain constant but the rates of formation of HFBT were higher than those of *trans* HTOB (Fig. 4. 10). Conversion of DBT to water-soluble compounds by GU110 is therefore a function of the DBT concentration and the rate of transformation is not inhibited by increasing amounts of DBT.

Kinetics of oxidation of aromatic substrates of Alcaligenes sp. strain GU110

Alcaligenes sp. strain GU110 was grown on a variety of compounds which included sodium lactate and glucose, simple aromatics like sodium benzoate and protocatechuate and polycyclic aromatics like naphthalene, phenanthrene and DBT. Cells from these flasks were harvested at log phase, pelleted and suspended in ASW to obtain an OD at 560nm of 5.0 units. Oxygen uptake of these cells due to oxidation of an array of

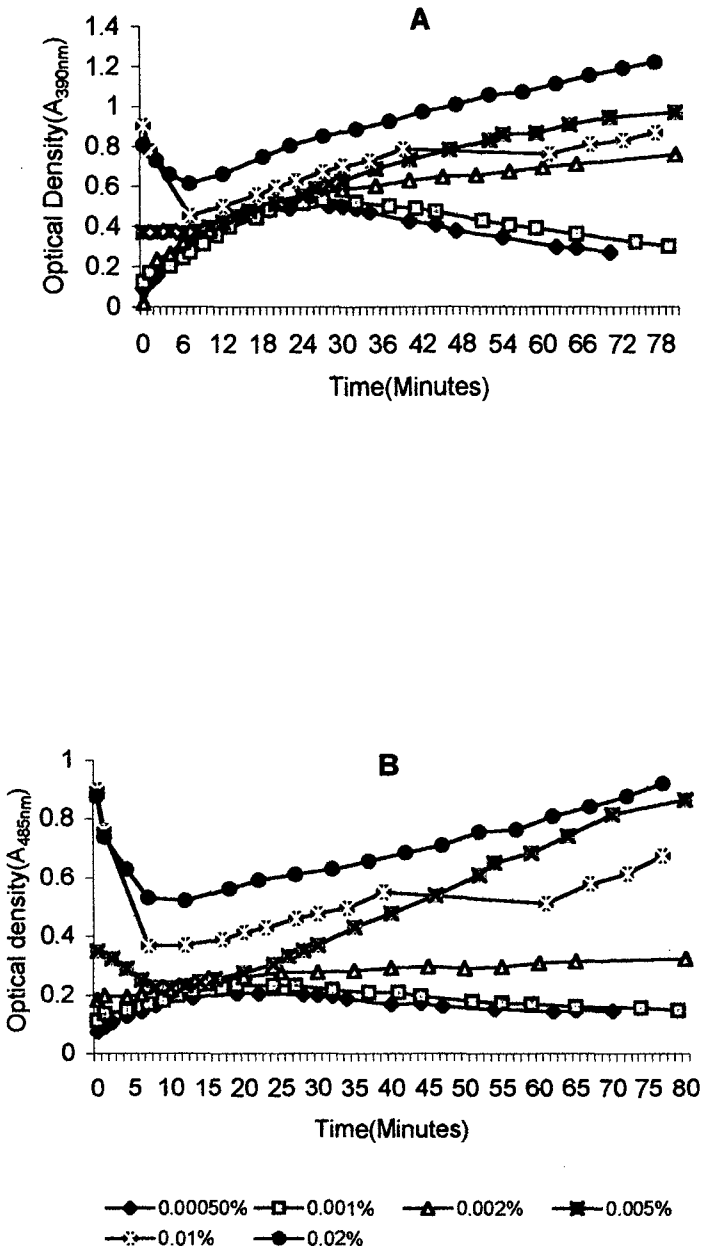


Fig.4.9 Formation of HFBT(A) and *trans* HTOB(B) by naphthalene grown washed cells of strain GU110 incubated with increasing concentrations of DBT.

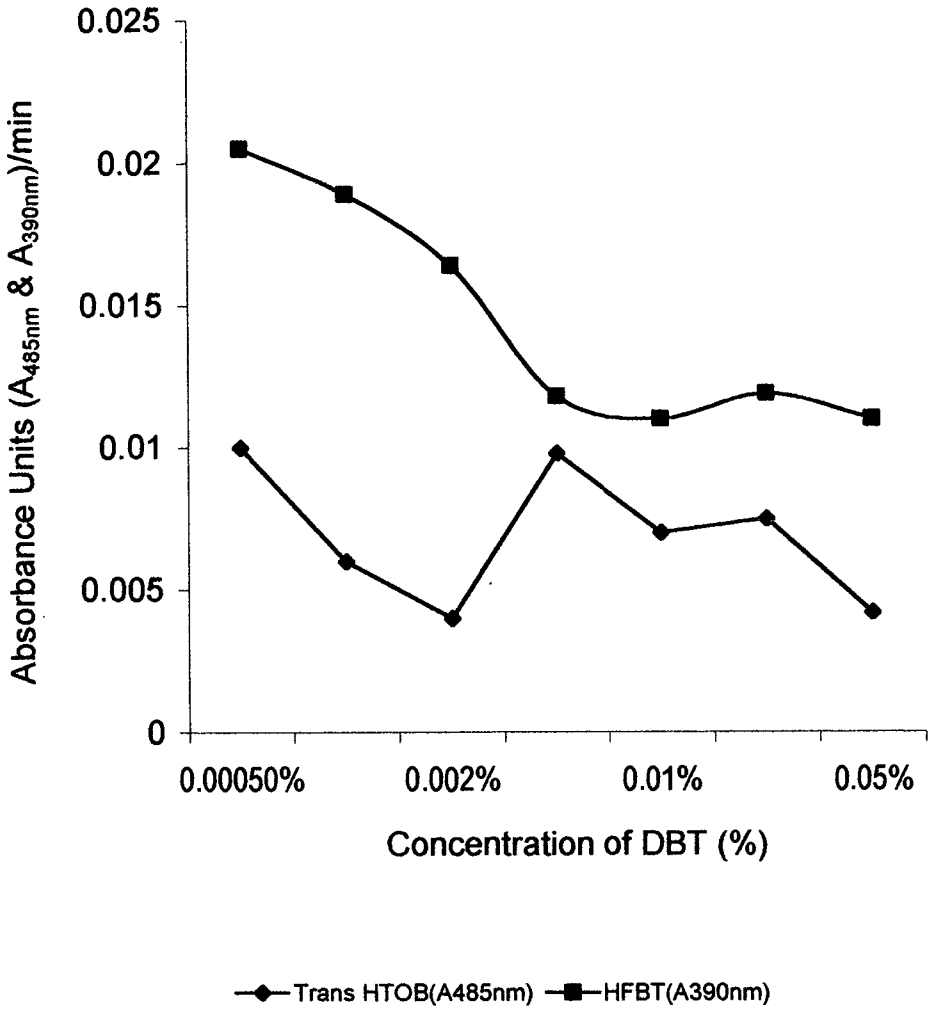


Fig.4.10 Rates of formation of metabolite II (A_{485nm}) and metabolite III (A_{390nm}) by naphthalene grown washed cells of strain GU110 (0.2mg/ml dry weight) incubated with increasing concentrations of DBT.

aromatic compounds was determined using a Clarkes electrode in a Gilson oxygraph as described in the Materials & Methods. Naphthalene breakdown product, i.e. naphthoresorcinol and phenanthrene pathway intermediate 1-hydroxy-2-naphthoic acid, and DBT-sulfone were also provided as substrates.

Table 4.2 gives the oxygen uptake rates for GU110 cells grown on polycyclic aromatic hydrocarbons compounds; DBT, phenanthrene and naphthalene. These cells showed rapid oxidation of polyaromatic compounds compared to that of cells grown on simple aromatic, sodium benzoate. DBT grown cells could also oxidize phenanthrene and naphthalene at almost identical rates. Phenanthrene and naphthalene grown cells showed preferential oxidation for the substrate on which they were grown. It was interesting to note that the reported intermediates of the DBT, phenanthrene and naphthalene breakdown pathway's such as DBT sulfone, 1H2NA and naphthoresorcinol were also oxidized at fairly high rates with naphthalene grown cells exhibiting the highest uptake rates on all the substrates in comparison with cells grown on DBT and phenanthrene.

Washed cells of GU110 grown on simple aromatic moieties like sodium benzoate and protocatechuate, were prepared and the rates of oxidation of higher aromatics or polycyclics by these cells was determined (Table 4.3). These cells revealed preferential uptake rates for the substrates on which they were grown. Oxygen uptake on DBT, phenanthrene and naphthalene was also noticed in such cells but these rates were lower than those obtained by cells grown on PAH's. These results indicate that the degradation of these aromatic compounds is dependent on the substrate on which it is grown.

Table No. 4.3 : Oxygen uptake rates of strain GU110 cells grown on SIMPLE AROMATICS

	Growth Substrates(Oxygen Uptake in nanomoles/min/mg dry weight)	
Substrates Oxidized	Benzoate	Protocatechuate
Benzoate	44.684	ND
Protocatechuate	N.D.	69.3
DBT	14.45	15.72
Phenanthrene	10.69	21.70
Naphthalene	18.09	24.50
DBT-Sulfone	10.14	ND
1-Hydroxy-2-naphthoic acid	17.26	16.91
Naphthoresorcinol	9.8	ND

Table No. 4.4 : Oxygen uptake rates of strain GU110 cells grown on LACTATE

	Growth Substrate(Oxygen Uptake in nanomoles/min/mg dry weight)
Substrates Oxidised	Lactate
Benzoate	38.97
Protocatechuate	ND
DBT	30.65
Phenanthrene	25.60
Naphthalene	25.47
DBT-Sulfone	33.08
1-Hydroxy-2-Napthoic acid	24.39
Naphthoresorcinol	18.145
Lactate	50.54

ND = Not Detected

Regulation of DBT oxidation

Oxygen uptake profiles were also determined for GU110 cells grown on non-aromatic substrates such as sodium lactate. When GU110 cells were subcultured on sodium lactate, high oxidation rates were seen on all aromatic substrates including naphthoresorcinol, 1H2NA, DBT sulfone and monoaromatic, benzoate. (Table 4.4). Results indicate that GU110 cells grown on a simple carbon source like lactate retained their ability to utilize PAH's.

To further confirm this observation, and the exact mode of regulation of oxidation of PAH's in GU110 cells, we cultured this *Alcaligenes* sp. strain GU110 on lactate and glucose, separately, for 3 successive subcultures in order to remove any traces of DBT, from the medium which may be responsible for inducing the production of DBT oxidizing enzymes in these GU110 cells. After the third transfer, cells from the late exponential phase were harvested by centrifugation at 8000rpm at 4°C in an RC5C Sorvall centrifuge and washed twice with ASW and resuspended in the same medium to an optical density of approximately 5 OD units at 560nm. These cell suspensions were separately treated with chloramphenicol to inhibit synthesis of protein in the cells and with DBT, to determine whether this molecule is responsible for induction of DBT-oxidizing enzymes. Untreated aliquots and aliquots treated with both CAM and DBT, together served as controls. 5ml volumes of these washed cell suspensions were divided into 4 different aliquots (A, B, C, D) which were treated in the following manner.

A: Plain cell suspension, untreated.

B: Cell suspension pre-incubated with 0.01% DBT (w/v).

C: Cell suspension pre-incubated with 100μg/ml of chloramphenicol (CAM).

D: Cell suspension pre-incubated with both CAM (100μg/ml) and inducer, i.e. DBT (0.01% w/v).

These aliquots were incubated at room temperature for 1 hour with shaking at 100 rpm. Volumes of these washed cells were then subjected to oxygen uptake studies using the Gilson Oxygraph and DBT and naphthalene were supplied as substrates from a methanol stock solution (0.002g/l). Oxidation was expressed as nanomoles of oxygen consumed per minute per mg of cell dry weight.

Both, glucose and lactate grown cells showed no induced oxygen uptake due to DBT when compared to CAM treated cell aliquots, or even untreated cell suspensions (Table 4.5 & 4.6). This confirms the constitutive nature of the DBT oxidizing enzymes. The differently treated aliquots of lactate grown cells were also capable of oxidizing naphthalene at similar rates, which were higher than those demonstrated on DBT. This indicated that the enzyme responsible for DBT oxidation could effectively oxidize naphthalene.

Stability of DBT degradative ability

To check whether the DBT and PAH degrading ability is stably maintained by the organism, the culture was repeatedly screened for degradation of DBT and other substrates at different stages of maintenance. It was observed that strain GU110 did not lose its DBT degradative phenotype even after culturing on rich media like Zobell's Marine media and other media which lacked the selective pressure of DBT.

Since the traits of degradation of polyaromatic compounds are normally encoded on transmissible plasmids, strain GU110 cells grown on different substrates were used for plasmid detection by the alkaline lysis method and also by simple lysis using 2 % SDS

Table No. 4.5 :Oxygen uptake rates of strain GU110 cells grown on LACTATE and subjected to CAM pre-treatment

Treatment of lactate grown washed cells		Oxygen Uptake in nanomoles/min/mg Substrates Oxidized		
		Lactate	DBT	Naphthalene
I)	Untreated	104.41	66.15	89.23
II)	Pre-incubated with 100μg/ml CAM	107.09	66.923	86.15
III)	Pre-incubated with 0.01% DBT	111.00	67.69	88.46
IV)	Pre-incubated with 100μg/ml CAM and 0.01% DBT	115.29	67.69	90.76

Table No. 4.6 : Oxygen uptake rates of strain GU110 cells grown on GLUCOSE and subjected to CAM pre-treatment

Treatment of glucose grown washed cells		Oxygen Uptake in nanomoles/min/mg Substrates Oxidized	
		Glucose	DBT
V)	Untreated	96.85	20.47
VI)	Pre-incubated with 100μg/ml CAM	95.51	21.70
VII)	Pre-incubated with 0.01% DBT	96.15	20.83
VIII)	Pre-incubated with 100μg/ml CAM and 0.01% DBT	96.49	22.80

CAM = Chloramphenicol

in TE buffer. *E.coli* harboring a broad host range plasmid, pCP13 (23.5kb) was used as a positive control. No mega plasmid could be detected in any of the cultures of GU110 grown on Zobell's media, DBT or naphthalene, but all such cells retained the ability to degrade DBT. The genes for DBT degradation could therefore be chromosomally encoded.

DISCUSSION

Alcaligenes sp. strain GU110 showed broad versatility in its action on aromatic and polycyclic aromatic compounds. In addition to the sulfur heterocyclic compound DBT, GU110 was also capable of growing on the di-aromatic compound, naphthalene and tri-aromatic compound, phenanthrene as sole source of carbon and energy. The biochemical pathway for the metabolism of DBT in this marine strain, was similar to that described by Kodama *et al.*, (1970 and 1973), as discussed in Chapter III. The Kodama pathway for DBT metabolism has biochemical similarities to the oxidative metabolic pathways of naphthalene (Eaton and Chapman, 1992), phenanthrene (Evans *et al.*, 1965) and anthracene (Evans *et al.*, 1965). Denome *et al.*, (1994) presented molecular evidence that DBT, naphthalene and phenanthrene were metabolized by a single set of upper pathway enzymes in a soil *Pseudomonad.*

Concentrations as high as 1% of DBT and phenanthrene, were not toxic to the growth of *Alcaligenes* sp. strain GU110 on these substrates. But, naphthalene at 1% (w/v) concentration exerted an inhibitory effect on GU110 such that cell densities achieved on this compound were very low. The magnitude of this decrement in cell density is therefore a function of both, the concentration of naphthalene and its inherent toxic

properties, which are enhanced due to its high solubility and volatility when compared to the aromatic hydrocarbons phenanthrene and DBT. This strain could also utilize reported intermediates of naphthalene (Davis & Evans, 1964) catabolism such as salicylaldehyde and catechol for growth. Naphthoresorcinol, a naphthalene-diol could also serve as growth substrate.

1-hydroxy-2-naphthoic acid, a key metabolite in the pathway proposed for phenanthrene utilization (Kiyohara *et al.*, 1976) could be used as sole source of carbon and energy by GU110. But other intermediates of phenanthrene degradation, by the pathway proposed by Evans *et al.*, (1965), such as 2-carboxybenzaldehyde and *o*-phthallic acid did not support growth of GU110. The similarity of products formed in the cell free supernatants of *Alcaligenes* sp. strain GU110 when on phenanthrene and naphthalene is further evidence for the utilization of these two compounds by a common pathway (Fig. 4.11).

Although many studies have demonstrated that bacteria grown on one aromatic substrate can transform other aromatic substrates as well (Barnsley, 1983; Boldrin *et al.*, 1993; Foght & Westlake, 1988; Heitkamp *et al.*, 1988; Saftic *et al.*, 1993), it is usually not known whether the same enzyme system is involved in multiple substrate transformation. It is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism (Stringfellow & Aitken, 1995).

