Impact of herbicide Butachlor on beneficial soil bacteria

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CERTIFICATE

This is to certify that the thesis entitled " **Impact of herbicide butachlor on beneficial soil bacteria"** submitted by **Arshbir Chandi (301701004)** in partial fulfillment of the requirement for the award of Degree of **Masters in Science** in Department of Biotechnology, Thapar Institute of Engineering and Technology (Deemed to be University), Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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DECLARATION

I hereby declare that the work presented in this thesis **"Impact of herbicide butachlor on beneficial soil bacteria"** submitted by me for the award of the degree of **Masters in Science** in Department of Biotechnology, Thapar Institute of Engineering and Technology (Deemed to be University), Patiala, is the true and original record of my own independent and original work carried out under the supervision of **Dr. Dinesh Goyal.** The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Arshbir Chandi

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301701004

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LIST OF CONTENTS

LIST OF SYMBOLS

%	Percent
℃	Degree celsius
C	Carbon
H	Hydrogen
N	Nitrogen
O	Oxygen
Cl	Chlorine
Hg	Mercury
P	Phosphorus
K	Potassium
Ca	Calcium
Na	Sodium

LIST OF ABBREVIATIONS

et al	And others
mg	Milligrams
g	Grams
kg	Kilograms
ha	Hectare
ml	Millilitres
L	Litres
min	Minutes
h	Hour
mm	Milli molar
µM	Micro molar
µl	Micro litre
nm	Nanometre
N	Normality
M	Molarity
a.i	Active ingredient
ppm	Parts per million
rpm	Revolutions per minute
FR	Field rate
CFU	Colony forming unit
TTC	2,3,5-triphenyltetrazolium chloride
HPLC	High performance liquid chromatography
GC-MS	Gas chromatography-mass spectrometry

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Bioremediation is one of the most important and promising methods for elimination of butachlor as it is cheap, effective and fastest route for degradation. Bioremediation of butachlor was carried out with eight different beneficial soil bacteria. Impact of butachlor on different beneficial soil bacteria in minimal and nutrient media was studied and it showed concentration dependent effect on the bacterial growth. Effect of butachlor on soil microbial activity was examined that involved effect on bacterial population, pH, organic carbon content, available phosphorus and soil dehydrogenase activity. pH of the soil showed initial increase, followed by a decrease till the end of the experiment. Bacterial count decreased with increasing concentration of butachlor; contrastingly organic carbon content and available phosphorus were enhanced even at higher concentration throughout the experiment. Soil dehydrogenase activity was enhanced initially at lower concentrations but decreased with increase in concentration and time. GC-MS data analysis showed complete degradation of 50 ppm butachlor in 7 days by bacterial isolate DGC (*Bacillus licheniformis* MS514).

INTRODUCTION

Pesticides are an important component of global agricultural system as they aid in increased food production (Carvalho 2017)They are applied in an effort to eliminate or prevent plant pathogens and act by inhibiting their biochemical processes. Pesticides cover a broad range of agrochemicals like herbicides, insecticides, fungicides and others. The global pesticides and other agricultural chemicals market was reported to be around $162 billion in 2017 with Asia being largest region for pesticides and other agricultural chemicals market accounting for 41% of the total market. In India pesticide use is about 0.29 kg ha $^{-1}$ which is comparitively lower as compare to other countries such as Japan (11.85 kg ha $^{-1}$), China (13.06 kg ha $^{-1}$) and Brazil (4.57 kg ha $^{-1}$) (Pesticides and other agricultural chemicals market report, 2018).

Herbicides are widely used pesticides for eradication of broad range weeds and can be categorized on the basis of chemical family, chemical structure, time of appication and mode of action. Chloroacetamides are among the most commonly used herbicides worldwide. These act by inhibiting synthesis of lipids or proteins and cell division in seeds (Gao et al, 2015).They tend to persist in the soil for a long duration of time and hence their residues damage crops and are also often detected in nearby water bodies.They have been reported to cause toxicity in marine life, carcinogenecity and stomach tumors in animals (Zhang et al, 2011).

Butachlor is one of the frequently used chloroacetamides and is widely employed in rice, barley and wheat cultivation. Due to its extensive use, it's residues can be often found in ground water and thus it poses a serious threat to agricultural ecosystems and human health (Debnath et al, 2002; Sinha et al, 1995). It is reported as a carcinogen (Dwivedi et al. 2012), neurotoxin (Rajyalakshmi et al. 1996) and genotoxin (Ateeq et al. 2005). Butachlor is known to cause reproduction and growth retardation in earthworms (Gobi and Gunasekaran 2010). It is suspected to induce malignant transformation in vitro (Xu et al. 2007b) and also reported to

immensely affect growth of aquatic macrophtyes (Pan et al, 2009). Therefore, removal of butchlor has become a necessary task.

Many remeditation strategies for removal of butachlor residues are available nowadays, such as adsorption, biodegradation, phytoremediation and nanoremediation. Clays like, bentonite and kaolin have been employed for adsorption of poor water soluble pesticides (Xu et al, 2005). Humic acids from different soils have also been reported to efficiently adsorb butachlor. *Acorus calamus*, was found to exhibit great degradation potential towards butachlor. Mahmoodi et al (2007) reported that titanium dioxide nanoparticle could effectively remove butachlor from polluted water bodies through nanophotocatalysis. *Catellibacterium caeni* sp. nov DCA-1T was able to degrade 81.2 % of 50 mg L^{-1} of butachlor in 84 h (Zheng et al, 2012) and in another study *Trichoderma viride* degraded 98 % of butachlor in 15 days (Alrahman and Bekhit (2013)
Bioremediation is the best method for butachlor removal as it is cost effective and breaks down compounds with higher molecular weight to compounds with lower molecular weight.

In this project, we have studied the effect of butachlor at different concentrations on different bacteria, effect on soil microbial systems which includes properties of soil such as pH, moisture content, organic carbon and phosphorus content, enumeration of bacteria and effect on dehydrogenase activity.

REVIEW OF LITERATURE

Pesticide is a broad term that includes vast variety of compounds like herbicides, insectides, rodenticides, plant growth regulators and molluscides (Aktar et al, 2009). Any substance or mixture of substances with a purpose of inhibiting, destroying, repelling, or weakening any pest is known as pesticide. Pesticides can be categorized in a number of ways on the basis of mode of action, use, chemistry, toxicity and formulations. Major classification of pesticide is on the basis of their target organism, i.e, insecticides, herbicides, fungicides, rodenticides.

2.1 Insecticides

Insecticides are formulated to kill, harm, repel or mitigate one or more species of insect.They cause harmful effects such as they can cause disruption of the nervous system, exoskeleton damage, (Ware and Whitacre, 2004; Bloomquist, 1996)**,** muscle damage and adversely affect physiological functions associated with growth, development and reproduction (Scharf and Suiter, 2011). Insecticides can be categorized on the basis of chemical structure for example, Organohalogen (Lindane), Organophosphorous (monocrotophos), Carbamates (cartap hydrochloride), Pyrethroids (allethrin), Neonicotinoids (thiacloprid) (Ware and Whitacre, 2004).

2.2 Fungicides

Fungicides are pesticides that are used to eradicate or prevent growth of fungi and their spores. They can be used to control fungi that are responsible for damage of plants such as rusts, mildews and blights. Fungicides act in different ways to kill fungi such as causing cell membrane damage, disrupting critical enzymes or proteins or by intrusion of metabolic processes like respiration. Some examples of fungicides are: carboxin, copper oxychloride, captan, ferbam, ziram, mancozeb, maneb, isoprothiolane (Mueller, 2006).

2.3 Rodenticides

Rodenticides are used for killing rodents such as mice and rats. Rodenticides can be categorized based on their mode of action : Anticoagulants that terminate normal blood clotting in the body of its target by interfering with function of vitamin K to produce blood clotting agents, thus causing uncontrollable bleeding that leads to death, other rodenticides act by different means such as

- Bromethalin that inhibits the ability of cells in the nervous system to produce energy, causing paralysis and eventually death;
- Cholecalciferol causes high buildup of calcium in the blood that can affect cardiovasular sytem, nervous system, kidneys of rodents;
- Zinc phosphide's property of converting phosphine gas into acid in the presence of water is employed to kill rodents as phosphine gas being toxic, obstructs cells from producing energy, leading to cell death;
- Strychnine works by causing breathing paralysis due to severe muscle spasms. Other examples of rodenticides are: Aluminium Phosphide, Magnesium Phosphide, bromadiolone, coumachlor, coumatetralyl (Peterson and Talcott, 2005).

2.4 Herbicides

Herbicides are the pesticides employed for eradication of unwanted plants such as weeds and grasses that adversely affect production and growth of desired crops. They can be broadly classified as selective (that target specfic weed species) and non-selective herbicides(that control broad range of weeds, grasses and plants) or according to chemical family, method of application, action site, activity or timing of application. Mode of action of herbicides depends on the chemical family they belong to such as: growth regulators damage hormone balance and protein synthesis (2,4-D), amino acid synthesis regulators inhibit enzyme responsible for synthesis of amino acids (glyphosate), seedling growth inhibitors that adversly affect cell division and lipid and protein synthesis in the seedling (butachlor), photosynthetic inhibitors disrupt electron transfer in photosynthesis (atrazine) and cell membrane disrupters that damage the cell membrane (paraquat) (Cobb and Reade, 2011).

2.5 Butachlor

Butachlor (N-butoxymethyl-2-chloro-N- 2,6-diethyl acetanilide) (Figure.1.) is a choloroacetanilide herbicide applied for the control of a wide variety of annual grasses and some broad leafed-weeds (Pal et al, 2006).

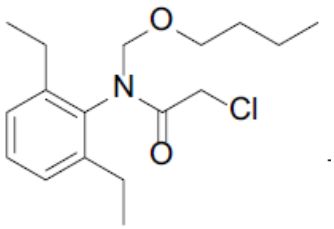

Figure.1. Chemical structure of Butachlor (Singh and Nandabalan, 2018)

It is most abundantly used in countries such as Asia, South America and Africa as a pre-emergence and/or post-emergence in cultivation of rice, wheat, cotton tea, beans, barley, rape, corn, beet, soybean and peanut (Dwivedi et al, 2012; 2010**)**. Butachlor was first produced by Monsanto Company (USA) and was launched in 1968 for the elimination of grasses and weeds (broad leafed) in direct as well as transplanted rice seeds and barley. Annual consumption of butachlor in Asia is approximately 4.5×10^7 kg (Ateeq et al, 2002). It is one of the heavily used herbicides in China, with yearly application reaching 10^7 kg (Zheng et al, 2012). It was initially the first herbicide for rice launched in India accounting for the usage of nearly 6750 metric tonnes per year (Verma et al, 2014; Tilak et al, 2007).

