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Biodegradation of Carbofuran by Naturally Occurring Soil Borne Bacteria

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A Thesis Submitted to the University of Rajshahi for the Degree of Master of Philosophy in Biological Sciences

By

Md.Akhtar-E-Ekram

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Rajshahi-6205, Bangladesh

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M. Phil. Thesis

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DEDICATED
To my
Daughter Rubai

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled **"Biodegradation of Carbofuran by Naturally Occurring Soil Borne Bacteria**" prepared for submission to the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh for the degree of Master of Philosophy, are the original research works of mine and have not been previously submitted for the award of any degree or diploma anywhere.

(**Md. Akhtar-E-Ekram**)
Signature of the candidate

CERTIFICATE

This is to certify that the thesis entitled "Biodegradation of Carbofuran by Naturally Occurring Soil Borne Bacteria" is the record of an original research done by Md.Akhtar-E-Ekram at the Institute of Biological Sciences, University of Rajshahi, Bangladesh, under our supervision for the degree of Master of Philosophy. All the data presented in this thesis are based on his own investigation. It contains no material previously published or submitted for any other degree.

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The Author

ABSTRACT

Biodegradation of carbofuran (Furadan) used as a pesticide, was investigated using microorganisms isolated from carbofuran treated rice field soil with a history of pesticide application. Samples of carbofuran treated soil were incubated in mineral salts medium supplemented with Standard carbofuran (99% purity) at 2 µg/ml,4 µg/ml, 8µg/ml and 16 µg/ml concentration for 7 days at 30°C and bacterial strain were isolated from the soil sample which were supplemented with standard carbofuran at 2 µg/ml and 4 µg/ml. No viable growths were found at 8µg/ml and 16µg/ml carbofuran concentrated MS media. The isolated bacteria were examined microscopically for their morphological and measured optical density (OD) to determine physiological characteristics at various temperature and pH. Morphological, physiological characteristics and biochemical properties indicated that the bacterium was gram negative, aerobic, lactose fermenting, catalase positive, oxidase negative, indole negative, methyl red negative, triple sugar iron test positive. The optimal culture condition of the bacterium for carbofuran degradation was p^H 7.0 and temperature at 30°C. The antibiotic resistance pattern is generally used for strain identification in ecological studies. It is evident from the present investigation that the isolated bacteria were resistance to nalidixic acid(NA30) and cefuroxime sodium(CXM30) and have the ability to utilize different carbohydrates viz.glucose, lactose, sucrose, mannose, arabinose and cellulose. The MIC of gentamycine against the isolated bacteria was also determined and the concentration was 400µg/ml. Viable cell counts were also done and 459×10^7 CFU/ml. Carbofuran toxicity was also evaluated on *Artemia salina* and LC_{50} value was 9.103558µg/ml for the *A. salina* and the regression equation was Y = 3.942777 + 1.102179 X, while the 95% confidence limits were 3.810799 to 21.74736 µg/ml for 12h of exposure only . The antagonistic effect of

the isolated bacteria was tested against some pure culture strain of bacteria and fungi. Unfortunately the bacteria did not show any antagonistic effect on plant pathogenic fungi and fortunately it exhibited no effect on Rhizobacteria. Thus, future application of the carbofuran degrading bacteria at field level would not be harmful against any plant growth promoting *Rhizobium* sp. Biodegrading ability of the isolated bacterium indicate a positive role of microorganism in pesticide contaminated sites bioremediation. So, the study recommends possible application of the isolate in the *In vivo* bioremediation of pesticide contaminated soils and assure pesticides hazards free better environment.

ABBREVIATION

EDTA	-	Ethylene diamine tetra acetic acid
TBE	-	Tris base boric acid EDTA
SDS	-	Sodium dodecyl sulphate
MS	-	Mineral salt
HPLC	-	High Performance Liquid Chromatography
PCR	-	Polymerase Chain Reaction
ICDDR,B	-	International Center for Diarrhea Disease Research,Bangladesh
OD	-	Optical density
mm	-	Millimeter
Kg	-	Kilogram
Kb	-	Kilo base
ppm	-	Parts per million
rpm	-	Rotation per minute
Viz	-	Vizard (namely)/ that is to say
Min.	-	Minute
hr.	-	Hour
No.	-	Number
µg	-	Micro gram
µl	-	Micro liter
mg	-	Milligram
ml	-	Milliliter
gm	-	Gram
Fig.	-	Figure
e.g.	-	For example
p^H	-	Negative logarithm of hydrogen ion concentration
i.e.	-	*id. est*, that is
etc.	-	*Exempli* gratia, for example
et al.	-	And others
ed.	-	Editor, edition, edited, edited by
%	-	Percentages
&	-	Punctuation