Oxygen uptake analyses was used to evaluate whether this bacterial strain isolated on DBT, could oxidize other PAH's by the enzymes responsible for DBT degradation. The first step in the aerobic degradation of PAH's is dependent on the presence of a

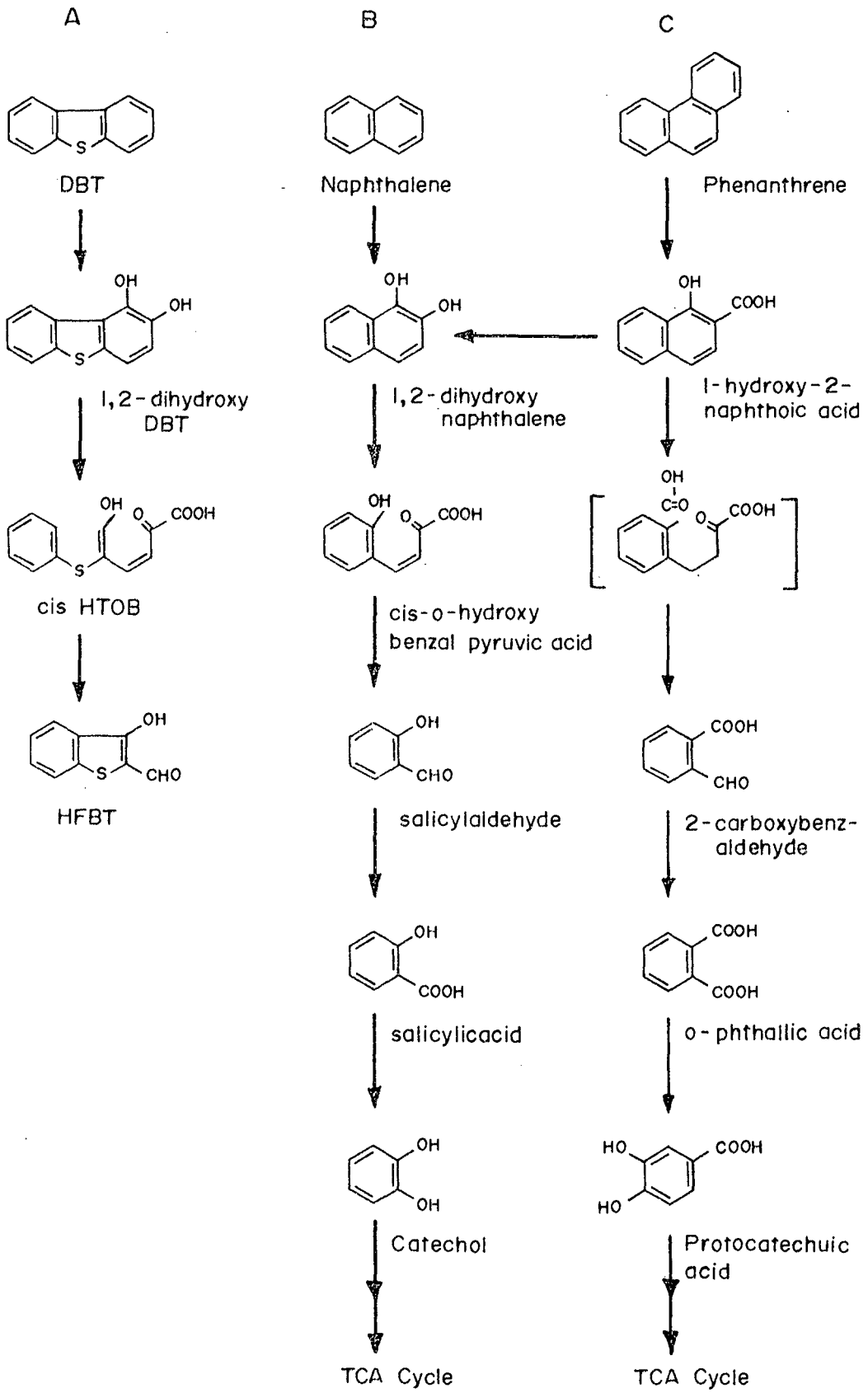


Fig. **4.11** Similarity of the pathways proposed for the breakdown of (**A**) DBT (Kodama *et al.*, 1970), (**B**) naphthalene (Davis & Evans, 1964), (**C**) phenanthrene (Evans *et al.*, 1965)

multicomponent enzyme system, the initial PAH dioxygenase (EC 1.14.12.12), catalyzing the hydroxylation of the substrate to the corresponding *cis*-dihydrodiol (Ensley *et al.*, 1982; Simon *et al.*, 1993; Williams & Sayers, 1994). The oxygen uptake rates on different substrates varied depending upon the type of aromatic compound on which GU110 was grown. These broad specificity, PAH oxidizing enzymes, were present even in strain GU110 cells grown a simple non-aromatic molecules like sodium lactate or on simple aromatic compounds like sodium benzoate and protocatechuate.

Little is known about the biodegradation of mixture of PAH's, especially the effect of one PAH on the biodegradability of another (Cerniglia, 1992). Studies on PAH degradation by mixed and pure cultures presented evidence that there are interactions between PAH's in mixtures that influence biodegradation but, exposure of *Alcaligenes* sp. strain GU110 to phenanthrene and naphthalene did not adversely affect the biodegradation of DBT by these cells.

When GU110 cells were supplied with mixtures of aromatic compounds which included DBT, phenanthrene and naphthalene, cell densities achieved on these three substrates were higher than when GU110 was grown on DBT alone, indicating that both phenanthrene and naphthalene are utilized for growth. Besides, GU110 cells grown on DBT, expressed similar oxygen uptake rates on phenanthrene and naphthalene. This broad specificity oxygenase in DBT grown GU110 cells, was also responsible for the oxidation and thereby utilization of other aromatic compounds at similar rates. This revealed that multiple PAH's are being possibly transformed by a common enzyme system in whole cells of GU110. Similar evidence has been reported by Stringfellow and Aitken, (1995) in two phenanthrene-degrading *Pseudomonads* which were capable

of metabolizing naphthalene, methylnaphthalenes and fluorene by a common enzyme pathway.

Alcaligenes sp. strain GU110 cells grown on phenanthrene and naphthalene respectively could also oxidize DBT with comparable ease although, they exhibited preferential oxidation of the substrate on which these cells were grown. Naphthalene grown cells could also oxidize 1-hydroxy-2-naphthoic acid, a reported phenanthrene pathway intermediate, at almost equivalent rates, as those obtained by phenanthrene grown cells. DBT grown cells also displayed this ability but at lower rates. DBT sulfone a reported end product in the degradation of DBT (Kodama *et al.*, 1973) could also be oxidized rapidly by these cells of without any lag period.

Naphthalene grown, washed cells of GU110 revealed higher oxygen uptake rates with DBT as substrate, than those achieved by DBT grown GU110 cells. Infact, naphthalene grown GU110 cells, exhibited the highest oxygen uptake rates on all the aromatic substrates tested for in the respirometric cell assays, compared to cells grown on DBT, phenanthrene and other substrates. It has been demonstrated that the *cis*-naphthalene dihydrodiol dehydrogenase oxidizes *cis*-dihydrodiols of several other PAH's (Patel & Gibson 1974, 1976). Denome *et al.*, (1993a) showed a 100% similarity between the DBT-oxidizing genes, *dox* ABD from *Pseudomonas* strain C18 and the *ndo* ABC genes that encode naphthalene dioxgenase of *Pseudomonas putida.*

Even after repeated subculture on naphthalene as sole source of carbon, GU110 cells retained their ability to transform DBT to water-soluble metabolites, HFBT and *trans* HTOB. The rates of conversion of DBT by these naphthalene grown washed cells increased with an increase in the density of cells. Higher transformation rates were

achieved at lower concentrations of DBT. An increase in DBT concentrations did not adversely affect the transformation of DBT by these washed cells, but conversion rates were maintained at a steady level at concentrations above 0.002%(v/v) of DBT, further confirming the fact that higher levels of DBT were not toxic to GU110.

Low DBT concentrations not only supported higher conversion rates of DBT to HFBT by GU110 cells, but also resulted in the depletion of HFBT within a period of 30 minutes. This further supported our observation in Chapter III that, HFBT, a reported dead-end product of the DBT degradative pathway (Kodama *et al.*, 1970) did not accumulate in cells of strain GU110 but was only transiently produced and was further converted to more polar metabolites. Therefore it was evident that *Alcaligenes* sp. strain GU110 cells grown on naphthalene possessed both;

(1) high levels of the DBT dioxygenase activity, the first enzyme responsible for DBT oxidation, and,

(2) the entire set of enzymes responsible for converting DBT to metabolites like HFBT and *trans* HTOB.

It can therefore be concluded that the pathway for degradation of DBT in *Alcaligenes* sp. strain GU110 is analogous to that of naphthalene degradation as was proposed by Kodama *et al.*, (1970 and 1973) (Fig. 4.11). The enzymes responsible for breakdown of naphthalene in strain GU110 are also probably involved in aromatic ring dioxygenation of DBT. Foght and Westlake (1990) have described a naphthalene degrading *P. putida* strain NCIB 9816 (NAH2) which could oxidize DBT to the pathway end product HFBT, with this oxidation being strongly stimulated by growth on naphthalene.

The high oxidation rates obtained by naphthalene grown cells with phenanthrene and the

phenanthrene pathway intermediate, 1-hydroxy-2-naphthoic acid, also confirmed that the enzymes responsible for degradation of naphthalene were also responsible for oxidation of phenanthrene via 1-hydroxy-2-naphthoic acid and salicylaldehyde to catechol (Fig. 4.11). Denome *et al.*, (1993a) reported that a single genetic system is responsible for the metabolism of DBT to 3-hydroxy-2-formyl benzothiophene, naphthalene to salicylate, and phenanthrene to 1-hydroxy-2-naphthoic acid in *Pseudomonas* strain C18 and that there is a high degree of similarity between its DNA sequence and the sequence encoding enzymes converting naphthalene to salicylate.

Regulation of DBT oxidation

Treatment of *Alcaligenes* sp. strain GU110 with chloramphenicol (CAM), did not reduce the rate of DBT oxidation when compared to that of untreated controls and DBT-induced cell aliquots. CAM-treated cell aliquots oxidized DBT at rates similar to those obtained by DBT-treated aliquots, indicating that protein synthesis was not required for DBT degradation and that it is not induced by DBT. Co-incidentally, CAM-treated GU110 cells could also oxidize naphthalene without any lag period and at a slightly higher rate than DBT. This implies constitutive degradation of both DBT and naphthalene in cells of *Alcaligenes* sp. strain GU110 and supports the possibility that the enzymes involved in oxidation of DBT are analogous to those involved in oxidation of naphthalene. *Pseudomonas* sp. HL7B was also reported to constitutively degrade DBT (Foght & Westlake, 1988) unlike *P. jianii* which is induced by DBT, naphthalene and anthracene (Kodama, 1977) and *P. alcaligenes* DBT2 (Monticello *et al.*, 1985a) which is induced by naphthalene, salicylate and DBT.

IN CONCLUSION

According to the results presented here, *Alcaligenes* sp. strain GU110 possesses a set of DBT degradative enzymes akin to those that degrade naphthalene. These enzymes are constitutive broad specificity oxygenases, rather than a suite of substrate-specific oxygenases, that permit the oxidation and ring cleavage of a number of aromatic compounds containing 1,2 or 3 aromatic rings. When these aromatic moieties were present as mixtures the degradation of these components could be achieved simultaneously by this marine strain without affecting the utilization of DBT. The rates of formation of the DBT oxidation metabolites were influenced by the increasing GU110 cell densities as well as the concentration of DBT. The PAH-degradative ability is stably maintained in strain GU110 cells and these degradative enzymes are constitutively produced with respect to the aromatic compounds, DBT and naphthalene. It can be concluded that PAH's, singly and in mixtures, are utilized by pathways that are common for certain substrates. Biotransformation is probably due to the broad specificity of enzymes and regulatory mechanisms of the pathways for the degradation of aromatic compounds. The advantages of organisms with wider substrate specificity and constitutive oxidative capability, to the development of bioremediation systems for PAH's is evident. The possibility to use such an organism for seeding environments where a recalcitrant compound or a mixture of such compounds has been spilled is quite promising. An understanding of the mechanisms involved in bacterial PAH metabolism can contribute to the development of better approaches for modeling and controlling PAH biodegradation within complex mixtures which occur in crude oil contaminated environments.

In the previous chapters we have determined the biotransforming ability of *Alcaligenes* sp. strain GU110 on DBT and various other PAH's and screened for its ability to grow on alkanes, and crude oils as sole carbon source. The biodegradation of such strongly hydrophobic, practically non-water-soluble compounds has a major ecological significance as it constitutes the major process for remediation of contaminated seawater environments.

Our objectives in this chapter were to determine;

1) The strategy by which GU110 grows on such poorly available substrates; two uptake modes were generally considered and these include,

a) the direct contact of the cell with the hydrocarbon which depends on the cell's surface hydrophobicity. This was measured by the bacterial adherence to hydrocarbon (BATH) assay.

b) hydrocarbon uptake by production of external emulsifying factor or biosurfactant, which was assayed for in cell free supernatants of *Alcaligenes* sp. strain GU110 by the D_{610} assay.

2) The efficiency of the *Alcaligenes* sp. strain GU110 to attack the different fractions of crude oil was determined and the ability of this strain to survive in artificially created microcosms when used as a member of a hydrocarbon utilizing mixed bacterial populations to attack crude oil or tarballs was also studied.

MATERIALS AND METHODS

The various carbon sources used as growth substrates included; sodium lactate, mannitol, yeast extract and tryptone from Hi Media Ltd. Sodium lactate and mannitol

CHAPTER V

EFFICIENCY OF *ALCALIGENES* SP. STRAIN GU110 IN THE MINERALIZATION OF CRUDE OIL AND CRUDE OIL COMPONENTS.

were used from a 20% autoclaved stock solution. The nutrient broth yeast extract peptone medium (NBYP) was formulated by incorporating yeast extract (0.2%) and peptone (0.3%) in nutrient broth (Hi Media). Alkanes; hexadecane and heneicosane and phenanthrene were of Sigma grade, DBT and fluorene from E Merck. Bombay High crude oil (BHCO) was obtained from ONGC, Bombay; parrafin oil (PCL laboratories, India); engine oil (*Servo* Indian Oil). All organic solvents (HPLC grade) were from E Merck. Silica gel (60-120 mesh) for column chromatography from S.D.Fine-Chem Ltd. and alumina from SRL Ltd.

A model hydrocarbon mixture was formulated as follows;

Nature of hydrocarbon	Name of component	Concentration used in ASW
Alkane	Hexadecane	0.1% (v/v)
Di-aromatic	Naphthalene	10mg%
Tri-aromatic	Phenanthrene	10mg%
	Fluorene	10mg%
Sulfur heterocyclic	DBT	10mg%

All solid hydrocarbon components were separately weighed, dissolved in hexadecane and then used as growth substrate.

Methods used

Alcaligenes sp. strain GU110 was grown on a host of compounds, as sole source of carbon and energy in an ASW containing media which included easily utilisable, non-aromatic carbon sources like lactate, mannitol, yeast extract, tryptone or on rich media like nutrient broth yeast extract peptone (NBYP). Besides, alkanes like hexadecane and heneicosane, simple aromatics like benzoate, PAH's; DBT and phenanthrene were used individually as growth substrates. Model hydrocrabon mixture, Bombay High crude oil (BHCO), paraffin oil, and engine oil also served as growth substrates. Incubations were

done at room temperature on a shaker (100 rpm) in conical flasks. Utilization of the various carbon sources, PAH's, alkanes and oils was determined as increase in biomass as measured by optical density at 560nm on a Milton Roy (Spectronic1201) spectrophotometer.

Volumes of samples (1ml) from these flasks were taken at different time intervals and centrifuged at 12,500 rpm for 10 minutes in a microfuge (Beckman E^{TM}), or centrifuged in a RC5C (Sorvall) at 8000rpm for 20 minutes at 4°C. The supernatants were subjected to the D_{610} assay (Reddy *et al.*, 1983, Roy *et al.*, 1979) and the cell pellets were washed and subjected to the BATH (Bacterial Adherence to Hydrocarbon) assay (Deziel, 1996; Rosenberg *et al.*, 1980). These assays were described as follows;

Assessment of Hydrocarbon Emulsifying ability by D_{610} assay:-

1ml volumes of the cell free culture supernatant was vigorously mixed with 10µl of hexadecane, in a quartz cuvette and the stability of the emulsion formed over a period of time was measured as turbidity at 610nm on a Milton Roy (Spectronic1201) spectrophotometer. Plain un-inoculated media's treated in a similar manner served as controls. The emulsifying activity of these cell free supernatants is a function of the degree of stability of the emulsion and is expressed as D_{610}.