Butachlor is classified under K3 class of herbicides that act by inhibiting enzymes such as elongase that synthesizes very long-chain fatty acids and the geranylgeranyl pyrophosphate cyclization enzymes (He et al, 2013; Götz and Böger, 2004). It also has adverse effect on redox homeostasis and targets other metabolic processes alongwith synthesis of lipids (Agrawal et al, 2014). Furthermore, butachlor also has an impact on cell division , germination of seeds, cell permeability, chlorophyll pigments and metabolism of lipids (Kearney et al, 1988).

Butachlor has a half life of 2.67 to 5.33 days in soil and 1.65 to 2.48 days in field water (Huarong et al, 2010) and its recommended field dosage is around 10 to 150 µM (Alla et al 2008; Chen et al 2007). Due to its brief half life it has been extensiveluy used in rice cultivation all over the world (Mohanty et al 2004; Ateeq et al 2002). Butachlor has an adsorption coefficient of

700.0 Koc and water solubility of 20 mg L^{-1} (Table 1), therefore it is persistent in soil and water bodies.

Table 1: Properties of Butachlor

Properties	Values
Chemical name	N-(butoxymethyl)-2-chloro- N-2,6-diethyl acetanilide
Molecular formula	$C_{17}H_{26}NO_2Cl$
Molecular weight	311.9
Physical state	Clear amber liquid at room temperature
Odor	Faint, sweet odor
Melting point (℃)	0.5-1.5
Boiling point (℃)	156
Density (g ml^{-1}) (at 25℃)	1.070
Water solubility (mg L^{-1}) (20℃)	20
Vapor pressure (mm Hg)(at 25℃)	1.8×10^{-6} at 25℃
Decomposing point (℃)	165
Viscosity (℃p) (at 25℃)	37
Adsorption coefficient (Koc)	700.0
Water partition coefficient (Kow)	4.5
Acute oral LD_{50}	2 g kg^{-1}

Source: Abigail et al, 2015

2.5.1 Environmental Fate and Toxicological Properties of Butachlor

Substantial use of this herbicide over a long period of time has led to damaging effects on soil flora and fauna, also its metabolites have been identified in diverse cultivable soils (Dwivedi et al 2010; Chiang et al 2001). Consisitent use of butachlor in fields tend to cause resistance in weeds over time. Therefore, farmers increase the dose of the herbicide for their eradication, resulting in increase in the concentration of residual herbicide in soil as well as adjacent water bodies (Zhang et al, 2013). Though being used for control of grasses and weeds, butachlor has an unfavourable impact on non-target organisms and the environment (Agrawal et al, 2015). It's residues have

been found in groundwater used for human consumption (Natarajan, 1993). Being a persistent pollutant, butachlor is a possible risk to the agricultural ecosystem and human health via food chains (Yu et al, 2003; Wilson and Takei 2000).

2.5.1.1 Microbial toxicity

Butachlor is known to cause toxicity and mutagenicity in *Nostoc muscorum* (Vaishampayana 1985). It also affects the growth, photosynthesis and nitrogen fixation of *Anabaena doliolum* and *Nostoc muscorum* (cyanobacteria) (Chen et al, 2007; Pandey and Rai, 2002).Butachlor is known to cause remarkable changes in the synthesis of pigments, growth rate and photosynthesis system (II) activities in cyanobacteria (*Nostoc* sp.) (He et al, 2013).

2.5.1.2 Phytotoxicity

Butachlor exposure to Italian ryegrass and cultivars of rice Zhejing 88 (ZJ 88) and Xiushui 134 (XS 134), caused significant cell damage in plant shoot and retarded the growth of the plant (Islam et al, 2016; Wang et al, 2013).Butachlor inhibited photosynthesis, alongwith synthesis of RNA, lipids and protein of isolated leaf cells of red kidney bean (*Phaseolus vulgaris* L.) at a concentration of 100µM (Chang et al, 1985). Butachlor was the cause of inhibition in synthesis of RNA and protein of rice (*Oryza sativa* L.) and barnyardgrass (*Echinochloa crusgalli* L.) root and shoot segments at a concentration of 50 µM (Chang et al, 1985).

2.5.1.3 Aquatic toxicity

Short term exposure of butachlor (30 days) caused impaired reproduction, affected the levels of thyroid hormones and sex steroids in adult Zebra fish (*Danio rerio)*(Chang et al, 2011). Exposure of butachlor at dosages of 3.2 and 0.64 µmol L^{-1} for 10 days in goldfish (*Carassius auratus*) caused extreme damage of gill filaments, disturbance of neurotransmitter balance, cellular edema in livers and kidneys, cellular and mitochondrial membrane and behavioral abnormalities and (Dong-Xu et al, 2014). In a study conducted by Farombi et al, (2008) on antioxidant enzyme system and lipid peroxidation formation in african cat fish (*Clarias gariepinus*), exposure to butachlor(1, 2 and 2.5 ppm) for 24h caused a significant increase in formation of malondialdehyde in the liver, kidney, gills and heart of the fish, suggesting butachlor induced oxidative stress in various tissues (especially kidney) of the fish. Butachlor has also been found

to cause extremely high acute lethality through impairment of the respiration of gills by a series of lesions such as edema, lifting and detachment of lamellar epithelium, breakdown of pillar cells, and blood congestion in marine flatfish flounder (*Paralichthys olivaceus*). In another research, butachlor was found to trigger extreme protein loss in *C. batrachus* at both lethal and sub-lethal concentration which might be due to increase in proteolysis (Muley et al, 2007) or by utilization of ketoacids to glucogenesis pathway for glucose synthesis (Rajput et al. 2012). Tantawy (2002) reported about the biological and biochemical toxicity of butachlor towards freshwater snails viz. *Pila globosa* and *Biomphalaria alexandrina*. On prolonged exposure, it exhibited toxic effects on spotted snakehead fish (*Channa punctata*) (Tilak et al, 2007).

2.5.1.4 Toxicity to mammals and other organisms

Several studies revealing adverse effect of butachlor on different animals have been conducted. Chromosomal aberrations and DNA strand breakage was observed in cultured mammalian cells on exposure to butachlor by Panneerselvam et al, (1999). Furthermore, butachlor is known to trigger necrosis in human peripheral blood mononuclear cells due to their oxidative role in DNA damage and production of intracellular reactive oxygen species (ROS), consequential mitochondrial dysfunction, and chromosomal breakage (Dwivedi et al, 2012).

Butachlor caused slight edema and erythema to New Zealand white rabbits when exposed for 24h. It is also contemplated to be dermal-sensitizer in guinea pig. Butachlor was found to cause mild anaemia (at 5000 ppm in males and 3000 ppm in females), toxicity in liver (only in males at 1000ppm) and decrease in body weight (at 1000 ppm) of F-344 rats. At a dosage level of 5000 -7500 ppm, butachlor was found to be responsible for liver and kidney damage, thyroid organ weight changes and changes in serum biochemistry and hematology in Sprague-Dawley (S-D) rats. Physical signs of toxicity (hair loss and emaciation) and mortality (one female) were also observed at dosage level of 15,000 ppm (Wilson and Takei, 1999). Butachor causes retardation in growth and cocoon production and cause damage to epithelial tissue of earthworm *Eisenia fetida* (Muthukaruppan and Paramasamy, 2009) and *Perionyx sansibaricus* (Muthukaruppan et al, 2005). In another study, butachlor caused mutagenicity in Chinese hamster ovarian cells and in primary rat tracheal epithelial cells (Hill et al, 1997). Butachlor is known to be a neurotoxin to land snails and as a genotoxin to toads, tadpoles, flounder and catfish (Rajyalakshmi et al, 1996; Ateeq et al, 2005; Geng et al, 2005b).

2.5.2 Effect of butachlor on soil microbial systems

Extensive research has been carried out to asses the affect of Butachlor on different microbial populations. It has been reported to enhance as well as inhibit the growth of microorganisms under optimal conditions. Butachlor has been proven to highly elevate growth of *azotobacter*, *arthrobacter*, actinomycetes and fungal sp. at an application rate of 1 kg ha^{-1} (Baboo et al, 2013). At higher application rates, i.e, 2 kg a.i ha^{-1}, high stimulation in growth of fungal sp. was recorded, whereas increase in actinomycete proliferation was observed at 0.5 kg a.i ha^{-1} application rate. Furthermore, it is known to cause significant reduction in heterotrophic bacterial as well as actinomycetes population when applied at 10 FR and 100 FR. Contrastingly, no noticeable destructive effect was observed in case of fungal population even at 100 FR (Latha and Gopal, 2010).

2.5.3 Effect of Butachlor on soil enzymes

Consecutive application of butachlor in soil is reported to effect various biochemical processes in soil, both positively and negatively, which in turn can affect microbial growth and availablity of nutrients to crops. It has inhibitory effect on dehydrogenase activity of soil when applied at high rates such as 1 kg a.i ha^{-1} and 2 kg a.i ha^{-1}, but enhances its activity at 0.5 kg a.i ha^{-1}. Distinctively, at 2 kg a.i ha^{-1}, stimulation of phosphatase activity and peroxidase activity has been observed (Chowdhary and Pal, 2018). Dehydrogenase activity increased initially after butachlor application (2.4 L ha^{-1}) till day 4 and then gradually decreased in subsequent days whereas no variation in urease activity was observed whereas phosphatase, invertase and protease activity followed an increasing trend throughout the experiment (Baboo et al, 2013). Min et al (2007) reported gradual increase in dehydrogenase activity upto 16 day after application of butachlor (22 µg g^{-1} soil) thereby followed by a successive decrease upto final day.

2.5.4 Effect of Butachlor on nutrient transformation in soil

Butachlor interferes with nutrinet content in soil both positively and negatively for instance, the oxidizable carbon content of soil is enhanced when butachlor is added at high concentration (2 Kg a.i ha^{-1}) and organic carbon content moderately increases followed by a decrease and then an increase. Furthermore, butachlor is reported to elevate levels of availaible nitrogen in soil due to

stimulation of microorganisms at high application rate (2 kg a.i ha^{-1}). Also, escalation in the amount of available phosphorus with gradual increase in the application rates was recorded through research and experimentation (Chowdhary and Pal, 2018).

2.5.5 Biodegradation of butachlor

Extensive experimental research has been carried out regarding degradation of butachlor through microbial transformation in recent years. A number of microorganisms have been reported for their capabilty of butachlor degradation (Table 2).