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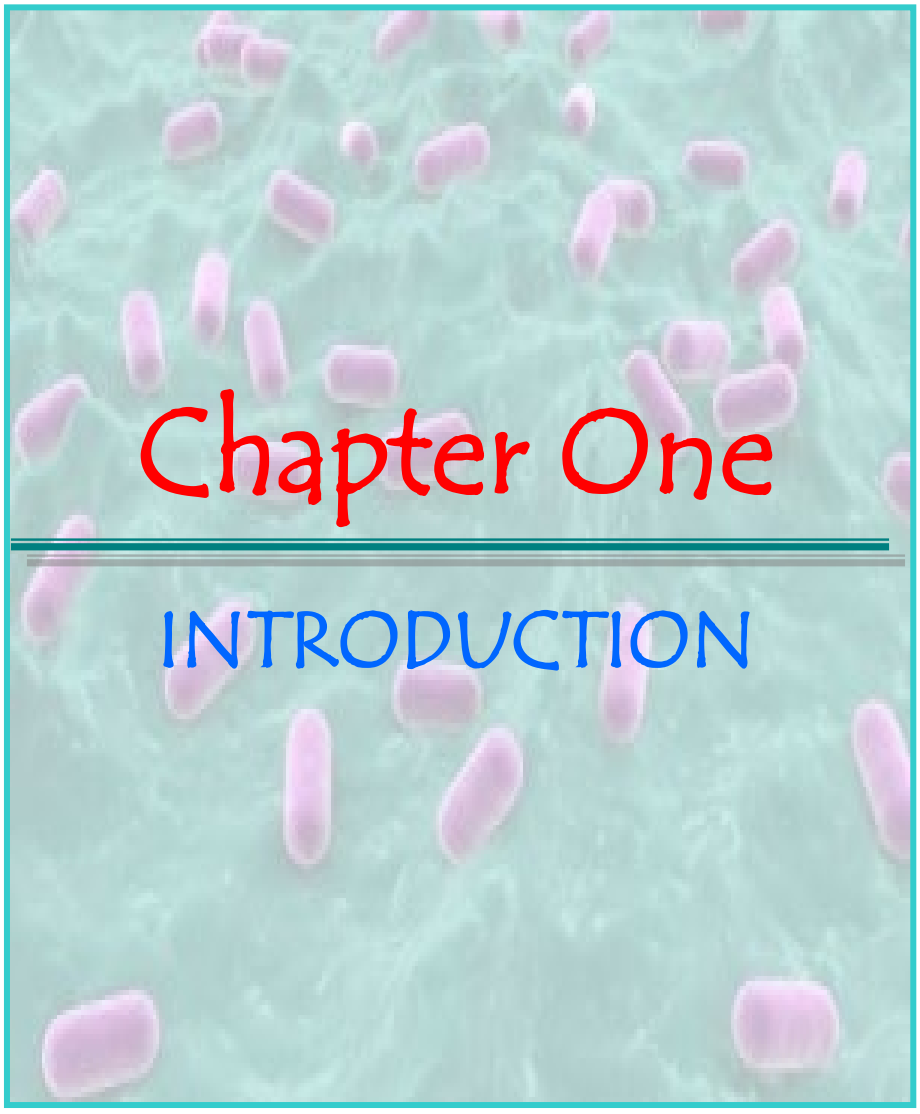
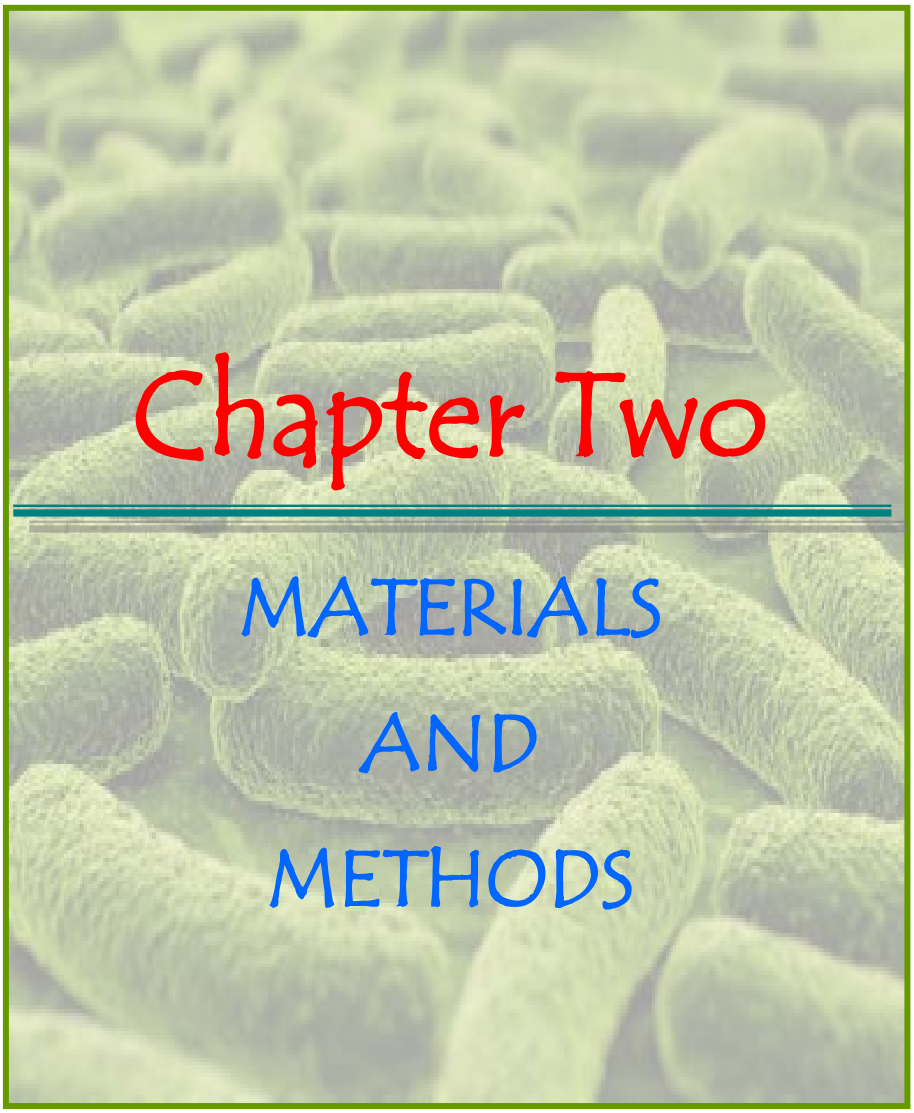