Determination of cell hydrophobicity by BATH assay:-

The cells of strain GU110 grown on the appropriate substrate were washed and resuspended in phosphate buffer (0.05M) or plain distilled water to give an OD at 560nm of 1.0. The cell suspension (2ml) in testubes, was vortexed with 500µl of hexadecane. After allowing the hexadecane and aqueous phases to separate (for 1 hour) the turbidity of the aqueous phase was measured at 560nm and hydrophobicity was

expressed as the percentage of adherence to hexadecane and calculated as follows;

100 x (1- OD at 560nm of the aqueous phase/OD at 560nm of the initial cell suspension).

Assessment of crude oil degradation by Alcaligenes sp. strain GU110

Alcaligenes sp. strain GU110 (2% v/v) from maintenance flasks was inoculated in 50mls of ASW with Bombay High crude oil (0.1% v/v) as sole carbon source and also with Bombay High crude oil supplemented with DBT (0.01% w/v) in 500ml conical flasks and incubated at room temperature on a shaker (100 rpm). Appropriate un-innoculated controls were also kept. Growth was monitored at 560nm and formation of polar hydroxylated aromatic metabolites was recorded in terms of RE (μg/ml) as discussed before.

Fractionation of crude oil by Column Chromatography

After allowing for growth of strain GU110 on crude oil, the entire flask contents were extracted with dichloromethane, dried over anhydrous sodium sulfate and the solvent removed by evaporation at 60°C. After drying, the residual oil samples were fractionated into the aliphatic, aromatic and NSO or polar components by an adaptation of the column chromatographic method described by ONGC (Dehradun) .

A 45 cms x 2.0 cms glass column was packed with activated silica gel (60-120 mesh) and alumina in an equal ratio to a height of 15 cms. The residual oil from the control and test flasks were adsorbed on 0.5gms of activated silica gel and overlayered on the column . The saturate, aromatic and polar fraction were eluted sequentially with 80ml each of petroleum ether, benzene and methanol respectively. Solvents were evaporated at 60°C and the residues were transferred to pre weighed vials and weighed on a Mettler

(AE 240) balance.

Gas Chromatographic analysis of crude oil fractions

The crude oil saturate and aromatic fractions were monitored before and after degradation by injection (0.5µl) into a Shimadzu Gas chromatograph (GC-14B) fitted with flame ionization detector (FID) and capillary column (DB-1). Carrier gas was nitrogen. The injector and detector were maintained at 320°C and the oven temperature was programmed to rise from 60°C to 300°C at the rate of 6°C per minute increment and then held at 300°C for 5 minutes.

Design of marine microcosms for oil spill simulation studies

Filtered natural seawater was filled in 4 glass tanks of 300 liter capacity equipped with mechanical stirrers and independent aeration line, and maintained at constant temperature (25°C) (Fig. 5.1). Seawater in these tanks was supplemented with nitrogen [8mM$(NH_4)_2SO_4$] and phosphorous [5mM each K_2HPO_4 and KH_2PO_4] when required.

Formulation of mixed marine bacterial consortium

A mixture of hydrocarbon degraders comprising of the *Alcaligenes* sp. strain GU110 and other marine bacterial strains that had been isolated and maintained in the laboratory was designed and referred to as **consortium 1** (Table 5.1) and **consortium 2** (Table 5.2) respectively. 500µl of each of the pure cultures in consortium 1 and 2, pre-grown on its principal substrate (in ASW) was inoculated into 100ml of ASW containing a mixture of m-toluic acid (0.07%), naphthalene (0.04%), xylene (0.04%), quinoline (0.02%) pyridine (0.04%), phenanthrene (0.08%) and DBT (0.05%) for the bacterial cultures in consortium 1 and on, m-toluic acid (0.07%), quinoline (0.02%), phenanthrene (0.08%) and DBT (0.05%) for the bacterial cultures composing

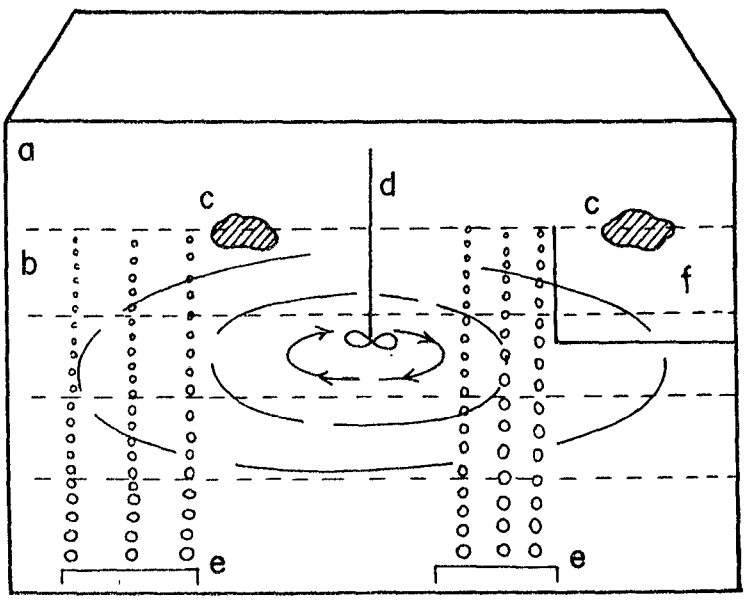


Fig.5.1 Line diagram of artificial microcosms used in this study (Coelho *et al.*, 1995); (a) 324 litre of glass tank, (b) filtered sea water, (c) tarballs/crude oil, (d) stirrer, (e) aerators, (f) sampling site

Table No. 5.1

Consortium I : List of bacterial cultures used in tarball simulation studies.

Place isolated	Strain No.	Principal Substrate	Strain identified as	Reference
Goa	GU 101	m-Toluic acid	*P.stutzeri*	Satyakam.C(1993)
Vengurla	GU 102	Naphthalene	Unidentified	J. Coelho *et al.*,(1995)
Vengurla	GU 103	Xylene	Unidentified	J. Coelho *et al.*,(1995)
Vengurla	GU 104	Quinoline	*Ochromobactrum sp.*	J. Coelho(1998) Ph.D thesis
Vengurla	GU 105	Pyridine	Unidentified	J. Coelho *et al.*,(1995)
Gulf of Kutch	GU 109	Phenanthrene	*P. putida*	N. Fernandes(2000) Ph.D thesis
Goa	GU 110	DBT	*Alcaligenes sp.*	J.Rodrigues(this study)

Table No. 5.2

Consortium II : List of bacterial cultures used in oil spill simulation studies.

Place isolated	Strain No.	Principal Substrate	Strain identified as	Reference
Goa	GU 101	m-Toluic acid	*P.Stutzeri*	Satyakam.C.(1993)
Vengurla	GU 104	Quinoline	*Ochromobactrum sp.*	J. Coelho(1998) Ph.D thesis
Gulf of Kutch	GU 109	Phenanthrene	*P.putida*	N.Fernandes(2000) Ph.D thesis
Goa	GU 110	DBT	*Alcaligenes sp.*	J.Rodrigues(this study)

consortium 2. Flasks were incubated at room temperature on a rotary shaker (200 rpm). These mixed cultures were then cryo-preserved at -70°C by a method developed in our laboratory (N. Fernandes, personal communication). This mixture was then amplified on the above substrates, and used as inoculum (0.5% v/v) in artificially created microcosms.

Determination of growth of Alcaligenes sp. GU110 as part of the consortium

50ml aliquots of seawater samples from each of the 2 inoculated tanks were filtered using a Millipore filter which was then dispensed in 5ml of ASW. Viable counts of all of the components of the mixed culture in consortium 1 and 2, including GU110 was established by the MPN (Most Probable Number) method by carrying out a series of 10 fold dilutions from these filtered seawater aliquots, in tubes containing 0.08% sodium benzoate as sole carbon source. To specifically determine the viability of *Alcaligenes* sp. strain GU110 , similar dilution's were performed using 0.05% (w/v) DBT as the sole carbon source. The highest dilution showing growth was taken as a measure of the DBT utilizing bacterial population. This was compared to the viable counts using sodium benzoate (0.08%) as sole substrate, since it supported the growth of all the cultures in the consortium.

RESULTS

The marine strain *Alcaligenes* sp. strain GU110 could effectively grow on a range of hydrocarbon and non-hydrocarbon compounds as sole carbon source.

Cell Surface Hydrophobicity (BATH) and Emulsifying ability (D_{610}) of Alcaligenes sp. strain GU110 grown on various aromatic and alkane moieties as carbon sources

The BATH for adherence to hydrocarbons and the D_{610} assay was performed during the growth of GU110 on different substrates, which represent the different components of crude oil. These included, the simple aromatic compound, benzoate; the PAH's, DBT and phenanthrene and components of the saturate alkane fraction, hexadecane and heneicosane. GU110 cells grown on these substrates displayed cell surface hydrophobicities ranging from 20% for phenanthrene grown cells to 80% for DBT grown cells but the emulsifying activities exhibited by strain GU110 grown on these different substrates were almost similar (Table 5.3).

A) Polycyclic aromatic hydrocarbons (PAH's): The PAH's, DBT and phenanthrene are prominent components of crude oil and serve as sole sources of carbon and energy when provided to *Alcaligenes* sp. strain GU110 in an ASW containing medium.

In the Chapter 3 we have proposed a model for utilization of DBT by *Alcaligenes* sp. strain GU110 based primarily on its ability to adhere to crystals to DBT as observed under the microscope. Like all other PAH's the DBT moiety, is hydrophobic and insoluble in nature. Growth of GU110 on 1% DBT, yielded cells with an increasing degree of hydrophobicity (Fig. 5.2A & Fig. 5.3). Infact these cells revealed cell surface hydrophobicities as high as 80%. Cell free culture supernatants obtained during different stages of growth on 1% DBT revealed low levels of extracellular emulsifying activity. Growth of GU110 at increasing concentrations of DBT revealed that the degree of emulsification activity and the hydrophobic nature of GU110 cells was not dependent on the increasing DBT concentrations (Fig. 5.4).

Table No. 5.3: ***Emulsifying ability and cell surface hydrophobicities of strain GU110 cells grown on aromatic and alkane carbon sources***

Carbon Source	Cell hydrophobicities (% adherence)	Emulsifying activity (D_{610})
Simple aromatic compound		
Sodium benzoate	57.83	0.109
PAH's		
Phenanthrene	23.77	0.156
Dibenzothiophene	86.4	0.182
n-Alkanes		
Hexadecane (C-16)	79.85	0.153
Heneicosane(C-21)	72.85	0.199

Table No. 5.4: ***Emulsifying ability and cell surface hydrophobicities of strain GU110 cells grown on easily utilisable carbon sources***

Carbon source	Cell hydrophobicities (% adherence)	Emulsifying activity (D_{610})
Sodium lactate	78.80	0.084
Mannitol	13.00	0.150
Yeast extract	24.00	0.339
Tryptone	15.5	0.475
NBYP medium	22.40	0.423
NBYP medium + n-hexadecane	27.76	0.737

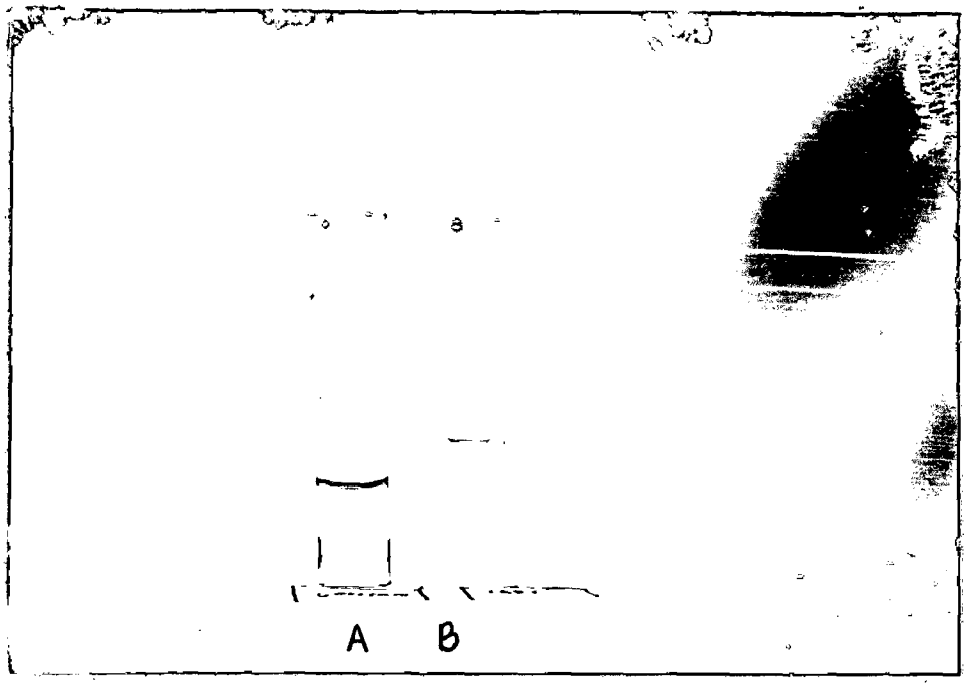


Fig.5.2A Bacterial adherence to hexadecane displayed by strain GU110 cells grown on DBT. (A) Control : DBT grown GU110 washed cell suspension (B) Test : DBT grown GU110 cell suspension vortexed with hexadecane. Note, the upper hexadecane layer exhibits a distinct red color due to adherence of cells from the lower aqueous layer.

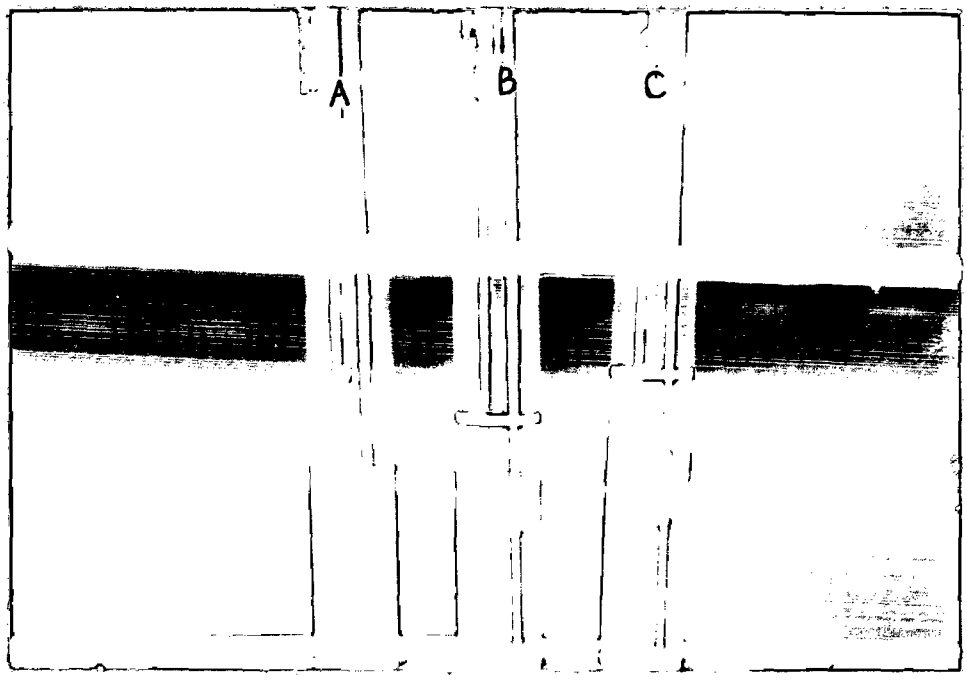


Fig.5.2B Bacterial adherence to hexadecane displayed by GU110 cells grown on hexadecane. (A) Control : Hexadecane grown GU110 washed cell suspension Test (B&C): Hexadecane grown washed cell suspension vortexed with hexadecane. The decrease in the turbidity of the lower aqueous cell suspension after vortexing with hexadecane, was due to adherence of cells to hexadecane