Singh and Nandabalan (2018) reported a bacterial strain, *Ammoniphilus* sp. JF that was capable of using butachlor and its metabolites as sole carbon source and showed complete degradation of butachlor(100 mg L^{-1}) within 24 h. *Enterobacter cloacae* and *Serratia ureilytica* strain AS1 were reported to degrade 2.08 mg L^{-1} h^{-1} and 500 mg L^{-1} (10days) of butachlor under optimal conditions (Mohanty and Jena, 2018). Other bacterial strains such as Hys-1 strain (*Bacillus* species), *Pseudomonas alcaligens*, Fusants F1 of *Rhodococcus* sp. BX2 and *Acinetobacter* sp. LYC-1, *Bacillus subtilis* were found to effectively degrade butachlor upto 83.6% (5 days), 94.7% (21 days), 61.53% (35 days), 86% (12 days) respectively (Gao et al, 2015; Alrahmn and Bekhit, 2013; Feng et al, 2012; Chen and Wu, 1978). A syntrophic pair of *Mycobacterium* sp. J7A and *Sphingobium* sp. J7B was reported by Kim et al (2013) that was capable of complete degradation of butachlor within 1 day over a broad temperature range of 10-37℃ without production of any residual compounds. Similarily another bacterial strain, *Catellibacterium caeni* sp. nov DCA-1T was found to degrade 81.2% of 50 mg L^{-1} of butachlor in 84 h over a temperature range of 15-35℃ (Zheng et al, 2012). Also, *Paracoccus* strain FLY-8 was found to be able to to degrade 25-95% of 100 mg L^{-1} of butachlor within 5 days followed by complete degradation of its metabolites in 7 days (Zhang et al, 2011).

Carascal et al (2017) reported fungal strains, *Neodeightonia subglobosa* (IFM 63572) and *Sclerotium hydrophilum* (IFM 63573) were capable of degrading 94.68% and 89.64% of butachlor (100 ppm) in 5 days. *Trichoderma viride* (Alrahmn and Bekhit, 2013), *Fusarium solani* and *F. oxysporum* (Chakraborty and Bhattacharyya, 1991) were able to degrade 97.6% and >50% of 50 mg kg^{-1} of butachlor in 5 days and three days respectively.

Table 2 : Microbial degradation of Butachlor

S. No	Microbe	Source	Findings	Reference
	Bacteria			
1	*Ammoniphilus* sp. JF	Agricultural fields, Gurdaspur district of Punjab, India	The maximum growth was observed at 108 h of incubation. Corresponding increase in cell density was observed with increase in time and decreasing butachlor indicating the capability of the bacteria to use butachlor and its metabolites as sole carbon and energy source. Two metabolites were obtained at the end of the degradation namely 2,4-bis(1,1-dimethylethyl)-phenol and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	Singh and Nandabalan , 2018
2	*Enterobacter cloacae*	Soil contaminated with effluents from pesticide formulation unit,Odisha,India	The microbial strain was able to tolerate up to 1000 mg L^{-1} of butachlor.Also, removal of butachlor was found to be 97.9% under optimal conditions.	Mohanty and Jena, 2018
3	*Serratia ureilytica* strain AS1	Agricultural field, Odisha,India	It was capable of complete biodegradation of butachlor upto 500 mg L^{-1} within 10 days and has an ability to withstand high concentration of butachlor upto 1000 mg $^{-1}$.	Mohanty and Jena, 2017

4	*Anabaena* sp. PCC 7120	Grown photoautotrophically in BG-11 medium	AKR17A1 enzyme of *Anabaena sp. PCC 7120* reduces acetamide substrates such as butachlor in the presence of cofactor NADPH. Two major metabolites were obtained 2,6 bis (1,1, dimethylethyl) 4,-methyl phenol and 1,2-benzene dicarboxylic acid	Agrawal et al, 2015
5	Hys-1 strain (*Bacillus* species)	Activatd sludge from Hangzhou Qingfeng Agro-chemical Co., Ltd., a pesticide manufacturer in Zhejiang province, China.	Under optimal conditions (temperature 30 °C; pH 7.0;butachlor concentration, 100 mg L^{-1}), hys-1 degraded butachlor rapidly, with 83.6% of the initial dose degraded within 5 days.	Gao et al, 2015
6	*Pseudomonas alcaligens*	Agricultural ground of Alkharj, Saudi Arabia	Reduction percent of butachlor(50mg kg^{-1}) was 94.7 when it was the only source of carbon and nitrogen(21 days)	Alrahmn and Bekhit, 2013.
7	Syntrophic pair of *Mycobacterium* sp. J7A and *Sphingobium* sp. J7B	Rice paddy soil from various locations in south korea	The syntrophic pair J7 completely degraded butachlor in 1 day over a broad temperature range of 10-37℃ without producing any residual compounds. *Mycobacterium* sp. J7A degraded butachlor to 2-chloro-N-(2,6-diethylphenyl) acetamide (CDEPA), which was subsequently degraded by strain *Sphingobium* sp. J7B	Kim et al, 2013

			through DEA.	
8	Fusants F1 of *Rhodococcus* sp. BX2 and *Acinetobacter* sp. LYC-1	Agricultural farm at the Northeast Agricultural University (Heilongjiang Province, China)	The degradation percentages of F1 to butachlor after 35 days were 61.53%. It can simultaneously degrade bensulfuron-methyl and butachlor.	Feng et al, 2012
9	B1 strain (*Rhodococcus* sp.)	Soil samples from a rice field in Changzhou, Jiangsu province, China	Strain B1 could degrade 100 mg L^{-1} butachlor within 5 days. As well as using it as its sole carbon source to support its growth. Two metabolites were produced: 2-chloro-N-(2,6-dimethylphenyl)- acetamide and butoxymethanol out of which only the latter could be degraded further	Liu et al, 2012
10	*Catellibacterium caeni* sp. nov DCA-1T	DSMZ (DSM 21823) and China General Microbiological Culture Collection Center (no 1.7745).	It degraded 81.2% of 50 mg/l butachlor in 84 h. Strain DCA-1T could efficiently degrade butachlor in a relatively broad range of temperatures from 15^0C to 35^0C with more than 59.8% of butachlor degraded in 84 h.	Zheng et al, 2012

11	*Paracoccus* strain FLY-8	Rice field in Jiangsu Province, China,	This strain was able to degrade about 25-95% of the initially added 100 mg L^{-1} butachlor within 5 days. Four metabolites of butachlor were degraded after 7 days	Zhang et al, 2011
12	*Stenotrophomonas acidaminiphila* JS-1	Herbicide-contaminated sandy loam soil of wheat rhizosphere	The HPLC analysis revealed almost complete disappearance of butachlor within 20 days in soil.	Dwivedi et al, 2010
13	*Bacillus subtilis*	Fermentaion Research Laboratory, Department of Agricultural Chemistry of this university.	It reduced butachlor by 86% in 12 days.	Chen and Wu, 1978
	Fungi			
14	i. *Neodeightonia subglobosa* IFM 63572 ii. *Sclerotium hydrophilum* IFM 63573)	Water and wood samples from Taal lake, Phillipines	Butachlor reduction of 94.68% and 89.64% was obtained by *N. Subglobosa* IFM 63572 *S. Hydrophilum* IFM 63573 respectively in 5 days of incubation.	Carascal et al, 2017

15	*Trichoderma viride*	Agricultural fields, Alkharj, Saudi Arabia	It was able to reduce butachlor (50mg kg^{-1}) by 97.6% in 15 days	Alrahmn and Bekhit, 2013.
16	*Fusarium solani* and *F. oxysporum*	Department of Plant Pathology, Faculty of Agriculture, BidhanChandra Krishi Viswavidyalaya, Kalyani,West Bengal, India.	Both the fungi could effectively degrade more than 50% of the total butachlor (50 ppm) applied within three days.	Chakraborty and Bhattacharya, 1991
17	*Mucor sufui* and *M. hiemalis* NTU-363	Fermentaion Research Laboratory, Department of Agricultural Chemistry of this university.	Both *Mucor sufui* NTU-358 and *M. hiemalis* NTU-363 were capable of complete degradation of butachlor into N-chloroacetyl-7-ethyl-2, 3-dihydroindole, 2-chloro-2',6'-diethylacetanilide, 2', 6'-diethylacetanilide and N-methyl-2-chloro-2',6'-Diethylacetanilide, 2-hydroxy-2', 6'-diethylacetanilide and 2, 6-diethylaniline within 12 days.	Chen and Wu, 1978
18	*Chaetomium globosum*	Laboratoire de Microbiologie des sols,INRA,Dijon-CEDEX, France	Butachlor undergoes degradation involving dechlorination in presence of this soil microbe in 7.5 days and production of more than ten metabolites taks place.	Lee, 1977

MATERIAL AND METHODS

3.1 Bacterial isolates

The following bacterial cultures were used in the present study.

Table 3: Bacterial isolates and their properties

S.No.	Bacterial isolates	Source	Name	Gram Character	Property
1.	NA15	Compost	*Bacillus subtilis* HB1	Positive	Cellulose degrading
2.	DGA	Sugarcane bagasse	*Lysinibacillus fusiformis* N139	Positive	Cellulose degrading
3.	DGC	Sugarcane bagasse	*Bacillus licheniformis* MS514	Positive	Cellulose degrading
4.	CT5	Tannary effluent	*Citrobacter* sp. IS2	Negative	Chromium removal
5.	CS8	Chrome sludge	*Citrobacter freundii* IS4	Negative	Chromium removal
6.	*Escherichia coli*	MTCC, Chandigarh	*Escherichia coli*	Negative	-
7.	*Pseudomonas* sp.	MTCC, Chandigarh	*Pseudomonas* sp.	Negative	Phosphate solublizer
8.	*Azotobacter* sp.	MTCC, Chandigarh	*Azotobacter* sp.	Negative	Aids in nitrogen fixation

3.2 Media

3.2.1 Nutrient Broth medium

Composition:	**Ingredients**	**g L^{-1}**
	Peptone	10
	Beef extract	10
	Sodium chloride	5

3.2.2 Nutrient Agar medium

Composition:	**Ingredients**	**g L^{-1}**
	Peptone	10
	Beef extract	10
	Sodium chloride	5
	Agar	12

3.2.3 Bushnell Haas medium

Composition:	**Ingredients**	**g L^{-1}**
	Magnesium sulphate	0.2
	Calcium chloride	0.02
	Monopotassium phosphate	1.0
	Dipotassium phosphate	1.0
	Ammonium nitrate	1.0
	Ferric chloride	0.05

3.3 Butachlor

Butachlor was procured from Chambal Fertilisers and chemicals limited under brand name Uttam Lido.

3.4 Soil

Garden soil from Thapar Institute of Engineering and Technology was procured for this project.

3.5 Effect of different concentrations of butachlor on growth of bacteria in nutrient broth

Requirements

Bacterial cultures: (DGC, CS8, CT15, *Escherichia coli*, *Pseudomonas* sp., *Azotobacter* sp.), test tubes,erlenmeyer flasks, nutrient broth, butachlor,

Procedure

1. Nutrient broth medium was prepared for inoculation of cultures as discussed in section 3.2.1
2. 2% inoculum was added to a flask containing 20 ml nutrient broth medium and was incubated for 24 h at 37℃.
3. Butachlor concentrations (2 ppm – 2048 ppm) were prepared in nutrient broth medium.
4. 120 µl of aliquot of butachlor spiked solutions was pipetted into a titre plate, followed by addition of 50 µl aliquot from overnight grown cultures (OD_{600} 0.01). The titre plates were incubated for 24 h at 37℃.
5. Absorption was recorded at 600 nm to study cell viability.

3.6 Effect of different concentrations of butachlor on growth of bacteria in minimal medium (Bushnell haas)

Requirements

Microbial cultures : DGC, CS8, CT15, *Escherichia Coli*, *Pseudomonas* Sp., *Azotobacter* sp., test tubes, erlenmeyer flasks, bushnell haas medium, butachlor, titre plate,

Procedure

1. Bushnell haas medium was prepared for inoculation of cultures as discussed in section 3.2.3
2. 2 ml inoculum was added to a flask containing 20 ml nutrient broth medium and was incubated for 24 h at 37°C.
3. Butachlor concentrations (2 ppm – 2048 ppm) were prepared in bushnell haas medium.
4. 120 µl of aliquot of butachlor spiked solutions was pipetted into a titre plate, followed by addition of 50 µl aliquot from overnight grown cultures (OD_{600} 0.01). The titre plates were incubated for 24 h at 37°C.
5. Absorption was recorded at 600 nm to study cell viability.

3.7 Effect of butachlor on soil microbial systems

Garden soil collected from Thapar Institute of Engineering and Technology was used in this experiment. Soil was dried and sieved. It was further spiked with 50 ppm butachlor. Control soil without addition of pesticide was maintained under same experimental conditions. Properties of soil such as pH, moisture content, organic carbon and phosphorus content alongwith estimation of dehydrogenase activity were studied in this experiment.

Requirements

Garden soil, butachlor, analytical balance, black polybags, distilled water

Procedure

1. 400 g of dried soil was weighed and spiked with different concentrations (10 ppm, 100 ppm,1000 ppm, 5000 ppm) of butachlor.

2. Soil samples were made in duplicates and transferred to black polybags.
3. These bags were kept in glass polyhouse and soil samples were withdrawn every 7 days till 21 days to determine the change in Viable cell count g^{-1} soil, total organic carbon, available phosphorus and dehydrogenase enzyme activity.
4. Control soil without the pesticide was studied for the same.

3.7.1 Properties of soil

3.7.1.1 Measurement of pH

Soil pH was determined as per the method given by Zhou et al, (1996) in a soil – water suspension of 1:2 ratio using an electronic pH meter.

Requirements

Soil sample, distilled water, beakers (100 ml), pipettes, buffer solutions for calibration of pH meter

Procedure

1. Before beginning the experiment, the pH meter was calibrated over the range of pH 4.0, 7.0, 9.2 using buffer solutions.
2. 5 g of soil was weighed and transferred to a beaker followed by addition of 50 ml distilled water
3. The suspension was stirred vigorously for 2 to 3 minutes and kept undisturbed for 5 minutes. It was stirred again. The experiment was performed in duplicates.
4. The sample was kept undisturbed for 30 minutes.
5. The pH of the soil - water suspension was read using pH meter. Results were recorded when the reading got stabilized.

3.7.1.2 Determination of moisture content

Moisture in soil was determined in percentage water content as per the method of black (1965)

Requirements

Soil sample, analytical balance, aluminium boxes, temperature controlled oven

Procedure

1. Weight of aluminium box was recorded and 10 g of soil sample was weighed in it afterwards.

2. The samples were heated in an oven at 50℃ for 24 h followed by cooling them to room temperature.
3. The change in the weight of the box was noted down and moisture content was calculated by the formula:

$$\text{Water content (\%)} = \frac{\text{Change in weight}}{\text{Initial weight (10g)}} \times 100$$

3.8 Butachlor spiked soil experiment

3.8.1 Enumeration of bacteria

Bacteria present in butachlor contaminated soil were isolated by serial- dilution and agar plating technique (Cappuccino and Sherman, 1987).

Requirements

Soil sample, analytical balance, autoclaved test tubes, saline (0.85%), pipettes, vortex machine, nutrient agar plates

Procedure

1. Nutrient agar plates were prepared as described in section 3.2.2.
2. 1 g of soil was suspended in 10 ml of sterile 0.85% saline.
3. 1 ml of this suspension was serially diluted five times using 9 ml of sterile 0.85 % saline.
4. Aliquots of 0.1 ml of each resting soil suspension were plated onto nutrient agar plates in triplicates
5. Plates were incubated at 37℃ and colonies were counted after 24 h and 48 h.

3.8.2 Estimation of Organic Carbon

Requirements

Soil sample, analytical balance, erlenmeyer flasks (500 ml), glass pipettes, burette, concentrated sulphuric acid (H_2SO_4 ; 98%), orthophosphoric acid (H_3PO_4), 1N potassium dichromate solution

($K_2Cr_2O_7$), 0.5 N ferrous (Fe^{2+}) ammonium sulphate solution, sodium fluoride (NaF), diphenylamine indicator

Procedure

1. 1g of soil sample was weighed and transferred to an erlenmeyer flask. Followed by addition of 10 ml of 1N potassium dichromate solution to the flask and swirled for proper mixing.
2. 20 ml of concentrated sulphuric acid was added through a glass pipette and the mixture was swirled gently. The mixture was allowed to stand for 30 minutes.
3. The suspension was diluted by addition of 200 ml distilled water so as to obtain a clear view of end point.
4. 10 ml of Orthophosphoric acid and 0.5 g of sodium fluoride were added to the mixture, followed by addition of 1ml of diphenylamine indicator.
5. The suspension was then titrated against 0.5 N ferrous (Fe^{2+}) ammonium sulphate solution till the dark blue suspension changed to a grassy green colour indicating end point.

3.8.3 Determination of available phosphorus

Requirements

Soil sample , analytical balance, erlenmeyer flasks (500 ml), whatman Filter paper, filter funnels, glass pipettes, volumetric Flasks (50 ml), orbital shaker, UV spectrophotometer, extracting solution (0.5 M sodium bicarbonate)

Acid molybdate stock solution :

Reagent A – Ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$]

Potassium tartrate ($K_2C_4H_4O_6$)

Sulphuric acid (2.5M)

Distilled water

Reagent B – Ascorbic acid $C_6H_8O_6$, Reagent A

Stock standard P solution : 50 ppm

Working standard P solutions : 2 ppm – 50 ppm

Procedure

1. 2.5 g of soil sample was weighed and transferred it to a flask (500ml), followed by addition of 50 ml extracting solution.

2. Mixture was kept on a shaker at 100 rpm for 30 minutes (25 - 28℃). It was filtered with the help of filter paper into another flask.
3. A 10 ml of aliquot of the filtrate was transferred to another flask. 1 ml of 2.5M sulphuric acid, 15.5 ml of distilled water, 8 ml of reagent B and again 15.5 ml of distilled water were added to the flask. Thorough mixing of the contents was carried out.
4. The mixture was allowed to stand for 10 minutes till blue color is clearly visible.
5. Absorbance was recorded at 882 nm with the help of UV - spectrophotometer
6. Standard curve for phosphorus was prepared by taking 10 ml aliquots of the working standards (2, 4, 6, 8, 10 ppm) and following the same procedure as above.This was used to estimate the amount of avaialable phosphorus in the soil samples.

3.8.4 Estimation of Dehydrogenase activity

Requirements

Soil samples, analytical balance, test tubes (22 ml), whatman filter paper, filter funnels, uv-spectrophotometer, calcium carbonate ($CaCO_3$), 3% triphenyl tetrazolium chloride (TTC), methanol

Procedure

1. 3 g of soil sample was taken in test tube and added 0.3 g of calcium carbonate to it.
2. Then, 1 ml of 3% Triphenyl tetrazolium chloride (TTC) was added to the test tube.The suspension was mixed thoroughly and incubated the mixture for 24 h at 37℃.
3. After incubation, 10 ml methanol was added and the test tube was shaken for 1-2 minutes.
4. The suspension was filtered with the help of Whatman filter paper into a flask. Again 10 ml of methanol was added to the test tube and the contents were filtered to obtain reddish coloured filtrate.
5. Intensity of the reddish coloured filtate indicated the amount of formazan and absorbance was measured with UV- spectrophotometer at 485 nm.

3.9 Degradation of Butachlor by different bacterial isolates

Requirements

Bacterial cultures- (NA15, DGA, DGC, CT5, CS8, *Escherichia coli*, *Pseudomonas* sp., *Azotobacter* sp.), erlenmeyer Flasks , bushnell haas medium, butachlor, test tubes

Procedure

1. Nutrient broth medium was prepared for inoculation of cultures as discussed in section 3.2.1
2. Bacterial cultures were pre inoculated in nutrient broth medium and incubated at 37℃ for 24 h.
3. Bushnell haas medium spiked with 50 ppm butachlor was prepared and inoculated with inoculum whose OD_{600} was adjusted to 0.01 with butachlor spiked medium
4. The flasks were then incubated in shaker at 37℃ for 5 days.
5. Control experiment without bacterial culture was carried out under same conditions.
6. After 24 h, sample aliquots were collected, and bacterial enumeration was done by serial dilution and agar plating.
7. Optical density at 600 nm was recorded every 24 h
8. Aliquot was centrifuged and absorption spectra at 200-800 nm was recorded to study butachlor degradation.

3.9.1 Enumeration of bacteria

Requirements

Bacterial samples (spiked with 50 ppm butachlor), nutrient agar plates**,** saline solution (0.85%)**,** eppendorfs**,** vortex, spreader**,** pipettes

Procedure

1. Nutrient agar plates were prepared as described in section 3.2.2.
2. 1 g of soil was suspended in 10 ml of sterile 0.85% saline.
3. 1 ml of this suspension was serially diluted five times using 9 ml of sterile 0.85 % saline.