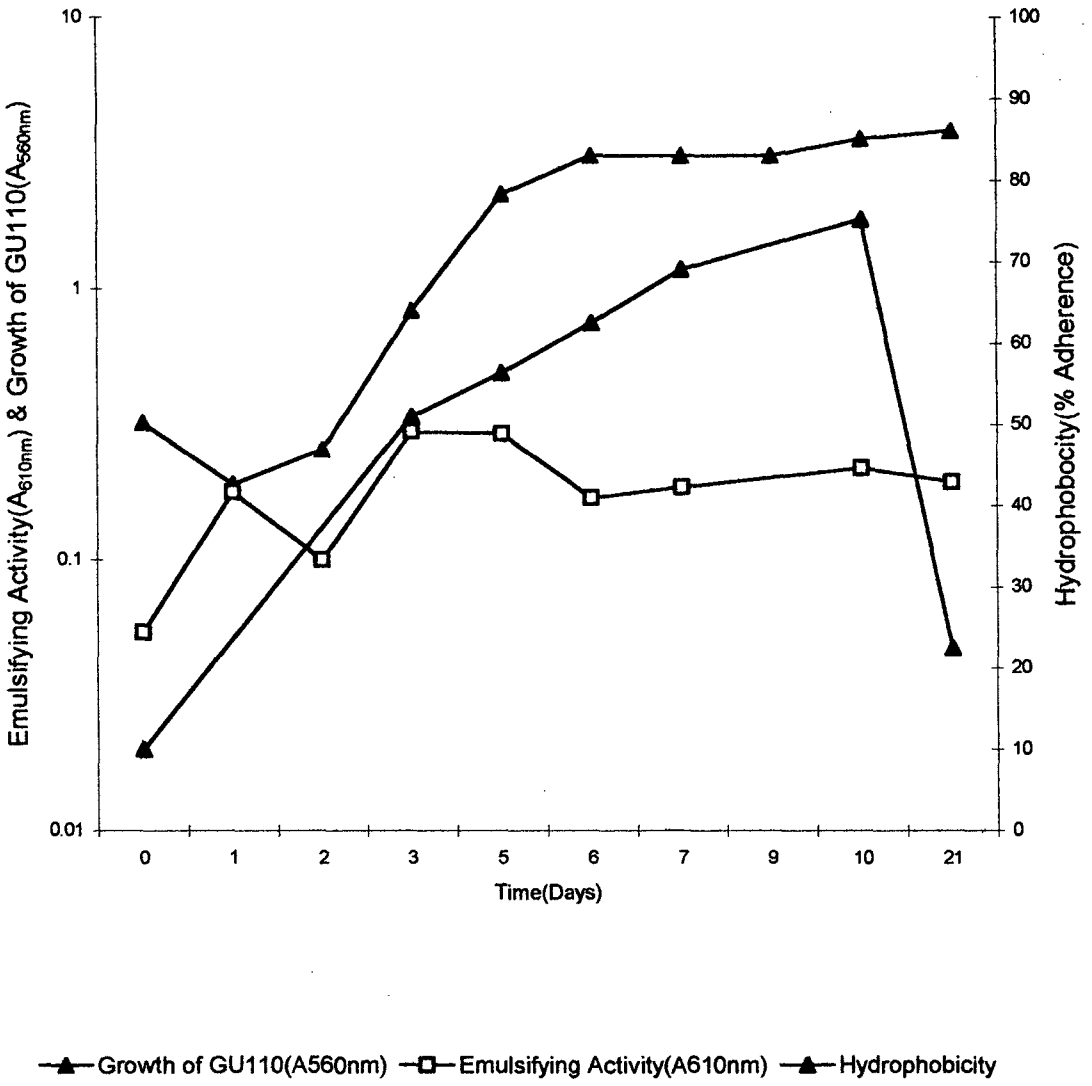


Fig.5.3 Cell surface hydrophobicities (% Adherence) and emulsifying activity (D_{610nm}) displayed by strain GU110 cells during growth on DBT(1%).

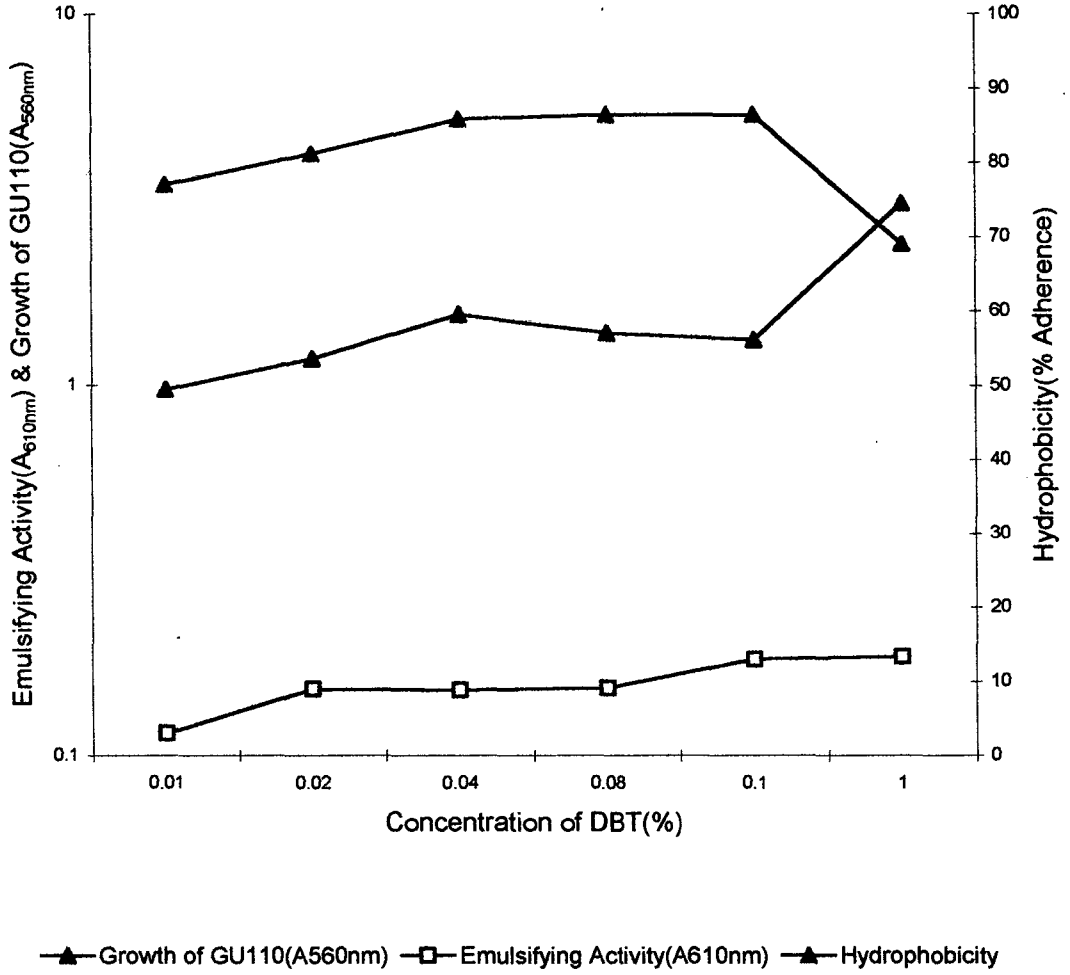


Fig.5.4 Cell surface hydrophobocities (% Adherence) and emulsifying activity (D_{610nm}) displayed by strain GU110 cells grown on different concentrations of DBT.

Growth of GU110 on 1% phenanthrene produced cells with a lower degree of adherence compared to cells grown on 1% DBT (Fig 5.5). Emulsifying ability (D_{610}) increased during initial log period and then remained steady.

B) Aliphatic hydrocarbons: Both hexadecane and heneicosane are representative alkane moieties present in the saturate fraction of crude oil and these differ only on the basis of the chain length.

Strain GU110 grows very rapidly on hexadecane (C-16) and greater biomass was obtained at higher concentrations. After 27 hours of growth on 0.1% hexadecane the optical density at 560nm decreases with the concomitant increase in the formation of orange colored waxy clumps of cells in the ASW media. These clumps tend to persist in the medium (even in the late stationary phase) and the turbidity increases in the lower aqueous phase abruptly, after 71 hours. When these orange clumps were observed under the phase contrast microscope (40X) they revealed aggregates of cells. Growth of *Alcaligenes* sp. strain GU110 on 1% hexadecane resulted in a rapid increase in the optical density at 560nm with the formation of a pinkish orange cellular aggregates or scum at the surface of the medium. When the culture broth was centrifuged at 8000 RPM in a Sorvall RC5C cooling centrifuge at 4°C at different time intervals, the pink scum along with the remaining hexadecane solidifies at the surface of the supernatant at this low temperature. The scum was collected and observed under the microscope and revealed high densities of cells in the hexadecane layer itself. The cell pellets obtained from the lower aqueous layer were washed and their cell hydrophobicities were determined. Although these cells, were not associated with the hexadecane, they had a high degree of adherence to hexadecane (Fig. 5.2B).

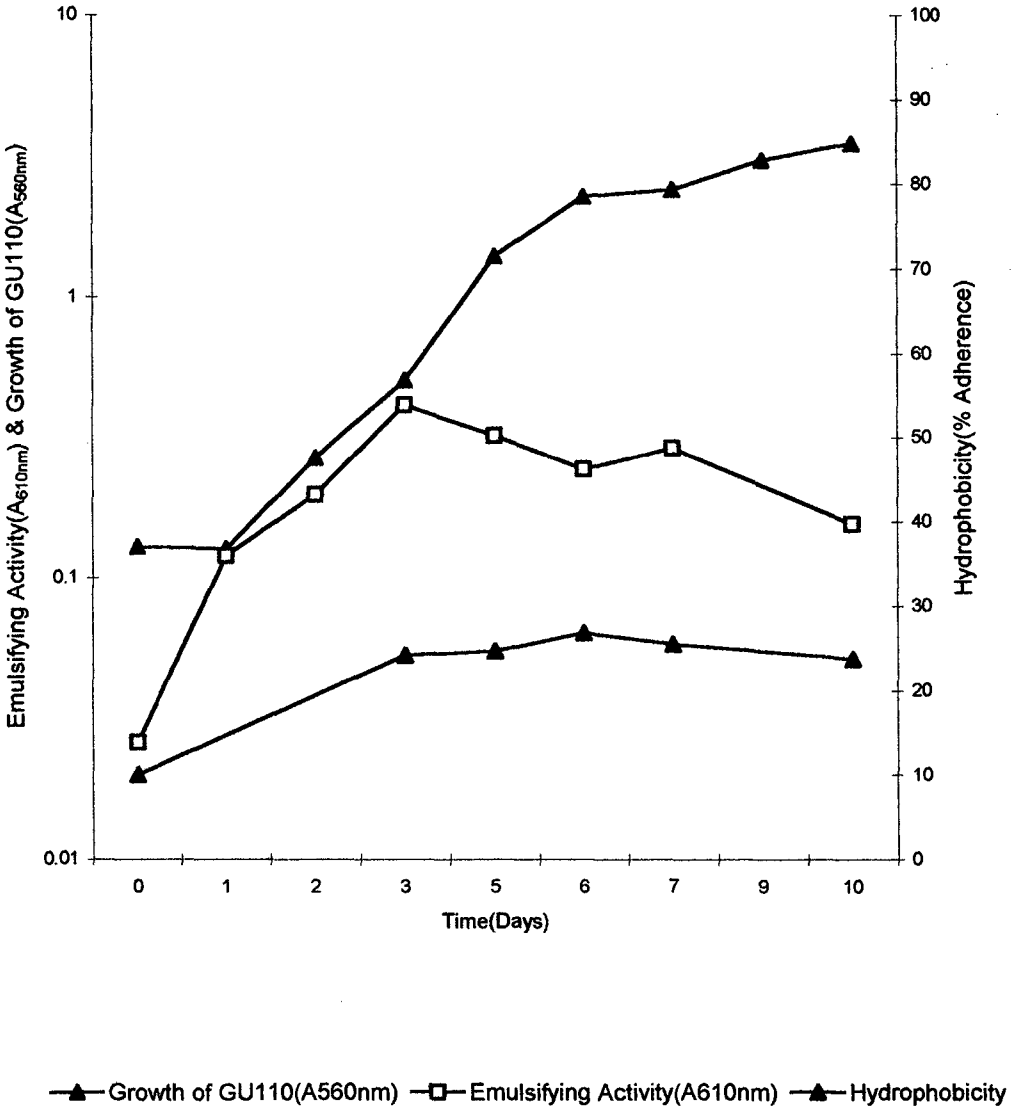


Fig.5.5 Cell surface hydrophobocities (% Adherence) and emulsifying activity (D_{610nm}) displayed by strain GU110 cells during growth on phenanthrene (1%).

Fig. 5.6 revealed that GU110 cells grown on 1% hexadecane exhibited hydrophobicities as high as 90 % which decreased on prolonged incubation of cells with hexadecane. The emulsifying ability or D_{610} was not very constant and varied with the growth of the culture on hexadecane (Fig. 5.6) but cell free supernatants of late stationary phase GU110 cultures grown at different hexadecane concentrations revealed the ability to form stable emulsions with hexadecane over a period of 30 minutes (Fig. 5.7).

Unlike hexadecane, utilization of heneicosane (C-21) carbon for growth of GU110 in ASW media resulted in very low levels of biomass being obtained over an extended period of time. These cells displayed hydrophobicities of 72% (Table 5.3).

Cell surface hydrophobicities (BATH) and emulsifying ability (D_{610}) during growth of GU110 on easily utilisable carbon sources

To determine whether *Alcaligenes* sp. strain GU110 displayed hydrophobicities and emulsifying activities in the absence of crude oil components, this strain was grown on simple carbon substrates such as lactate and mannitol, yeast extract and tryptone as well as a rich medium like NBYP. Growth on sodium lactate yielded GU110 cells which exhibited the highest degree of hydrophobicity compared to cells grown on all the other simple carbon sources tested but emulsifying activities in these lactate grown cell free supernatants were considerably lower than those obtained by growth of strain GU110 on other substrates (Table 5.4). On yeast extract, tryptone and NBYP media, cell hydrophobicities and D_{610} values were similar. When the NYBP medium was supplemented with hexadecane, emulsifying activity was higher than on NYBP without hexadecane although the cell hydrophobicities remained same.

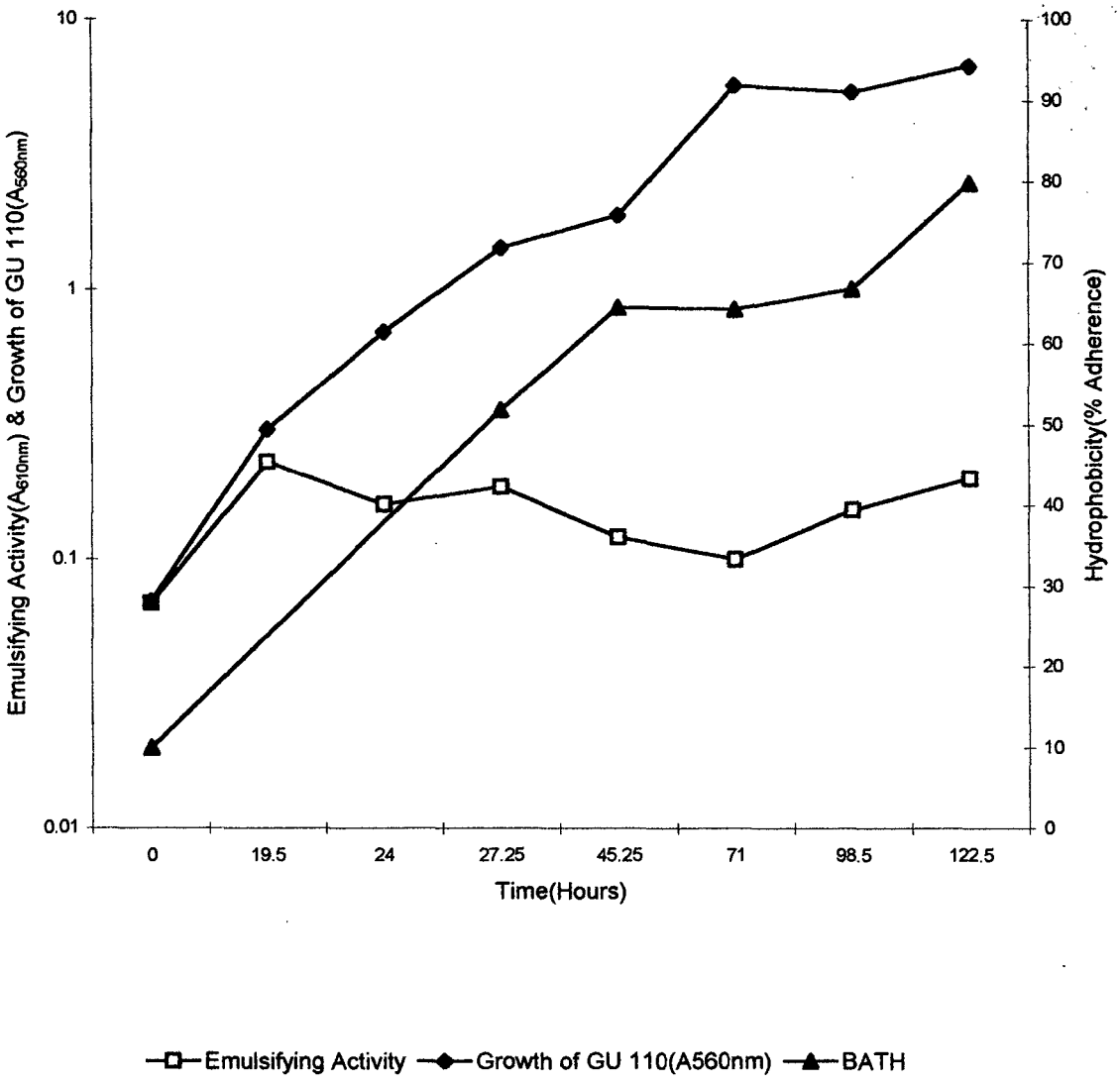


Fig.5.6 Cell surface hydrophobicities (%Adherence) and emulsifying activity (D_{610nm}) of strain GU110 cells during growth on 1% hexadecane.