4. Aliquots of 0.1 ml of each resting soil suspension were plated onto nutrient agar plates in triplicates.
5. Plates were incubated at 37℃ and colonies were counted after 24 h and 48 h.

$$\text{CFU count} = \frac{\text{No. of colonies} \times \text{Dilution factor}}{\text{Volume of culture plated}}$$

3.9.2 Extraction of Butachlor

Requirements

Bacterial sample spiked with butachlor (50 ppm), eppendorfs, pipettes, dichloromethane, acetonitrile, separating funnel

Procedure

1. 2 ml aliquot was taken from the sample and centrifuged at 12000 rpm for 5 min.
2. The supernatant was transferred to the separating funnel and equal volume of dichloromethane was added to it followed by vigorous shaking.
3. The separating funnel was left undisturbed for 3-5 minutes till two distinct layers were clearly visible.
4. The top layer was collected in a separate flask and bottom layer was extracted thrice with dichloromethane.The top layer collected in three trials was combined and dried over anhydrous sodium sulfate.
5. The resultant residue was re-dissolved in HPLC grade acetonitrile and stored for GC- MS analysis.
6. The GC-MS analysis was achieved in electron ionization mode (70 eV) with Perkin-Elmer clarus 680 GC equipped with Elite-5MS column. The column temperature system was programmed from 100 °C (2 min hold) to 280 °C at10 °C min^{-1} and then held for 20 min. The helium was used as the carrier gas at a constant flow of 1 mL min^{-1}. The samples were analyzed in split mode (1:20) at an injection temperature of 280 °C and detected in the mass range from m/z 30 to 650 (Kim et al, 2013)

RESULTS AND DISCUSSION

4.1 Effect of different concentrations of butachlor on growth of bacteria in nutrient rich medium (Nutrient broth) and minimal medium (Bushnell haas)

At different concentrations, butachlor exhibited both inhibitory and stimulatory effect on different microbial cultures. Figure.2. shows decreasing trend in growth of DGC upto 512 ppm (OD_{485} 0.349) followed by a gradual increase upto 2048 ppm (OD_{485}1.134). Similarily in Figure.3. and Figure.4, CT5 and *Escherichia coli* follow an increasing trend in growth after 64 ppm (OD_{485} 0.206 and 0.039) till 2048 ppm (OD_{485}1.285 and 1.377). The cause of such trends can be credited to adjustive behaviour of the bacterial cells. CS8, (Figure.5.) shows an increasing trend upto 256 ppm (OD_{485} 1.740) followed by a decrease till 1024 ppm (OD_{485} 1.139) and again followed by an increase at 2048 ppm (OD_{485} 1.529). *Pseudomonas* sp. (Figure.6.) showed a steep increase in growth after 512 ppm. Growth of bacterium *Azotobacter* sp. (Figure.7.) follows a steep increase from 64 ppm (OD_{485} 0.674) to 512 ppm (OD_{485} 1.374) followed by stagnation in growth upto 2048 ppm (OD_{485} 1.360).

Bacterial isolates exerted different levels of tolerance capacities towards butachlor concentrations based on their capacity to utilize butachlor as the sole carbon source in minimal medium. Figure.2. and 3. showcase the growth of DGC and CT5 respectively in presence of butachlor at different concentration. Butachlor enhances their growth at concentration higher than 128 ppm and 64 ppm respectively, which depicts that they were able to utilize butachlor as a sole carbon source in bushnell haas minimal medium. In contrast, CS8 (Figure.4.) shows a gradual increase in growth followed by a decrease and again an increase till the end. *Escherichia coli* (Figure.5.), *Pseudomonas* sp. (Figure.6.) and *Azotobacter* sp. (Figure.7.) follow an increasing trend after 64 ppm, illustrating their tolerance towards butachlor and also their capability to use butachlor as an carbon source.

Table 4: Growth of bacteria (OD_{600}) in presence of different concentrations of butachlor after 24 h incubation at 37℃(Nutrient broth)

Butachlor (ppm)	**DGC**	**CT5**	**CS8**	***Escherichia coli***	***Pseudomonas* sp.**	***Azotobacter* sp.**
Control	0.79 ±0.04	0.50±0.04	1.36±0.04	0.86±0.03	1.45±0.04	0.86±0.04
2ppm	0.69 ±0.02	0.33±0.01	1.38±0.03	0.25±0.05	1.25±0.05	0.64±0.01
4 ppm	0.56±0.03	0.37±0.01	1.18±0.02	0.23±0.04	0.81±0.03	0.70±0.02
8ppm	0.60±0.01	0.53±0.01	1.28±0.01	0.29±0.08	0.78±0.03	0.79±0.06
16ppm	0.57±0.01	0.48±0.008	1.44±0.02	0.28±0.08	0.87±0.01	0.85±0.02
32ppm	0.56±0.01	0.36±0.04	1.37±0.005	0.13±0.05	0.79±0.02	0.76±0.03
64ppm	0.54±0.007	0.21±0.01	1.33±0.02	0.04±0.005	0.67±0.005	0.67±0.001
128 ppm	0.57±0.004	0.33±0.01	1.43±0.01	0.26±0.03	0.61±0.01	0.85±0.009
256 ppm	0.42±0.03	0.42±0.01	1.74±0.04	0.76±0.08	0.50±0.01	1.40±0.005
512 ppm	0.35±0.01	0.62±0.05	1.25±0.03	0.83±0.05	0.48±0.01	1.37±0.01
1024 ppm	0.75±0.05	1.07±0.06	1.14±0.02	1.09±0.08	0.82±0.03	1.30±0.01
2048 ppm	1.13±0.07	1.28±0.05	1.53±0.04	1.38±0.03	1.48±0.009	1.36±0.02

Values are average of three replications

Table 5: Growth of bacteria (OD_{600}) in presence of different concentrations of butachlor after 24 h incubation at 37℃(Bushnell haas)

Butachlor (ppm)	**DGC**	**CT5**	**CS8**	***Escherichia coli***	***Pseudomonas* sp.**	***Azotobacter* sp.**
Control	0.42±0.02	0.29±0.05	0.30±0.04	0.57±0.02	0.26±0.01	0.13±0.01
2ppm	0.27±0.03	0.10±0.05	0.37±0.03	0.23±0.04	0.21±0.02	0.19±0.06
4 ppm	0.19±0.04	0.21±0.04	0.35±0.08	0.11±0.03	0.16±0.05	0.24±0.05
8ppm	0.21±0.01	0.14±0.04	0.57±0.04	0.27±0.06	0.15±0.06	0.32±0.01
16ppm	0.21±0.007	0.11±0.02	0.62±0.1	0.36±0.01	0.17±0.05	0.24±0.07
32ppm	0.12±0.004	0.16±0.05	0.17±0.07	0.31±0.05	0.22±0.07	0.19±0.07
64ppm	0.09±0.005	0.09±0.02	0.06±0.01	0.02±0.02	0.04±0.01	0.02±0.007

128 ppm	0.06±0.01	0.18±0.01	0.24±0.01	0.12±0.02	0.12±0.0.5	0.13±0.02
256 ppm	0.29±0.01	0.37±±0.04	0.46±0.01	0.48±0.008	0.46±0.02	0.56±0.03
512 ppm	0.51±0.04	0.47±0/06	0.73±0.08	0.79±0.05	0.72±0.08	0.68±0.06
1024 ppm	0.90±0.05	0.90±0.08	1.53±0.08	1.12±0.04	1.50±0.06	1.62±0.13
2048 ppm	1.59±0.06	1.63±0.1	1.99±0.07	1.87±0.02	1.95±0.05	2.20±0.10

Values are average of 3 replications

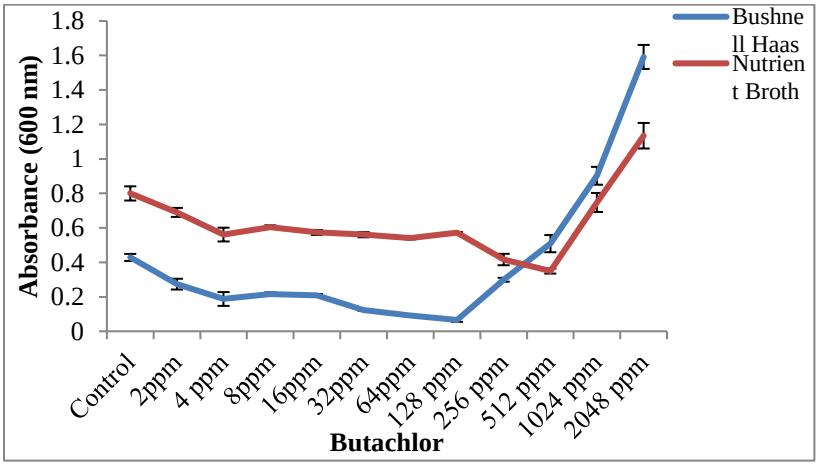

Figure.2. Growth of bacterial strain DGC in presence of different concentrations of butachlor

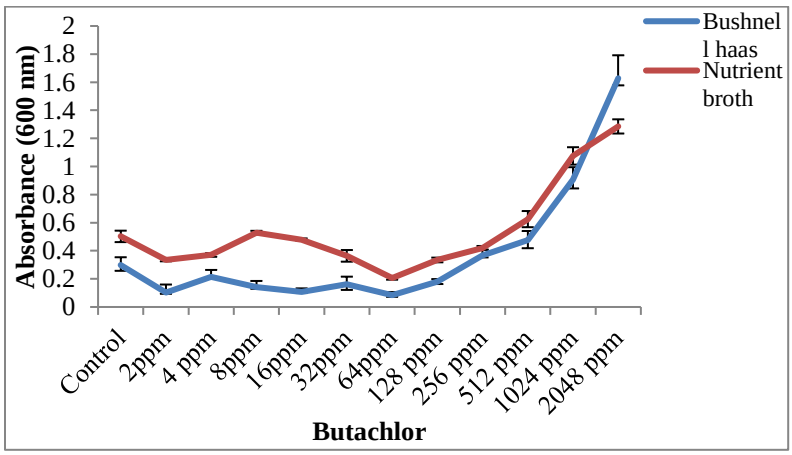

Figure.3. Growth of bacterial strain CT5 in presence of different concentrations of butachlor

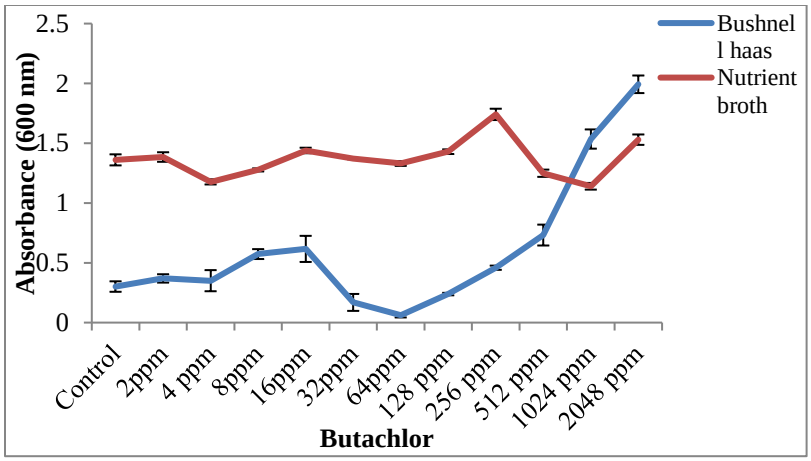

Figure.4. Growth of bacterial strain CS8 in presence of different concentrations of butachlor

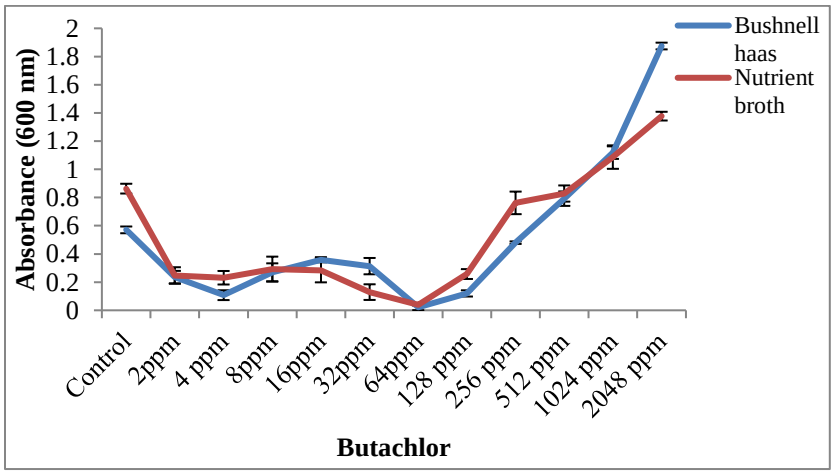

Figure.5. Growth of bacterial strain *Escherichia coli* in presence of different concentrations of butachlor

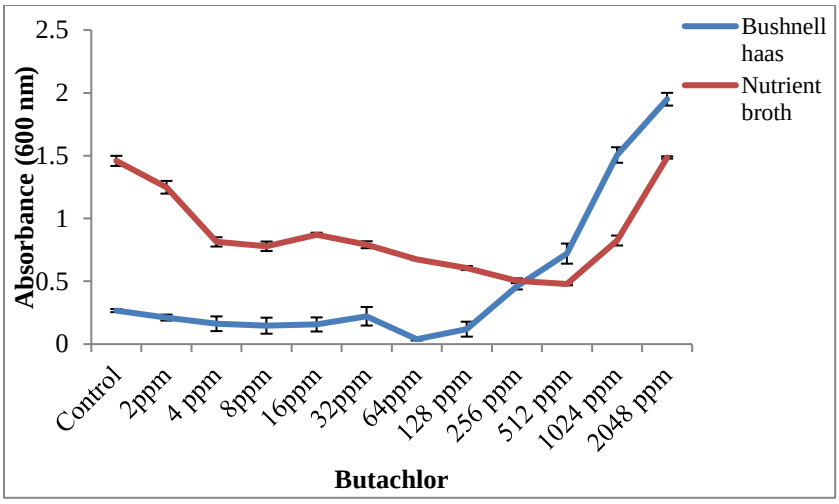

Figure.6. Growth of bacterial strain *Pseudomonas* sp. in presence of different concentrations of butachlor

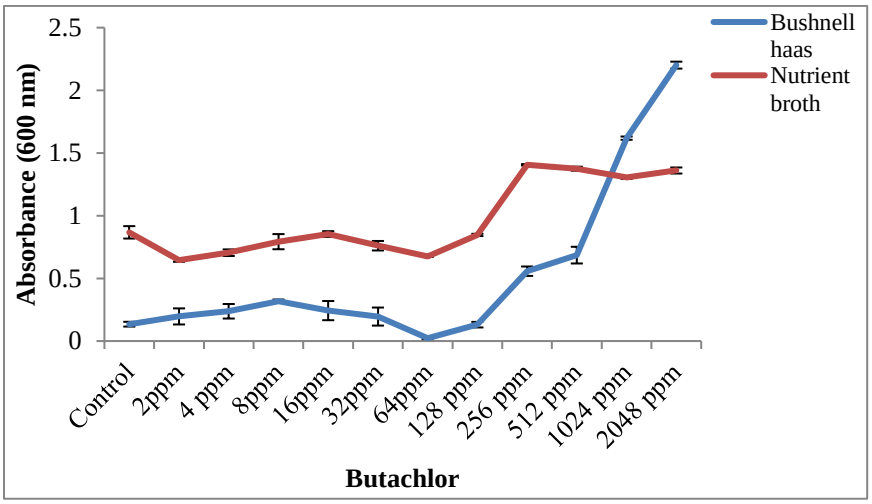

Figure.7 Growth of bacterial strain *Azotobacter* sp. in presence of different concentrations of butachlor

Figure.8. Soil Samples Spiked with different concentrations of butachlor

4.2 Measurement of pH

pH of fresh soil was recorded to be 8.39 and no distinctive change in pH was observed in control soil sample over a period of 21 days. pH of soil containing 10 ppm and 100 ppm butachlor exhibited a decrease followed by an increase at day 21. At initial stages, soil with 1000 ppm and 5000 ppm showed highest pH levels followed by a decrease in later stage.Variations in pH levels may have been due to consumption of the herbicide butachlor as an energy source, leading to decrease in its concentration and hence fall in pH.

Table 6 : pH of soil at different concentration of butachlor at different time intervals

Sampling days	Control	10 ppm	100 ppm	1000 ppm	5000 ppm
Day 0	8.43±0.04	8.41±0.02	8.51±0.007	8.64±0.01	8.72±0.007
Day 7	8.49±0.007	8.52±0.007	8.35±0.007	8.34±0.007	7.83±0.007
Day 14	8.49±0.007	7.88±0.007	7.51±0.01	8.25±0.07	8.5±0.14
Day 21	8.55±0.07	8.33±0.007	8.35±0.007	8.3±0.014	8.12±0.007

Values are average of three replications

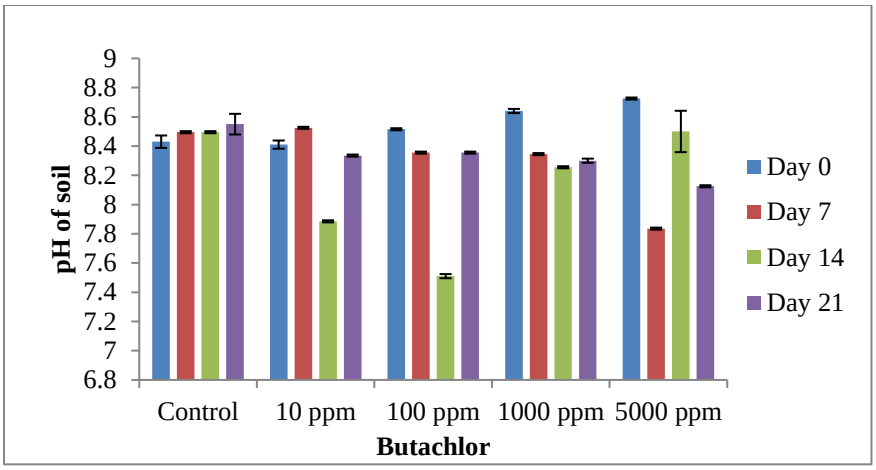

Figure.9. pH at different butachlor concentrations at different intervals

4.3 Determination of moisture content

Moisture content of fresh soil was recorded as 15.78 % and dropped to 6.37 % after the soil was dried in sunlight. During spiking of soil with butachlor, the moisture content was increased by 16.45% and decreased to 5% at the end of the experiment.

4.4 Enumeration of bacteria

Growth of bacteria in control soil followed an increasing trend until the end of experiment in contrast to soil spiked with 10 ppm and 1000 ppm butachlor which exhibited a decrease in growth, followed by subsequent increase and then again declined at the end. Growth of bacteria in soil spiked with 100 ppm declined subsequently throughout the experiment in contrast to growth in soil spiked with 5000 ppm butachlor which gradually increased till the end.

Table 7: Viable cell count g^{-1} soil (x 10^6) at different intervals

Butachlor (ppm)	**0 day**	**7 day**	**14 day**	**21 day**
Control	2.43±0.04	2.52±0.06	2.63±0.07	2.48±0.09
10 ppm	2.29±0.08	1.06±0.09	1.37±0.04	1.21±0.0
100 ppm	2.32±0.08	1.87±0.05	1.18±0.08	1.07±0.07
1000 ppm	2.36±0.05	1.08±0.03	1.18±0.06	1.15±0.07
5000 ppm	2.41±0.09	2.58±0.06	2.70±0.08	1.12±0.04

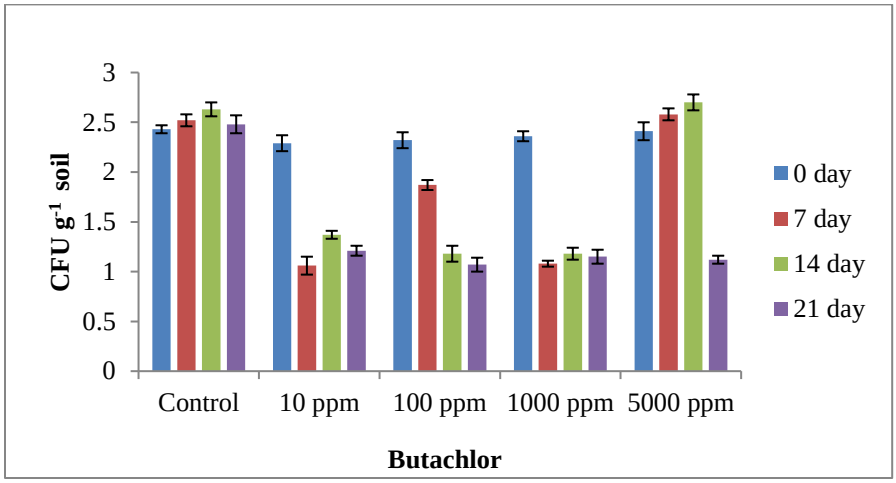

Figure.10. Viable cell count g^{-1} soil (x 10^6) at different concentration of butachlor

4.5 Estimation of organic carbon

Organic carbon content (%) of soil was initially found to be 1.52, but gradually decreased in 21 days to 1.28 (Figure.14.).Soil spiked with 10 ppm butachlor (Figure.15.) exhibited an increasing trend upto 1.76 (14 day) followed by a decrease (1.40) at 21 days. In comparison to control, the carbon content in soil spiked with 100 ppm, 1000 ppm, 5000 ppm increased gradually upto the end of the experiment.This may be due to the ability of the microorganisms in soil to use butachlor as an energy source and carry out various biochemical activities.