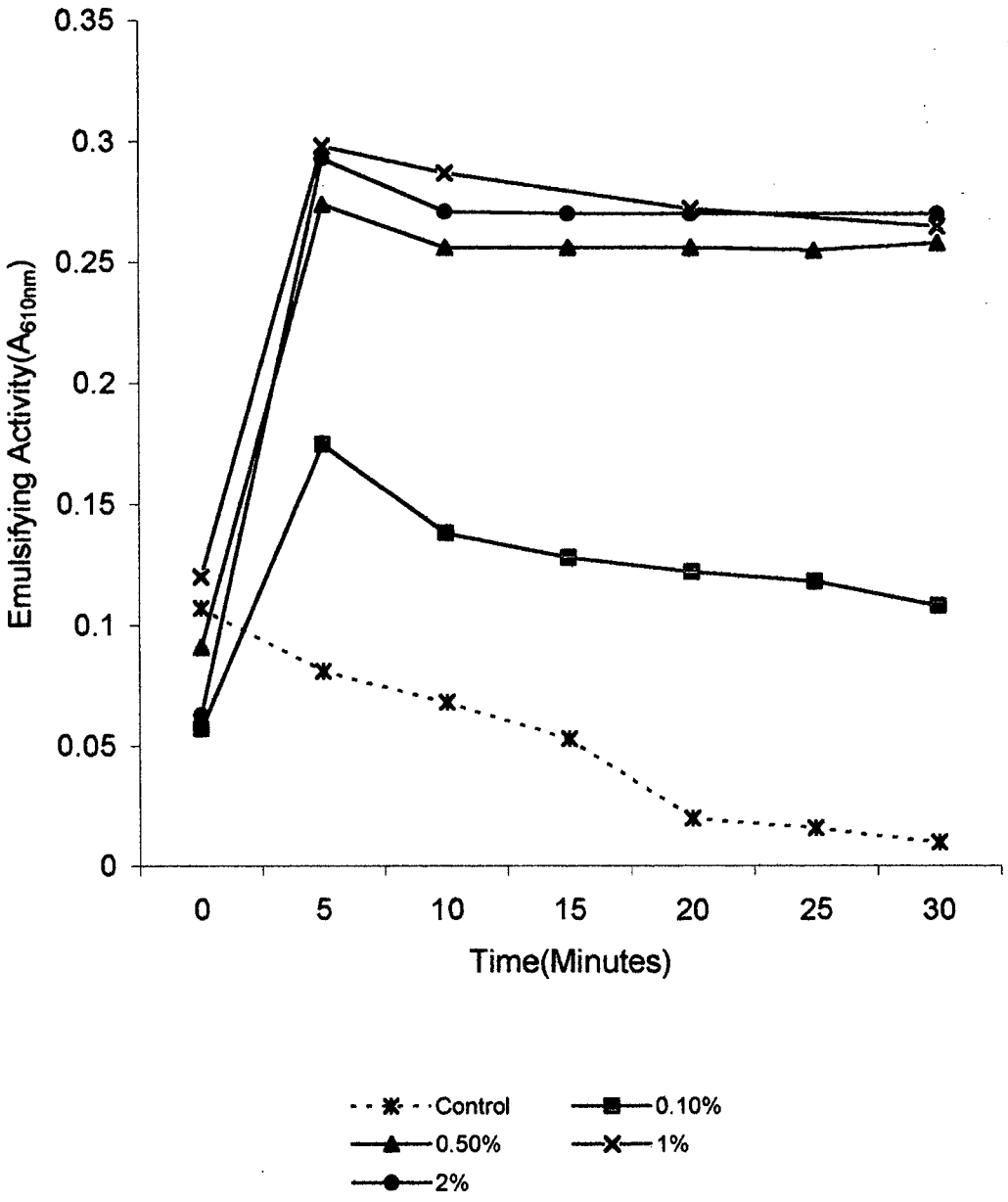


Fig.5.7 Emulsifying ability (D_{610nm}) of GU110 cell free supernatants after growth on ASW with different concentrations of hexadecane.

Mode of Utilization of crude oil and other oils by GU110

Alcaligenes sp. strain GU110 could utilize Bombay High crude oil, paraffin oil and engine oil as growth substrates (Fig. 5.8) and the cell surface hydrophobicities and emulsifying abilities of this marine strain after growth on these different oils was determined (Table 5.5). Uniform turbid GU110 cell suspensions were not observed on all these hydrophobic substrates and cells formed aggregates or clumps.

Growth of GU110 on crude oil and tarball resulted in a breaking up or emulsification of oil into small brown clumps (Fig. 5.9). Microscopic examination revealed the presence of cellular aggregates within these clumps. Higher cell densities were achieved by strain GU110 on increasing the concentration of crude oil in the medium. Cell hydrophobicities varied from between 10 to 20% and increased with growth of the culture on 1% crude oil (Fig. 5.10). Low degrees of emulsifying activity were also observed in these cell free supernatants.

Degradation of DBT in presence of crude oil

We determined the utilization of sulfur heterocycle DBT, in presence of crude oil by growing the culture on crude oil supplemented with DBT. Growth was recorded as an increase in biomass at 560nm with the concomitant production of a visible vermilion color in the ASW medium which is characteristic of DBToxidation products and is measured as Resorcinol equivalents or RE (μg/ml). Fig. 5.11 shows the comparison of the phenolic metabolites formed during growth of GU110 on crude oil in the presence and absence of DBT. Results clearly indicate that DBT is attacked by *Alcaligenes* sp. strain GU110 even in presence of crude oil.

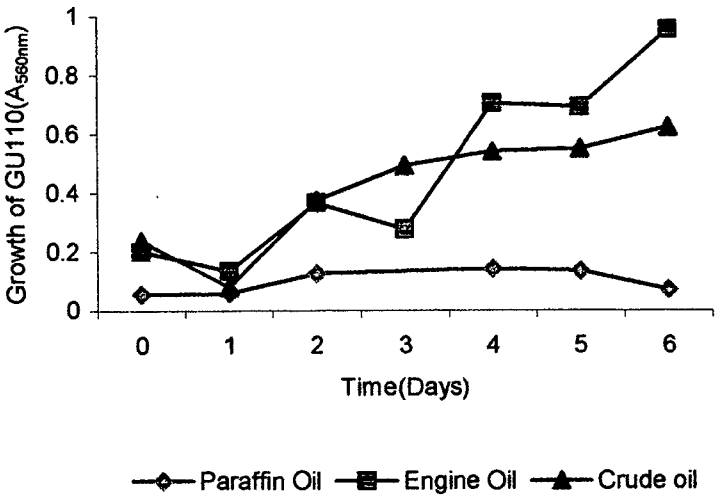


Fig.5.8 Comparison of growth of GU110 on different oils.Paraffin oil, Crude oil and engine oil were used as sole source of carbon (0.1%).

Table 5.5 : Cell hydrophobicities and emulsifying ability of GU 110 cells grown on different oils

Oil Used	Concentration used at	Emulsifying activity(D_{610})	Hydrophobicity (% Adherence)
Crude Oil	0.1%	0.293	38.61
	1%	0.208	31
Engine Oil	0.1%	0.136	23.12
	1%	0.140	29.60
Paraffin Oil	0.1%	0.083	16.01
	1%	0.076	5.3

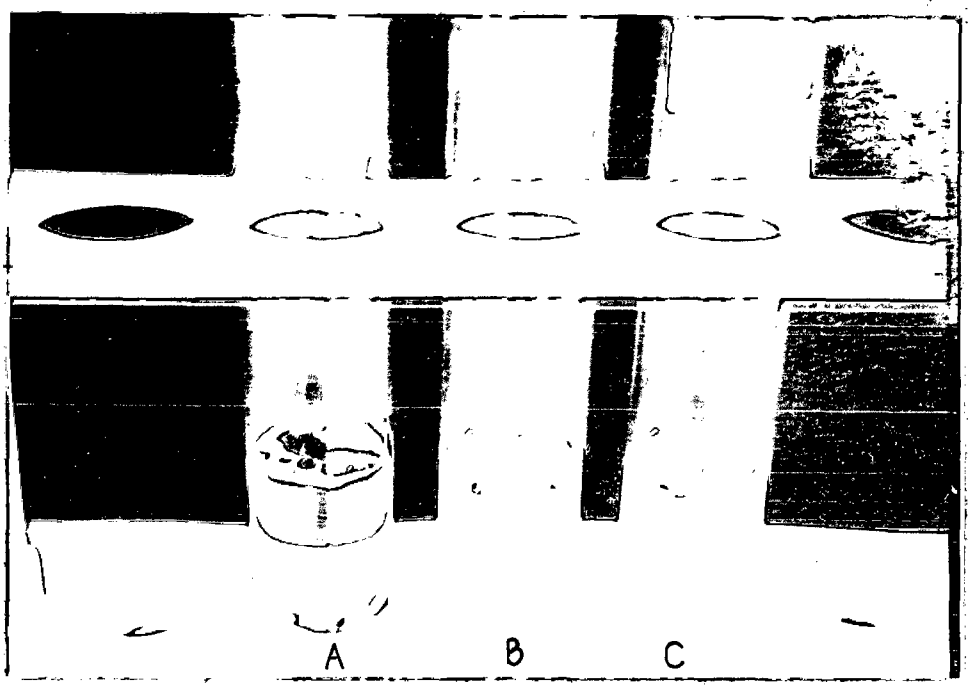


Fig.5.9a Growth of strain GU110 on crude oil (A) Control : Un-inoculated ASW with crude oil. Test with 0.1% (B) and 1% crude oil (C) inoculated with strain GU110.

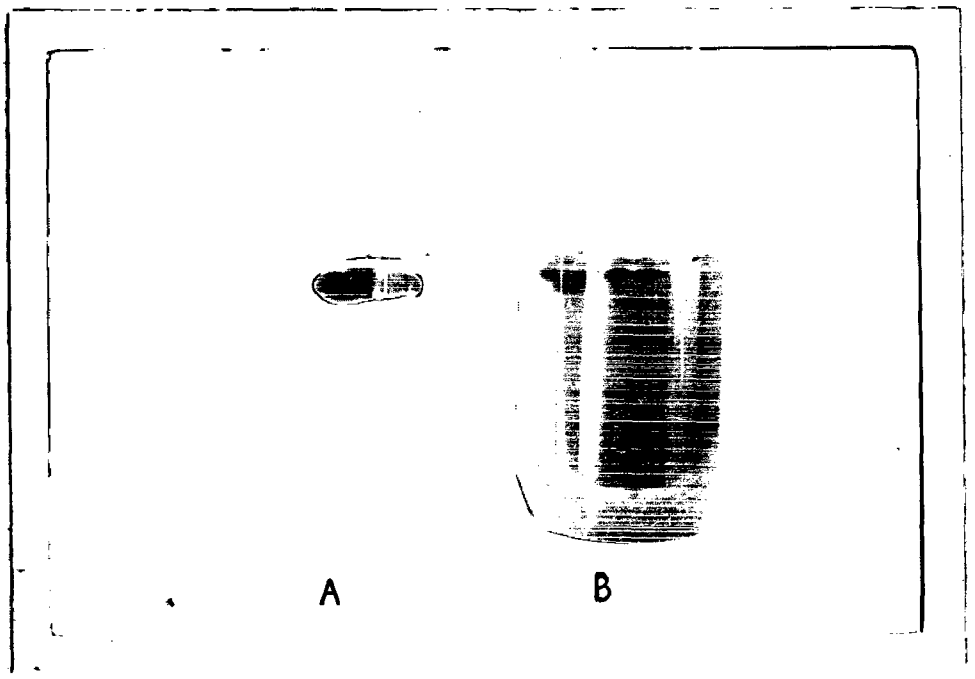


Fig.5.9b Growth of strain GU110 on tarball.
(A) Control : Un-inoculated ASW with tarball.
(B) Test : Growth of strain GU110 on tarball resulted in the emulsification and dispersion of the tarball and in the formation of colored polar metabolites which is typical of this strain.

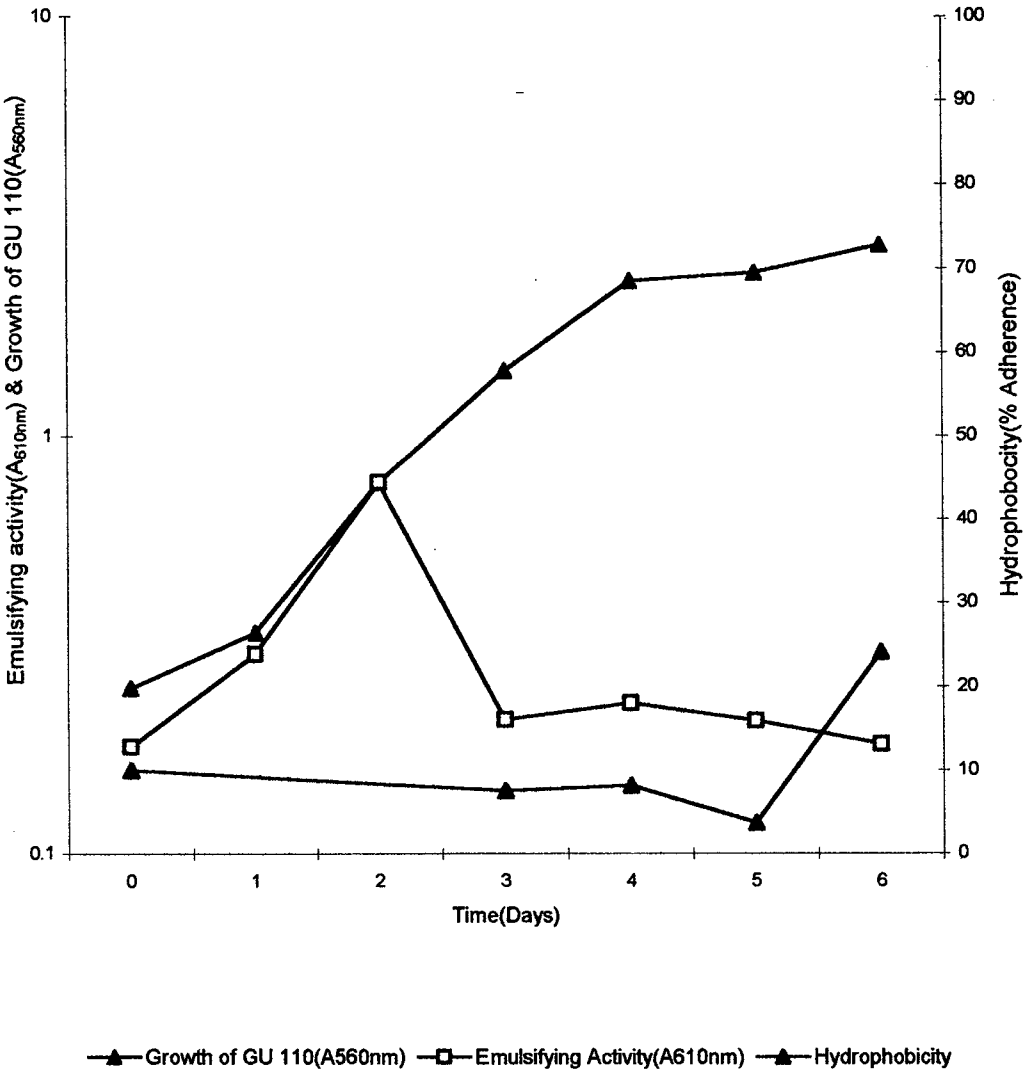


Fig.5.10 Cell surface hydrophobicities (% Adherence) and emulsifying activity (D_{610nm}) displayed by GU110 cells during growth on 1% crude oil.

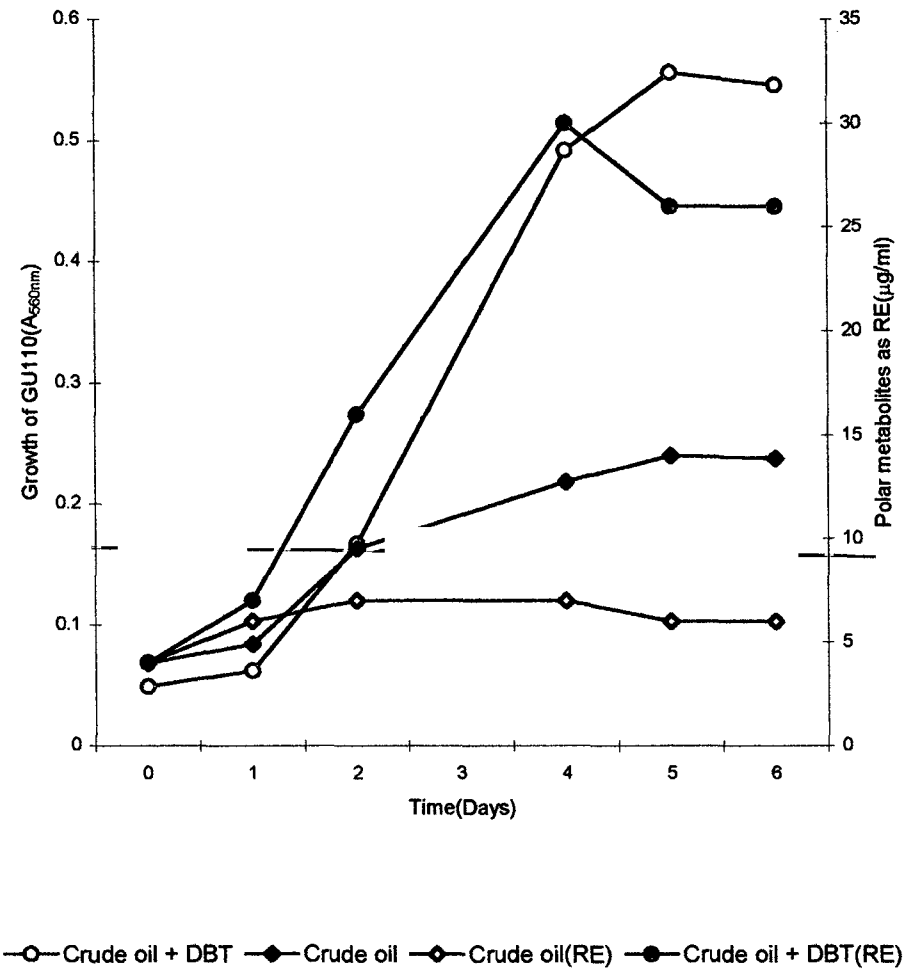


Fig.5.11 Growth(A_{560nm}) and formation of polar metabolites (Total phenolics as RE) by strain GU110 cells grown on crude oil and crude oil supplemented with DBT.

Efficacy of strain GU110 in the degradation of crude oil fractions

To determine the extent of degradation of crude oil by *Alcaligenes* sp. strain GU110, the extracted residual crude oil from GU110 grown flasks, were analyzed separately for the three different fractions, as described in Methods.

Table 5.6 shows the gravimetric analysis of the crude oil components after growth of strain GU110 on crude oil and on crude oil supplemented with DBT. Both, the alkane and the aromatic fractions were reduced considerably but the polar or NSO fraction was enriched when compared to sterile un-innoculated controls.

To confirm the contribution of GU110 towards the mineralization of crude oil we analyzed the different fractions on the gas chromatograph (GC). Gas chromatographic analysis of the alkane fraction revealed depletion of all the major peaks in comparison to sterile controls, and few of these peaks could be observed after increasing the sensitivity by a factor of 10 (Fig. 5.12). In comparison only a few of the peaks were removed in the aromatic fraction of degraded crude oil (Fig. 5.13). GC analysis of the fractions obtained after growth of GU110 on crude oil supplemented with DBT revealed that DBT eluted out in the alkane saturate fraction of crude oil. Fig 5.14 reveals the simultaneous removal of DBT along with the other peaks from the saturate fraction.

Utilization of Model Hydrocarbon mixture

Utilization of the artificially formulated mixture of hydrocarbon components consisting of DBT and different aromatic substrates, including the alkane, hexadecane (described in Materials) by *Alcaligenes* sp. strain GU110 was assessed by an increase in the optical density at 560nm as well as by determining the release of water-soluble phenolic polar

Table No. 5.6 : Degradation of Bombay High Crude Oil by *Alcaligenes sp.* strain GU110

Fraction	Amount remaining in			
	Control sample		Degraded sample	
	mg	%	mg	%
Culture grown on crude oil				
Alkanes	16.9	100	6.3	37.27
Aromatics	8.6	100	7.3	84.88
NSO	12.7	100	15.5	122
Culture grown on crude oil and DBT				
Alkanes	15.7	100	7.6	48
Aromatics	9.6	100	6.2	64.58
NSO	7	100	11.2	166

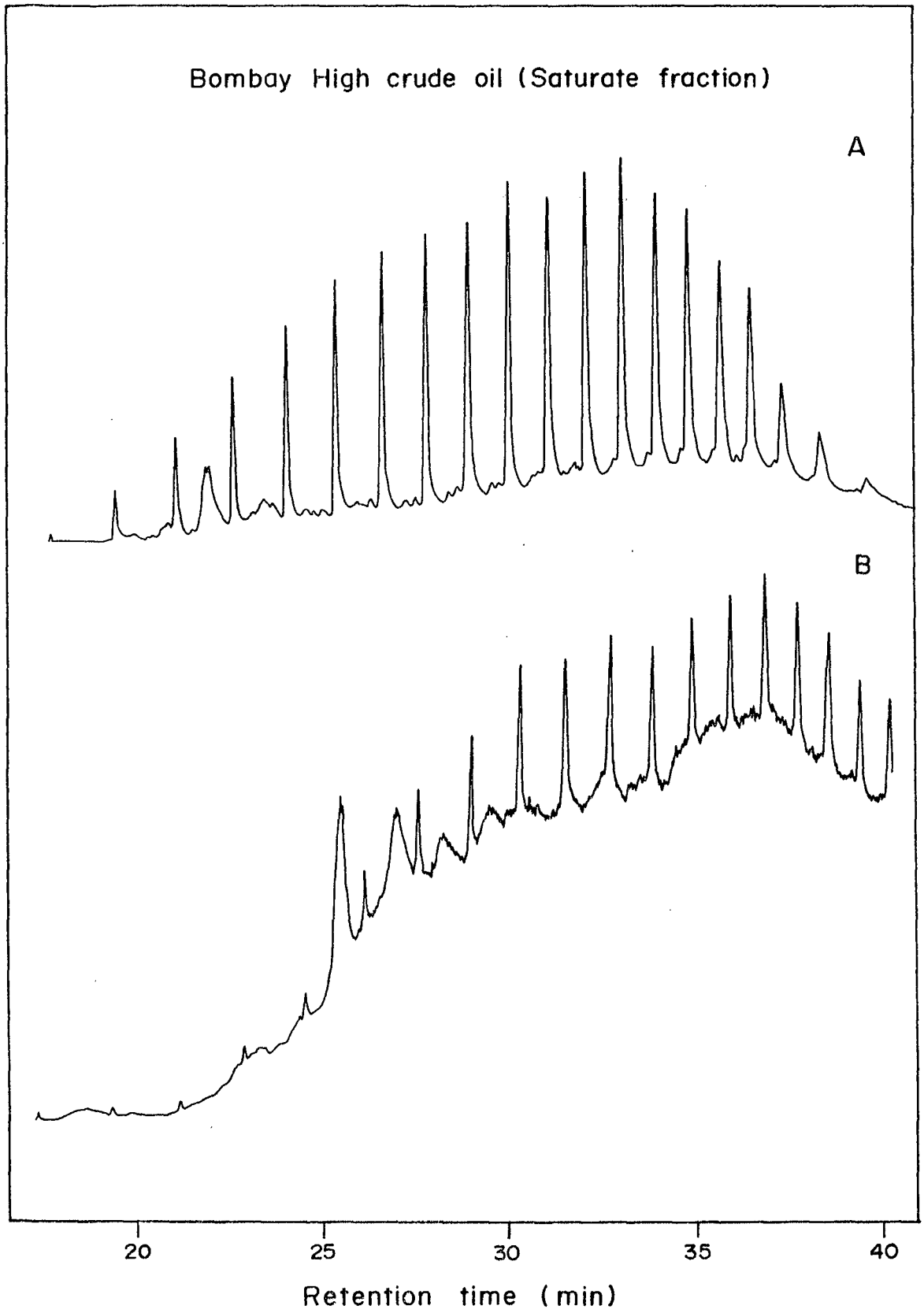


Fig.5.12 GC profile of n-alkane fraction of Bombay high crude oil (BHCO)
(**A**) Un-inoculated sterile control.
(**B**) Test : After growth of strain GU110 (14 days). Recorder sensitivity was increased 10 fold to depict residual peaks. Complete removal of certain peaks may be noted.

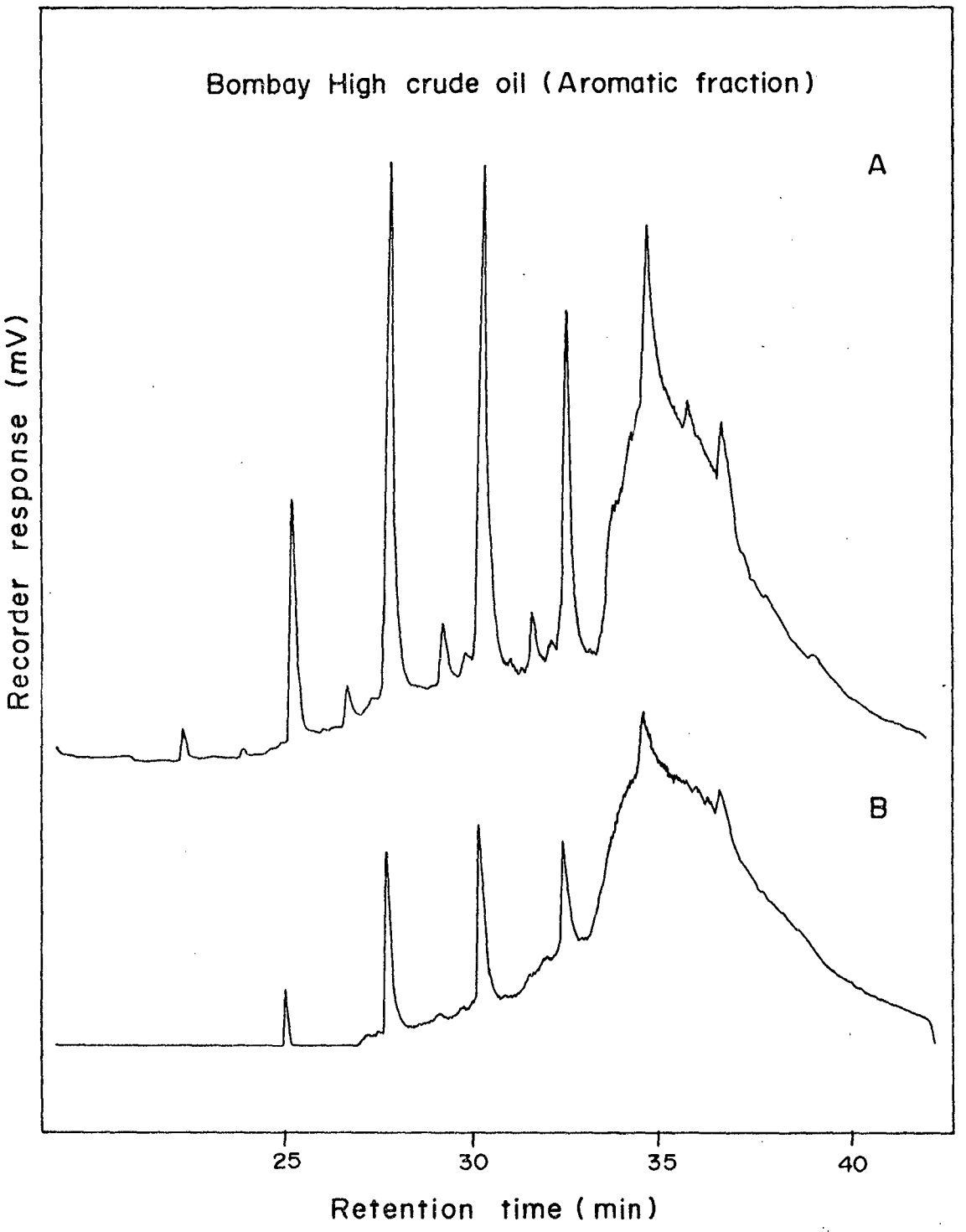


Fig.5.13 GC profiles of aromatic fraction of BHCO
(**A**) Un-inoculated sterile control.
(**B**) Test : After growth of strain GU110 (14 days).

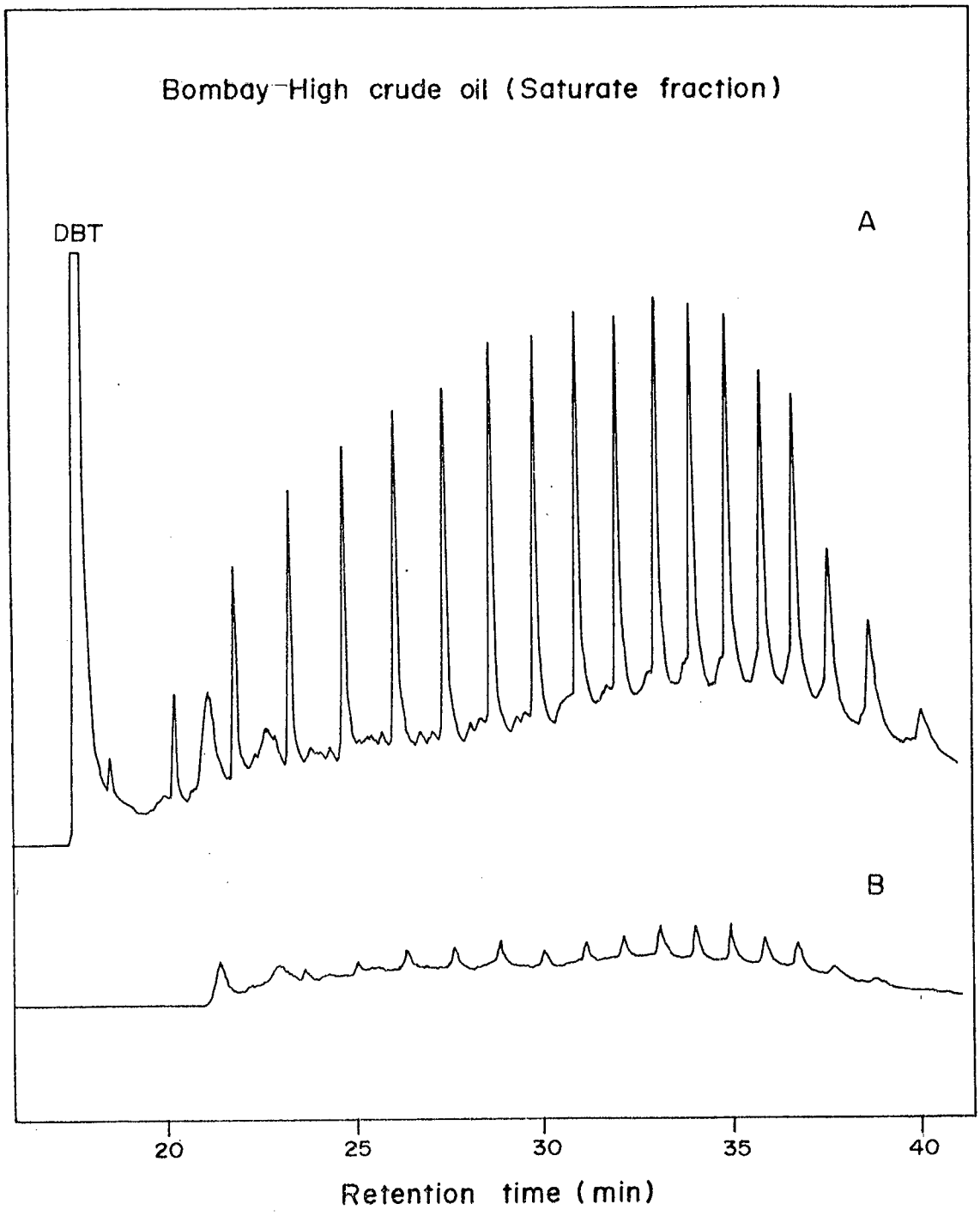


Fig. 5.14 GC profiles of n-alkane fraction after growth of strain GU110 on BHCO supplemented with DBT.
(A) Un-inoculated sterile control
(B) Test: After growth of strain GU110 (14days).
DBT eluted out in the alkane fraction by the crude oil fractionation method employed. Test samples revealed absence of the DBT peak after degradation.

metabolites into the medium which were estimated as Resorcinol Equivalents (RE in µg/ml). Growth and utilization of this model hydrocarbon mixture by *Alcaligenes* sp. strain GU110 resulted in an increase in biomass and in the formation of water-soluble polar metabolites (Fig. 5.15). The whole cell culture broth was then extracted with ethyl acetate, the organic extract dried over anhydrous sodium sulfate and injected into a GC (NETEL Omega QC^{+}) fitted with a stainless steel (SE 30) packed column and FID. Carrier gas was nitrogen. GC analysis of these organic extracts revealed removal of these hydrocarbon substrate peaks when compared to sterile control, as shown in Fig. 5.16. It was interesting to note the removal of fluorene by strain GU110 cells when it was provided as part of the hydrocarbon mixture since fluorene did not serve as growth substrate for GU110 when supplied as the sole carbon source.