Table 8: Organic carbon (%) of soil at different butachlor concentration at different intervals

Butachlor (ppm)	**Sampling Days**			
	0 day	7 day	14 day	21 day
Control	1.52±0.09	1.41±0.07	1.39±0.07	1.28±0.08
10 ppm	1.36±0.08	1.51±0.08	1.76±0.09	1.40±0.07
100 ppm	0.87±0.06	0.60±0.05	1.08±0.08	1.24±0.06

1000 ppm	1.02±0.06	1.07±0.05	1.36±0.08	2.03±0.06
5000 ppm	3.63±0.05	3.69±0.06	3.76±0.09	3.81±0.06

Figure.11. Titration of soil for organic carbon

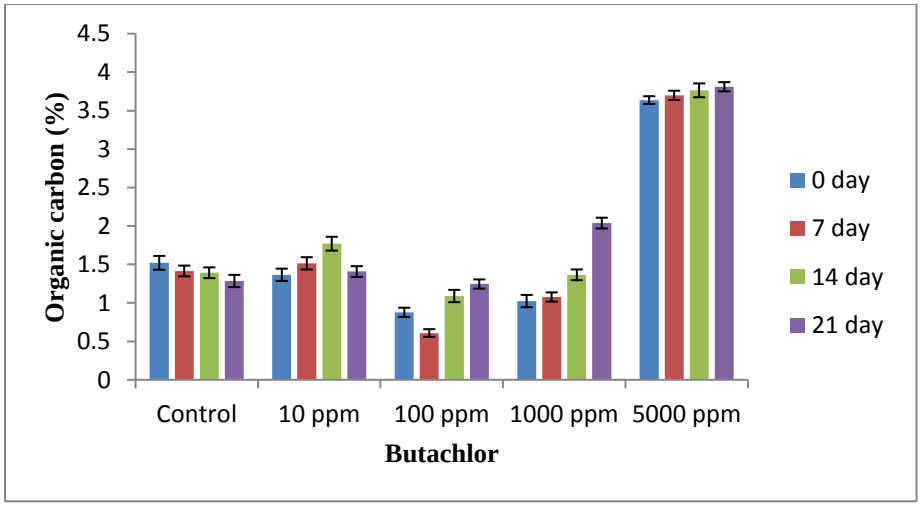

Figure.12. Organic carbon content (%) of soil at different intervals

4.6 Determination of available phosphorus

The available phosphorus content showed an increase (14 day-19.89 ppm) followed by a decrease (21 day – 19.84 ppm in control soil sample as compared to soil sample with 10 ppm ,100 ppm and 1000 ppm in which the phosphorus content decreases after day 7 till the end of the experiment. Figure.22. shows available phosphorus content in soil spiked with 5000 ppm

butachlor that shows an increasing trend upto day 7, followed by a decrease and then again an increase at the final stage.

Table 9: Available Phosphorus (ppm) of soil at different butachlor concentration at different interrvals

Butachlor (ppm)	**0 days**	**7 days**	**14 days**	**21 days**
Control	19.76±0.07	19.94±0.04	19.89±0.08	19.84±0.05
10 ppm	19.88±0.04	19.95±0.07	19.92±0.07	19.88±0.06
100 ppm	19.85±0.03	20.23±0.09	19.89±0.08	19.90±0.06
1000 ppm	19.89±0.08	19.94±0.08	19.92±0.05	19.91±0.04
5000 ppm	19.79±0.04	19.92±0.07	19.87±0.09	19.90±0.07

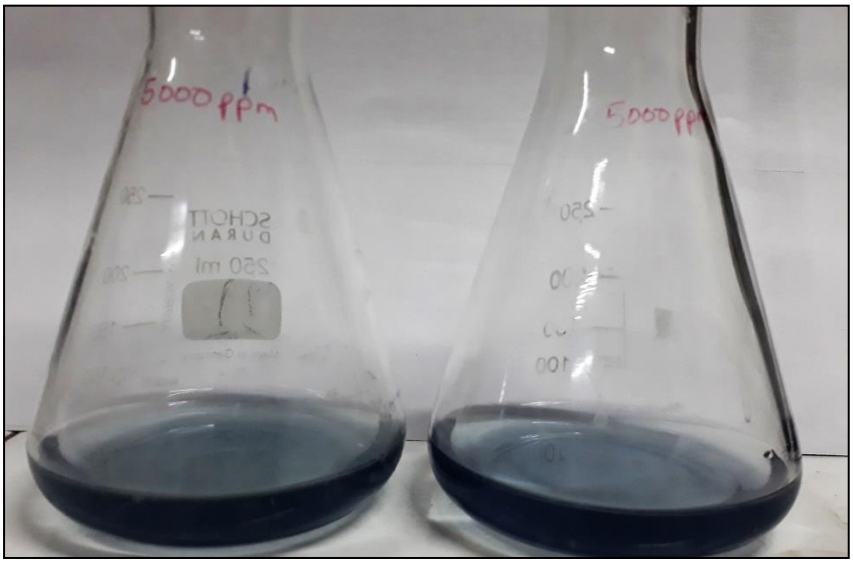

Figure13. Phosphorus estimation of butachlor spiked soil

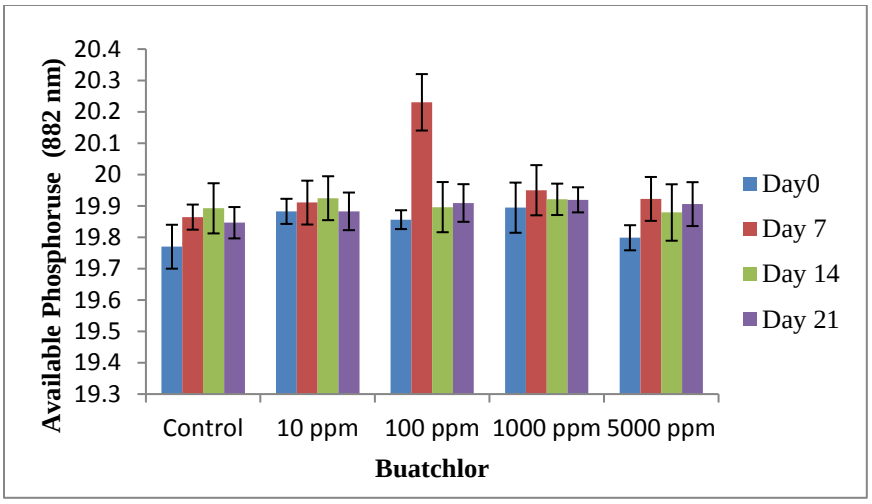

Figure.14. Available Phosphorus (ppm) of soil at different intervals

4.7 Estimation of dehydrogenase activity

Dehydrogenase activity in soil depicts oxidative activities of soil microflora. It was estimated by amount of formazan produced at different intervals. Figure.18. shows the trend of dehydrogense activity which tends to increase at 10 ppm concentration till the 21st day but as butachlor concentration increases the dehydrogenase activity follows a decreasing trend towards the final stage.Hence, butachlor enhances the activity at lower concentrations but inhibits it at higher concentrations.

Table 10: Formazan (mg g^{-1} soil) at different butachlor concentration at different intervals

Butachlor (ppm)	**Sampling Days**			
	0 day	7 day	14 day	21 day
Control	1.8759±0.03	1.8761±0.08	1.8764±0.03	1.8762±0.07
10 ppm	1.8758±0.05	1.8762±0.02	1.8763±0.04	1.876±0.03
1000 ppm	1.8757±0.02	1.8761±0.05	1.8763±0.04	1.8761±0.06
1000 ppm	1.8758±0.01	1.8761±0.06	1.8764±0.07	1.8763±0.06
5000 ppm	1.8757±0.07	1.876±0.02	1.8765±0.01	1.8761±0.07

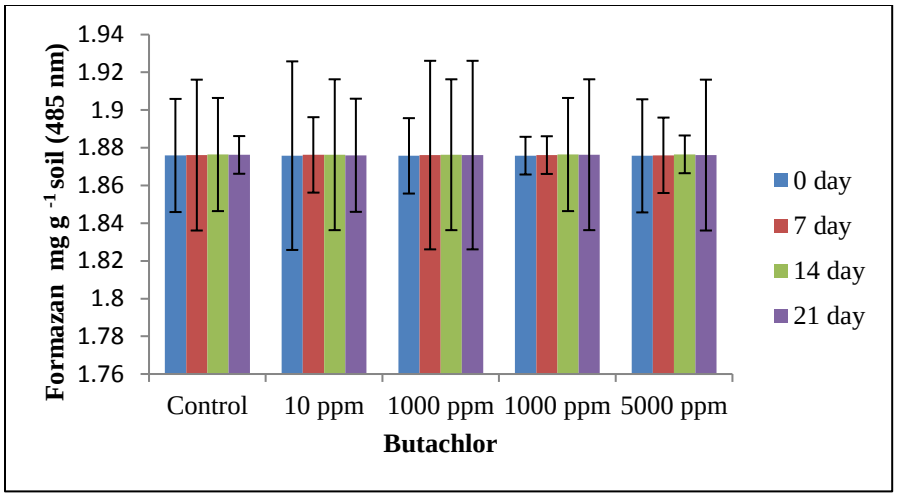

Figure.15. Dehydrogenase activity (mg g^{-1}) of soil at different intervals

4.8 Degradation of butachlor by different bacterial isolates

4.8.1 Enumeraion of Bacteria

Growth of NA15 increased initially followed by a decrease and then again increased till the end; DGA followed a trend of increased and decreased growth throughout the experiment.DGC exhibited the best growth trend among all bacteria as it increased with time. CT5 and CS8 and *Escherichia coli*. do not follow any specific growth trend. Growth of *Pseudomonas* sp. Initially increased but declined towards the end of the experiment. *Azotobacter* sp. Growth was pronounced during initial days but decreased towards the end.