Oil Spill Simulation studies

Two sets of experiments were separately designed using tarballs and crude oil as growth substrates. **Set 1**: Tarball's from Bombay High was used as substrate and introduced into the 'test' and 'control' tanks at a concentration of 0.15% wt/v. The test tanks (with and without N & P) were inoculated with a mixture of seven different bacterial cultures, which we termed as consortium 1.

Set 2: Bombay High crude oil (0.1% v/v) was used as substrate in the tanks in a similar manner as described above, and the test tanks were inoculated with a mixture of four different bacterial cultures which we termed as consortium 2.

The 4 different glass tanks used for both of these experiments were designated as follows;

1) Tank A [Control]: Seawater + crude oil / tarball + nitrates + phosphates.

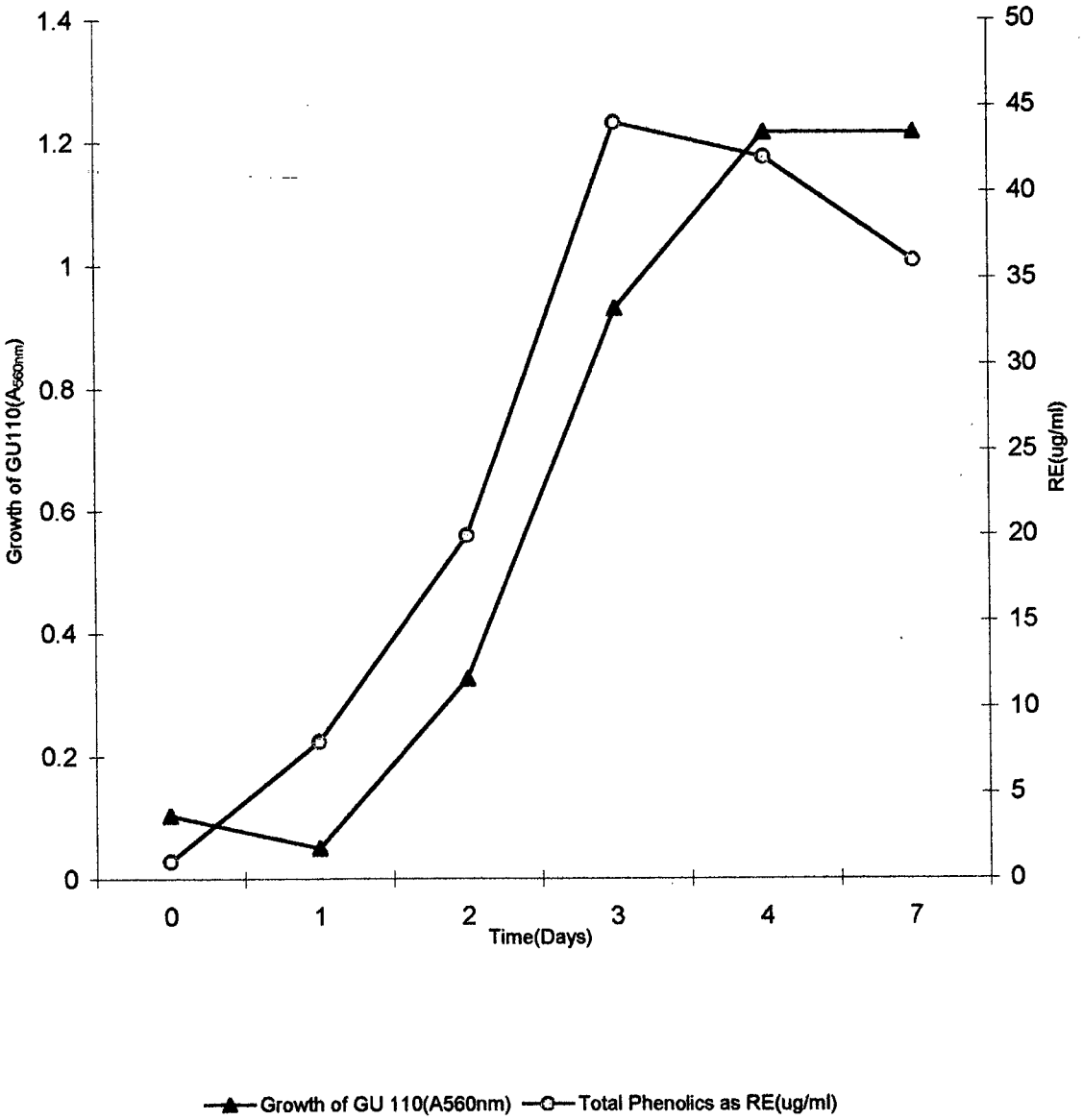


Fig. 5.15 Growth of strain GU110 on a Model Hydrocarbon mixture as determined by an increase in turbidity and in the formation of polar phenolic metabolites (Resorcinol Equivalents,RE)

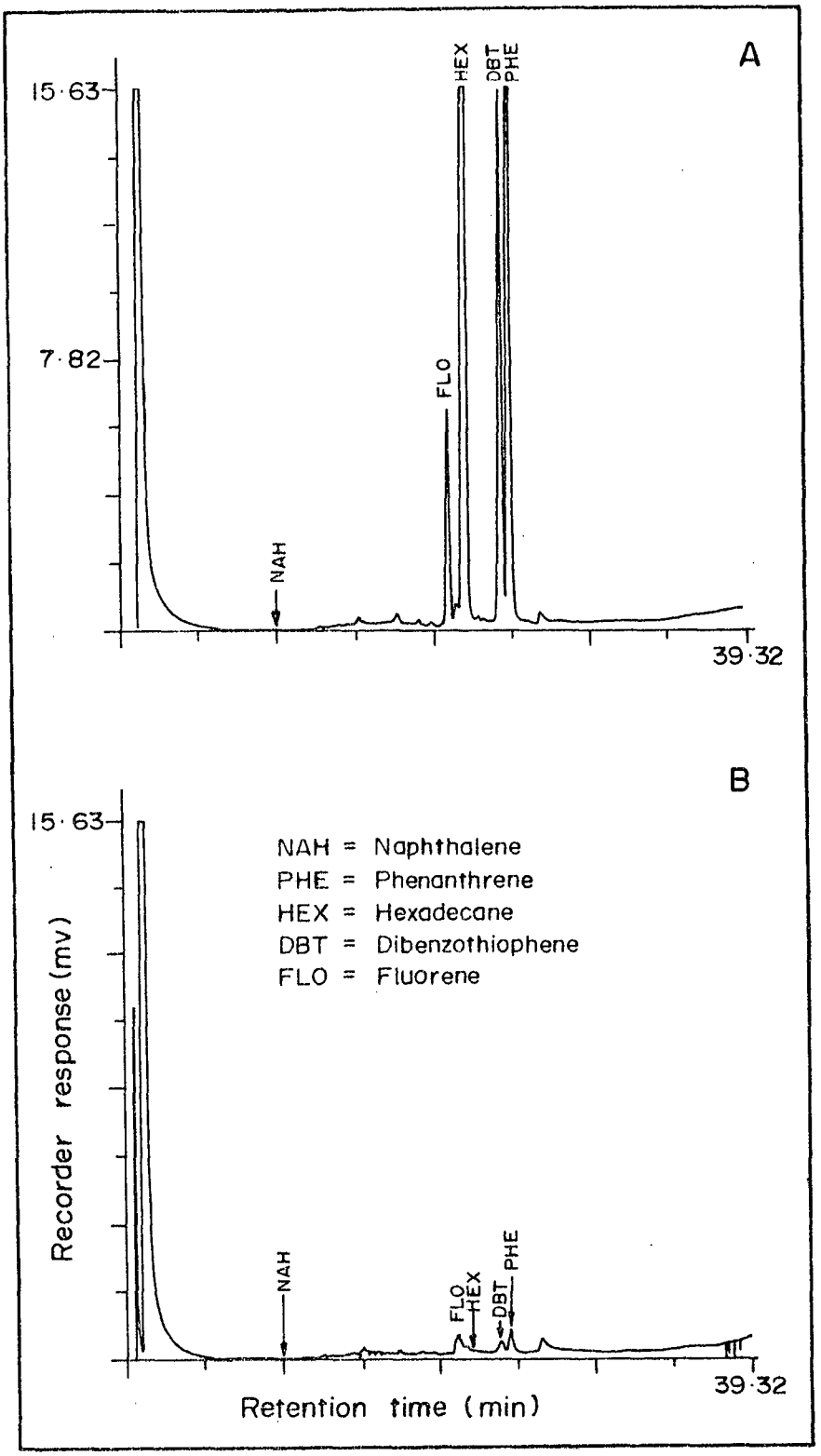


Fig. **5.16** GC profile of **Model Hydrocarbon** Mixture before (**A**) and after (**B**) growth of strain GU110. DBT and all other components within this mixture were utilized. Note: Although fluorene is not used individually as a sole source of carbon by strain GU110 it is attacked when present within a mixture of other HC compounds.

2) Tank A [Test]: Seawater + crude oil/ tarball + nitrates + phosphates + microbial inocula.

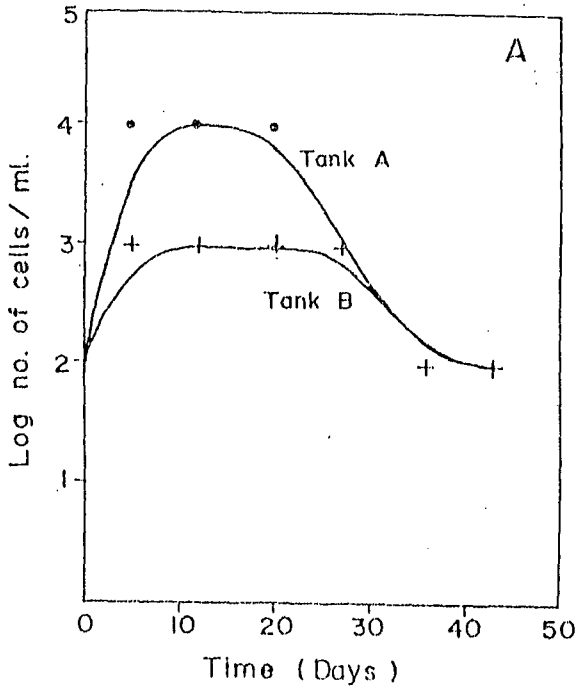
3) Tank B [Control]: Seawater + crude oil / tarball.

4) Tank B [Test] : Seawater + crude oil / tarball + microbial inocula.

Fig. 5.17A shows the growth and survival of *Alcaligenes* sp. strain GU110 on tarballs as part of a seven membered consortium (consortium 1) in an artificially created microcosm with and without nitrogen and phosphorous when compared to the growth of all of the bacterial components of consortium 1 as described in Fig. 5.17B. Similarly, the increase in the GU110 bacterial population on crude oil, when used as a member of consortium 2 was seen in Fig. 5.18A. Survival of all of the bacterial components from consortium 2 was determined on sodium benzoate as shown in Fig. 5.18B. *Alcaligenes sp.* strain GU110 cells remained viable for more than 20 days on both crude oil and tarballs.

DISCUSSION

The biodegradation of hydrocarbons has a high ecological significance as it constitutes the major process for remediation of sites, which are contaminated with hydrocarbons. A better understanding of the fate of polluting hydrocarbons in the natural environments would help in the biotechnological improvement of the biodegradation processes thereby enabling the use of engineered bioremediative processes in such ecosystems. A key point which is only partially understood is the mechanism of uptake by microorganisms of, of these strongly hydrophobic compounds (Bouchez, 1999). Two uptake modes are generally considered for the biodegradation of PAH's (Deziel *et al.*,



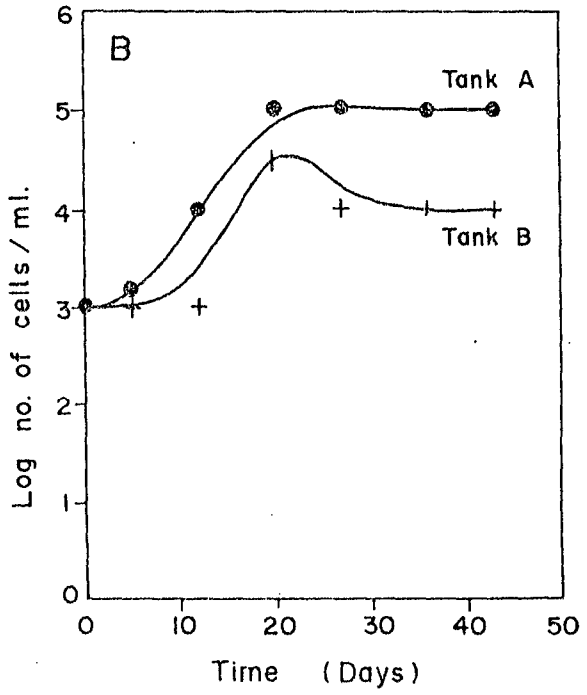


Fig. **5.17** (**A**) Growth and proliferation of strain GU110 on tarball in artificially created microcosms as part of **consortium 1**. Bacterial counts were performed using the MPN method with DBT as the growth substrate.
(**B**) Growth of 7 membered mixture of bacterial HC degraders (consortium 1) on tarball in artificial microcosms containing seawater (**Tank B**) and seawater supplemented with nitrates and phosphates (**Tank A**). Growth was estimated using benzoate as substrate by the MPN method.

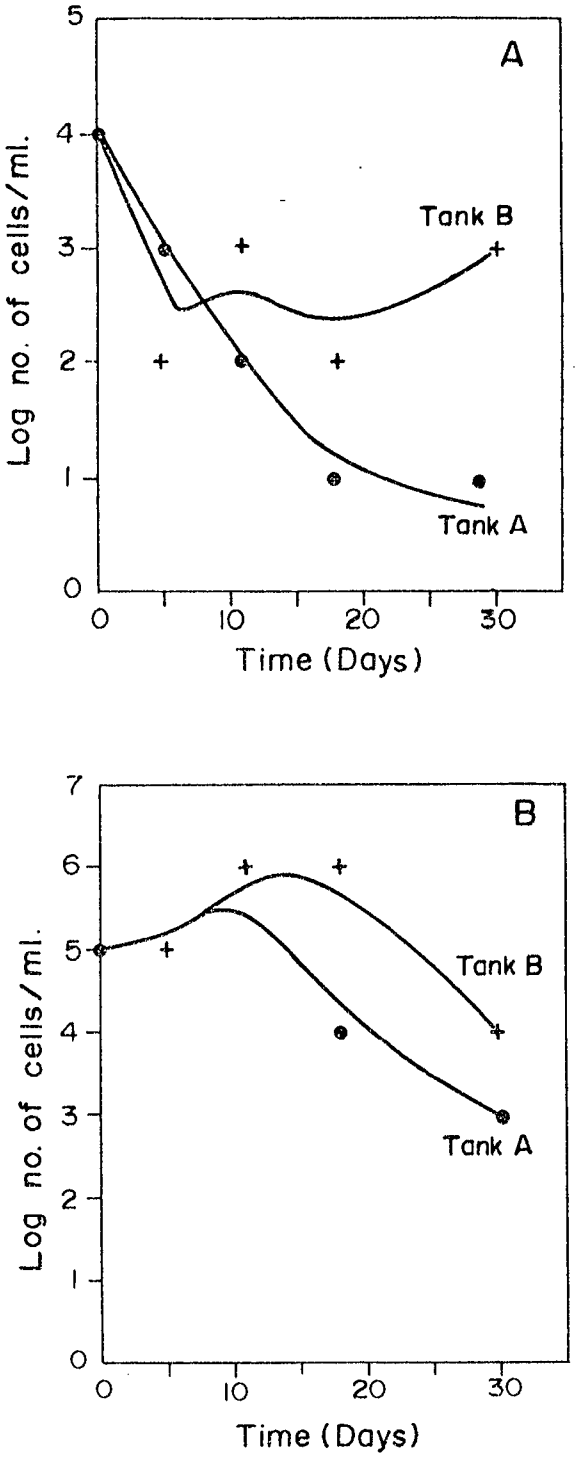


Fig. 5.18 (**A**) Growth of strain GU110 on crude oil, in artificially constructed microcosms as part of **consortium 2**. Bacterial counts were performed using the MPN method with DBT as the growth substrate.
(**B**) Growth of the 4 membered mixture of bacterial HC degraders on crude oil in artificially constructed microcosms containing seawater (**Tank B**) and seawater supplemented with nitrates and phosphates (**Tank A**) estimated by the MPN method using benzoate as the carbon source.

1996; Bouchez *et al.*, 1997) and long chained alkanes (Boulton and Ratledge, 1984; Singer and Finnerty 1984; Haferburg *et al.*, 1986; Hommel, 1994). These include;

1) Interfacial accession or direct contact of cells with hydrocarbon, a function of the cell surface hydrophobicity expressed in terms of % adherence. The main characteristics of interfacial uptake were a high cellular hydrophobicity to allow cell adherence (to the hexadecane phase) and a high interfacial tension of culture supernatant fluid proving that there was no biosurfactant production. (Bouchez *et al.*, 1999).

2) Biosurfactant-mediated HC uptake or cell contact with pseudosolubilized or solubilized hydrocarbons; hydrocarbon uptake is most often associated with the production of surface active compounds called biosurfactants, that enable (pseudo) solubilization and or emulsification of hydrocarbons (Foght *et al.*, 1989; Goma *et al.*, 1973; Goswami and Singh 1991; Reddy *et al.*, 1983; Rosenberg 1986; Zhang and Miller 1992; Deziel *et al.*, 1996). Several researchers have reported the production of biosurfactant by marine bacteria (Reisfeld *et al.*, 1972; Floodgate 1978; Zajic *et al.*, 1974; Rosenberg 1986). These surface active molecules are either released extracellularly or are bound at the bacterial cell surface (Rosenberg, 1986). This extracellular emulsifying agent was assayed for by measuring the ability of the cell free supernatants to form stable emulsions with hexadecane over a period of time (D_{610} assay). *Alcaligenes* sp. strain GU110 was isolated from the marine environment, primarily for its ability to attack a sulfur heterocycle, DBT, but it also possessed the ability to grow on other water-immiscible hydrocarbon substrates.

By growing this marine bacterial isolate on a variety of hydrocarbon substrates, we tried

to discern the mode of hydrocarbon uptake in this strain.

Our studies revealed that;

(i) *Alcaligenes* sp. strain GU110 possessed high degrees of cell surface hydrophobicities but these levels depended on the nature and type of substrate on which strain GU110 was grown.

(ii) External emulsifying activity was low but not absent and could only lead to enhanced hydrocarbon uptake.

This confirmed the fact that adherence or direct contact of cell with hydrocarbon is the primary mechanism of uptake of these strongly hydrophobic compounds by GU110.

GU110 cells grown on DBT and hexadecane revealed high degrees of hydrophobicities compared to cells grown on other aromatic or alkane compounds. Deziel *et al.*, (1996) have reported a *Pseudomonas aeruginosa* strain19SJ which exhibited surface hydrophobicities of the order of 50% when grown on 2% (w/v) naphthalene. The *Alcaligenes* sp. strain GU110 revealed surface hydrophobicities as high as 80% when grown at different concentrations of DBT ranging from 0.01% to 1.0%. The cell surface hydrophobicities displayed by this strain are therefore independent of the concentration of the hydrocarbon on which it is grown.

Growth on n-hexadecane was very rapid, as seen by the high turnover of bacterial cells within 24 hours when grown in an ASW medium containing hexadecane as sole source of carbon and energy. n-Hexadecane has been shown to enhance oxygen supply to organisms, resulting in rapid growth. (Rols and Goma, 1991). Growth of GU110 on longer chained alkanes (C-21) was considerably slower.

The growth of GU110 on hexadecane was accompanied by breakage of the upper

hydrocarbon layer into droplets and the formation of a pink colored surface cream. Microscopic examination of this upper phase indicated that these droplets were covered with patches of adhering cells. These hexadecane droplets were not observed in the aqueous phase. As growth increases, the turbidity of the lower aqueous phase also increases and this was due to the unbound cells. The values presented for growth of GU110 on hexadecane (Fig. 5.6) should be considered minimal, since surface adhered bacteria did not contribute to turbidity at 560nm. Although these cells were unbound, they still possessed the ability to adhere to hydrocarbon and exhibited % hydrophobicities as high as 80%. A similar phenomenon was exhibited by *Acinetobacter calcoaceticus* RAG-1 cells grown on hexadecane (Rosenberg and Rosenberg, 1981) but this organism was known to produce a potent extracellular emulsifying agent, referred to as emulsan. Hexadecane grown *Alcaligenes* sp. strain GU110 cells also produced emulsions which remained stable for more than 30 minutes at 610nm when vortexed with hexadecane and we could therefore infer the production of external emulsifying activity in these cell free supernatants. Bouchez *et al.*, (1999) have described an *Alcaligenes faecalis*, isolated from soils polluted by hydrocarbons, which exhibited a 49% hydrophobicity when grown on hexadecane as sole carbon source. The high adherence (80%) to hexadecane, by GU110 cells could be attributed to the fact that these cells had already been exposed to hexadecane containing medium, when it was used as sole carbon source by the cells.

Microorganisms are known to produce surface active compounds not only during growth on hydrophobic substrates but also on non-hydrocarbon substrates (Cooper and Goldenberg 1987; Guerra-Santos *et al.*, 1994; Mulligan *et al.*, 1989; Reiling *et al.*,

1986). These surface active compounds may therefore be divided according to the type of carbon source used to produce them, such as hydrocarbons, water-soluble molecules, or both (Haferburg *et al.*, 1986). When the *Alcaligenes sp.* strain GU110 was grown on a variety of water-soluble carbon sources, excreted biosurfactant as assayed by the D610 assay, was low except when cells were grown on tryptone. Surface hydrophobicities of these cells, which were grown on hydrophillic moieties, varied depending upon the type of the substrate and its concentration in the media. Lactate grown GU110 cells were highly hydrophobic at low concentrations of lactate (0.2%) and exhibited hydrophobicities as high as 70% but when these cells were grown on other easily utilisable carbon sources the hydrophobicities ranged from 10 to 20 %. According to Rosenberg & Rosenberg (1985) adherence prevails in natural environments (open systems) and emulsification under artificial closed systems. But the hydrophobic nature of GU110 cells prevailed even under in vitro flask conditions.

Growth of *Alcaligenes* sp. strain GU110 on different oils was observed as turbidity, but greater biomass turnover was produced on crude oils, and engine oil than on paraffin oil. Visual examination of flask contents during growth of GU110 on these different oils, as well as on n-alkane hexadecane, as described above, revealed a common phenomenon; the oil-water interface is a hydrophobic interface and after inoculation of bacterial culture GU110, into such a closed system containing oil and ASW, the physical nature of these oils change, where in they tend to get dispersed within the media into fine fiber like threads, immediately followed by an increase in turbidity of the aqueous media. Obviously, cell proliferation is preceded by an emulsification of the

hydrophobic moiety, whereby the increased surface area of the oil droplets leads to greater surface area being made available for better cell adherence. As the cells gain access to these molecules substrate utilization is enhanced and therefore biomass increases. Microbial surface active compounds are known to be ideally suited to mediate the interaction (adhesion and de-adhesion) between micro-organisms and such hydrophobic interfaces (Neu, 1996). *Alcaligenes* sp. strain GU110 grown on the different oils revealed low emulsifying activities but these cells did display surface hydrophobicities ranging from 10 to 30%.

Prominent amongst the oil degrading capacities of strain GU110 is its ability to grow on and utilize crude oil as sole source of carbon and energy. Rambeloarisoa *et al.*, (1984) have shown that crude oil degradation is inversely proportional to the concentration of oil in the medium. The marine species we isolated could produce higher biomass at higher concentrations of crude oil, thus proving that increasing concentrations of crude oil do not adversely affect degradation by strain GU110.

Crude oil is a major sea pollutant and its susceptibly to attack by indigenous marine microbial populations will depend on its chemical composition. Studies concerning the relationship of chemical composition and biodegradability of crude oil have shown that crude oil containing a higher concentrations of alkanes was found to be more susceptible to microbial attack (Walker *et al.*, 1975, 1976). However PAH components of crude oil are toxic and carcinogenic, and their biodegradability decreases with increase in the number of benzene rings (Herbs & Schwall, 1978) viz. ; mono-aromatics > di-aromatics > tri-aromatics > tetra-aromatics > penta-aromatics. Aromatic nuclei containing sulfur were twice as refractory as non-sulfur analogs

(Walker *et al.*, 1975).

Crude oil and hydrocarbon degradation by heterotrophic consortia has been extensively reviewed by Leahy and Colwell (1990). It has been observed that individual organisms could metabolize only a limited range of hydrocarbon substrates (Britton 1984). Few environmental microbial isolates have been isolated that could degrade both alkanes and PAH compounds. Atlas & Bartha (1972a) have isolated a *Brevibacterium* and *Flavobacterium,* which were capable of degrading only the aliphatic fractions of crude oil. Foght *et al.*, (1990) screened 138 isolates for degradation of hexadecane and phenanthrene and observed that none of the isolates mineralized both these compounds. Several environmental strains such as *Acinetobacter calcoaceticus* and *Alcaligenes odorans* (Lal & Khanna, 1996) *Arthrobacter* sp. (Efroymson and Alexander, 1991) and *Rhodococcus* strains (Malachowsky *et al.*, 1994) and a *Pseudomonas* sp. (Whyte *et al.*, 1997) were found to degrade both.

The results obtained in the present study are in contrast to the findings of Atlas & Bartha (1972a) and Foght & Westlake (1990). *Alcaligenes* sp. strain GU110 was capable of degrading both the alkane and aromatic fractions of BHCO with preferential and considerable degradation of alkane fraction as compared to the aromatic fraction. Similar findings have been reported by Lal & Khanna (1996) where-in bacterial soil isolates *Acinetobacter calcoaceticus* S30 and *Alcaligenes odorans* P20 degraded BHCO by 50% and 45% respectively, with both strains showing preferential degradation of n-alkane fraction.

Only 15% (w/w) of the aromatic fraction of BHCO was degraded by strain GU110 when crude oil was used as sole carbon source. When strain GU110 was supplied with

crude oil in combination with S-heterocycle DBT, we observed a greater degradation (35.42%) of aromatic fractions compared to when crude oil was provided alone. The depletion of dibenzothophene from such a crude oil supplemented medium was concomitant with the decrease in the aromatic fraction of BHCO and the increase of polar water-soluble metabolites in the NSO fraction.

In the previous chapter we have seen that *Alcaligenes* sp. strain GU110 possesses a constitutive broad specificity dioxygenase enzyme which encompasses a broad range of aromatic moieties which include mono-aromatics, di and tri-aromatics. When confronted with both DBT and alkane rich crude oil in the medium, the culture attacks the alkanes as well as constitutively attacks the DBT molecule. The same set of enzymes involved in DBT degradation is also responsible for the removal of other aromatic moieties present in crude oil, thereby accounting for the increased degradation of the aromatic crude oil fraction in the presence of DBT. The fact that strain GU110 could remove all the components, including DBT, when grown on the model hydrocarbon mixture, which we formulated in the laboratory, is further evidence that this marine strain could attack alkanes as well as di, tri and substituted aromatics with comparative ease.

After the strain GU110 degraded crude oil, the weight of the NSO or polar fraction increased. Similar findings were reported by Zajic *et al.*, (1974) and Jobson *et al.*, (1972). Increases in this asphaltene fraction after microbial degradation of crude oil probably arise from the production of extracellular compounds during growth which include phenolic acids, carboxylic acids, esters and ketones (Walker *et al.*, 1975).

After degradation of crude oil supplemented with DBT, by strain GU110, the weight of

the polar fraction increases considerably, than when it was grown on crude oil alone. We could attribute this to the formation of polar metabolites or water-soluble compounds produced by strain GU110 on utilization of DBT. Oxidation of other aromatic compounds present in the crude oil by GU110 in the presence of DBT, would also contribute to the enhanced levels of phenolic polar intermediates, when compared to those obtained by growing the culture on crude oil alone. These water-soluble metabolites did not elute out from the silica gel column with the aromatic fraction and thus became part of the polar fraction in crude oil. Similar findings have also been described by Sagardia *et al.*, (1975), Malik (1978) and Fedorak & Westlake (1983) when studying the fate of sulfur heterocycles in the environment.

The complete removal of DBT, which is a representative of the organic sulfur containing component of crude oil, by *Alcaligenes* sp. strain GU110, helps to predict the fate of sulfur heterocycles within crude oil, when it is attacked by such a DBT degrading marine microbe. Biodesulfurizations, where-in bacterial cultures specifically cleave only the sulfur atom from the thiophenic moiety without attacking the carbon ring, is the commercially useful and preferred alternative for removal of sulfur compounds from crude oil. But the role of microbial systems like the *Alcaligenes* sp. strain GU110 to desulfurize crude oil cannot be underestimated as such microorganisms formed water-soluble compounds by the oxidation of sulfur heterocycles like DBT, which could be readily separated from crude oil, thereby enhancing the sulfur removal process.

In situ experiments wherein oil spills are simulated help to predict the fate of oil when it is spilt in the open marine environment, as well as help to determine the remediation

capabilities of microbial seed cultures. In our laboratory, we have isolated, maintained and preserved a host of marine bacterial isolates capable of attacking different toxic hydrocarbon moieties present in crude oils. It has previously been reported (Lal & Khanna, 1996) that a faster rate of degradation of crude oil is achieved by the action of a combination of microorganisms with different hydrocarbon degrading capabilities rather than by a single versatile organism with the capability to degrade both alkanes and aromatic compounds. When these cultures were used as members of a mixed microbial consortium (consortium 1 & 2) to seed oil spills in closed systems, we could track down the culture of our choice and assess its growth by its unique degradative ability. The fate of GU110 in such a closed system, in presence of other aromatic hydrocarbon degraders is quite prominent as seen by the high bacterial counts obtained over a period of more than 30 days. The bacterial numbers obtained were also a measure of the growth of culture on crude oil / tarball and therefore of their degradation. GU110 was therefore not only capable of surviving as a part of the mixed microbial consortium but it was also capable of proliferating on both crude oil and tarball as carbon source.

IN CONCLUSION

Alcaligenes sp. strain GU110 was capable of growing on and emulsifying a variety of hydrophobic moieties ranging from straight chain alkanes to polyaromatics and sulfur containing compounds, and different types of oils. The increasingly hydrophobic property of these cells when grown on such water insoluble molecules confirms that a specific adhesion mechanism is involved in HC uptake wherein the cell comes in direct

contact with the hydrocarbon.

This marine bacterium is capable of growing effectively on crude oil but biodegradation of the crude is by preferential attack of the alkane fraction, although S - heterocycle, DBT when present, is simultaneously removed.

Bioremediative and biodegradative potential of GU110 was evident even when it was present as a member of a mixed marine bacterial population for seeding crude oil contaminated closed aquatic systems, therefore confirming its ability to be used commercially to enhance the oil spill clean up processes.

CHAPTER VI

SUMMARY

The contamination of marine ecosystems with petroleum results in the introduction of a multitude of aromatic and polycyclic aromatic hydrocarbons (PAH's) into the oceans. As a consequence indigenous marine bacterial populations capable of hydrocarbon degradation are enriched. Amongst these aromatic compounds, the thiophenes are the predominant component of the organic sulfur fraction of crude oil. Using dibenzothiophene (DBT) as a representative sulfur heterocycle the distribution of DBT degraders in the water column of the Bay of Bengal was studied. The ubiquity of these bacterial degraders in these open seas was a function of the degree of exposure of these marine ecosystems to hydrocarbons. A marine bacterial strain, which could utilize DBT as sole source of carbon, sulfur and energy was isolated from the coastal waters of the Arabian Sea. After extensive physiological, morphological and biochemical characterization this strain was identified and designated it as *Alcaligenes* sp. strain GU110 (MTCC 3317).

The biotransformation of DBT by this strain was by a set of constitutive enzymes analogous to those described for the biodegradation of naphthalene. Metabolites formed by the oxidation of DBT by this microorganism were similar to those obtained by the carbon-destructive pathway for DBT degradation as described by Kodama *et al.*, (1970 & 1973). These oxidized compounds were more water-soluble than the parent thiophene and did not accumulate in the medium but were produced only transiently.

One of the driving forces behind the studies of biotransformation of hydrocarbons is to comprehend the feasibility of bioremediation in environmental clean up. The *Alcaligenes* sp. strain GU110 possessed a broad specificity enzyme which was capable of oxidizing not only DBT but a variety of hydrocarbon moieties which ranged from

mono-aromatics, di and tri-aromatics to heterocyclic compounds. These compounds were attacked when present individually as well as when present as mixtures in crude oil, tarballs or in artificially formulated hydrocarbon mixtures. The mode of uptake of these water-insoluble hydrocarbon moieties by this marine strain was by interfacial accession as revealed by the high degrees of cell surface hydrophobicities when grown on these substrates. Extracellular emulsifying activity was low but helped to enhance the degradation of these compounds by this bacterial culture. Utilization of crude oil was by preferential attack of the alkane fraction but when crude oil was supplemented with sulfur heterocyles like DBT, this aromatic moiety was also simultaneously removed. This organism could also successfully survive as a member of a microbial consortium comprising of a mixture of bacterial hydrocarbon degraders, when used to seed artificially created seawater microcosms exposed to crude oil and tarballs.

The high degree of adaptability of this naturally occurring strain as revealed by its capacity to survive under nutrient limiting seawater conditions and also by its ability to degrade a broad spectrum of hydrocarbon moieties makes it potentially feasible for seeding of petroleum contaminated sites in the open environment. Our attempts at the isolation of a truly oil degrading marine bacterial isolate have thus been successfully accomplished.

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