Table 11: Viable cell count g^{-1} soil (x 10^8) of bacterial cultures at different intervals

Sampling Days		**Bacterial cultures (x 10 ^{8})**						
	NA15	DGA	DGC	CT5	CS8	*Escherichia coli*	*Pseudomonas* sp.	*Azotobacter* sp.
Day 1	23±0.003	9±0.007	11±0.01	18±0.02	4±0.02	29±0.02	10±0.006	9±0.04
Day 2	1±0.3	3±0.01	21±0.01	10±0.02	21±0.03	13±0.04	10±0.05	19±0.02
Day 3	12±0.007	3±0.01	25±0.01	14±0.02	20±0.04	20±0.01	11±0.01	8±0.01

Day 4	22±0.01	42±0.008	34±0.01	16±0.005	14±0.09	30±0.02	14±0.005	22±0.01
Day 5	26±0.06	29±0.03	38±0.03	11±0.02	12±0.05	23±0.02	11±0.006	21±0.006

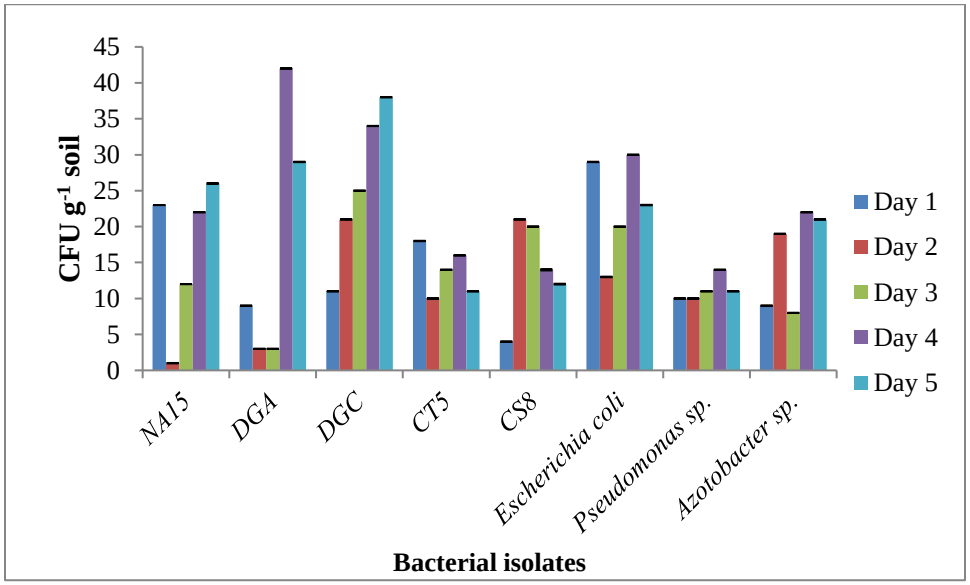

Figure.16. Viable cell count g^{-1} soil (x 10^8) of bacterial cultures at different intervals

4.8.2 Residual concentration of butachlor

Table 12: Residual concentration of 50 ppm butachlor

Sampling Days		**Absorbance (265 nm)**						
	NA15	DGA	DGC	CT5	CS8	*E.coli*	*Pseudomonas* sp.	*Azotobacter* sp.
0 day	0.56±0.04	0.56±0.02	0.56±0.07	0.56±0.02	0.56±0.03	0.56±0.04	0.56±0.01	0.56±0.05
1 day	0.40±0.02	0.18±0.01	0.15±0.03	0.17±0.06	0.24±0.01	0.12±0.08	0.13±0.01	0.12±0.04
2 day	0.29±0.04	0.18±0.03	0.19±0.01	0.17±0.07	0.23±0.01	0.14±0.02	0.15±0.03	0.13±0.07
3 day	0.32±0.06	0.21±0.07	0.22±0.01	0.21±0.03	0.26±0.01	0.16±0.08	0.14±0.05	0.15±0.05
4 day	0.33±0.05	0.23±0.01	0.24±0.05	0.23±0.01	0.29±0.06	0.17±0.07	0.17±0.07	0.14±0.05
5 day	0.29±0.02	0.21±0.06	0.20±0.08	0.19±0.03	0.22±0.02	0.13±0.01	0.12±0.03	0.06±0.01

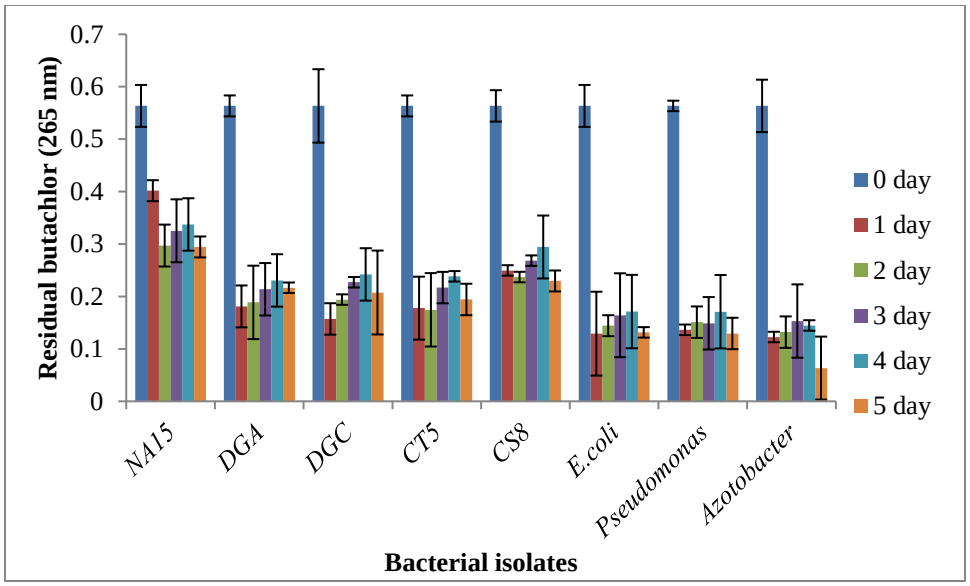

Figure.17. Residual concentration of Butachlor after 5 days

5.0 Extraction of Butachlor

Bacillus licheniformis MS514 (DGC) was chosen for butachlor degradation as it was able to grow remarkably well in presence of 50 ppm butachlor. Butachlor degradation after seven days was studied by GC-MS.

Only one peak [Rt 17.21] was found in the control, which was identified as butachlor using the identification program of the NIST library. No major metabolites were detected in degraded sample, suggesting complete degradation of butachlor into other metabolites.

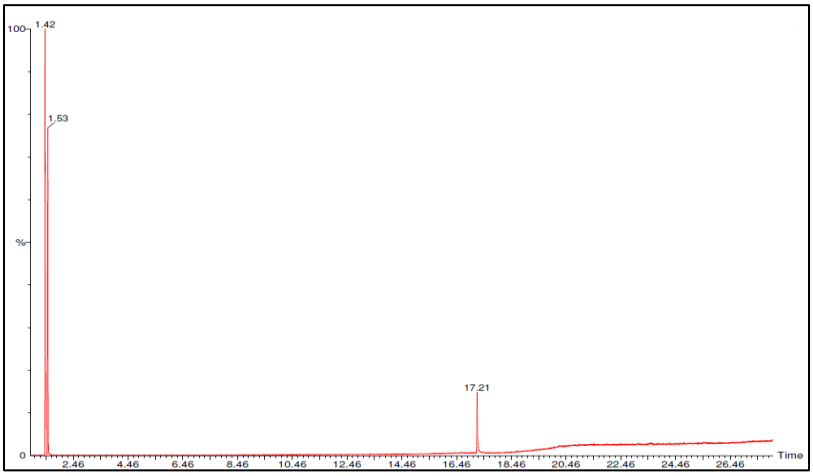

Figure.18. GC-MS of Butachlor

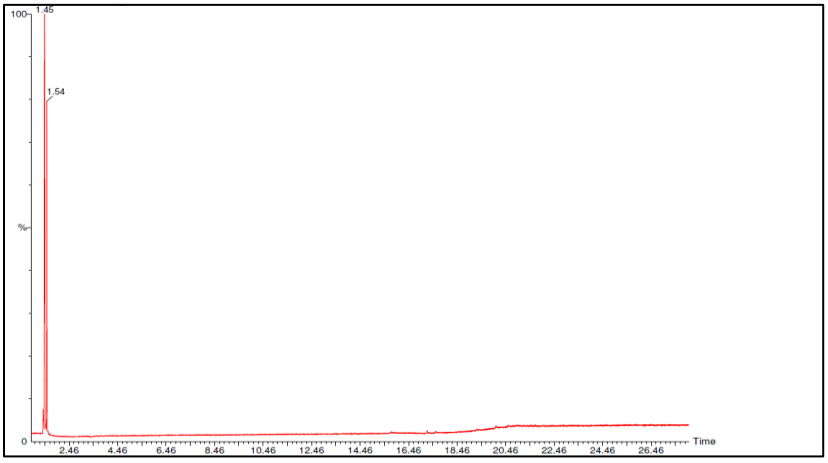

Figure.19. GC-MS of butachlor spiked bacterial culture

CONCLUSION

1. At different concentrations, butachlor exhibited both inhibitory and stimulatory effect on different microbial cultures. Growth of some bacteria in nutrient broth as well as bushnell haas broth is enhanced at higher concentration of butachlor and this can be accounted to the fact that more amount of butachlor is available to the microbial cells to utilize as an energy source**.**
2. pH of soil was highest immediately after addition of butachlor but decreased with time. The main reason for this may be that butachlor dissolves in water and dissociates into ions. Gradual decrease in pH over time can be accounted to the fact that the microbes present in soil consume the butachlor as the main carbon source thus causing decrease in available ions from butachlor.
3. Growth of bacteria in butachlor spiked soil at 10 ppm and 1000 ppm initially decreased follwed by an increase and then again declined at the end. This may be due to the reason that initially growth decreased because bacteria were not able to cope up wth the environment and after sometime, when they were able to utilize butachlor, growth increased and when their was accumulation of toxic metabolites, the growth declined.
4. The carbon content in soil spiked with 100 ppm, 1000 ppm, 5000 ppm increased gradually till the end . This may be due to the ability of the microorganisms in soil to use butachlor as an energy source and carry out various biochemical activities and release organic carbon. The available phosphorus content followed a non uniform trend throughout the experiment.
5. Dehydrogenase activity decreases with increase in butachlor concentration. As it depicts the oxidative activities in soil , it can be inferred that high concentration of butachlor is harmful for the soil microflora.
6. *Bacillus licheniformis* MS514 (DGC) effectively degraded butachlor in 7 days without production of any residual metabolites.

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