Chapter One

INTRODUCTION



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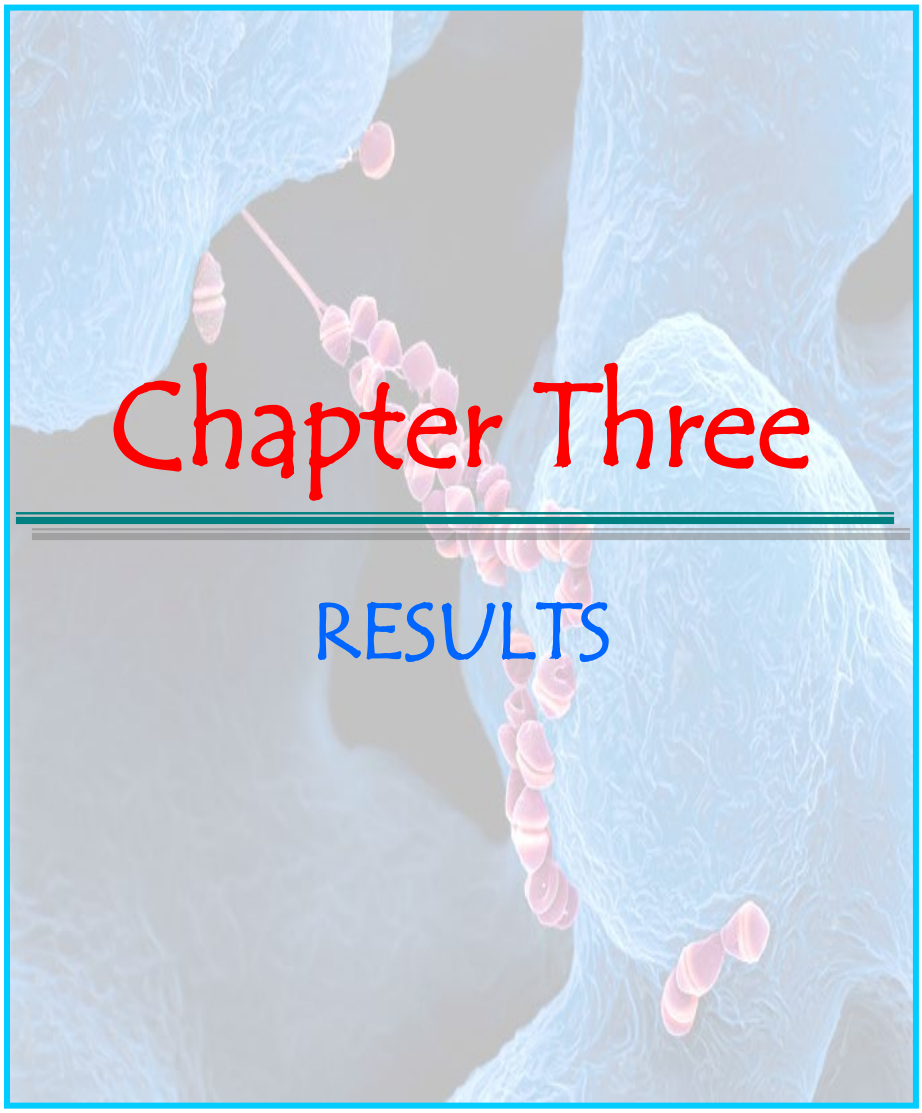
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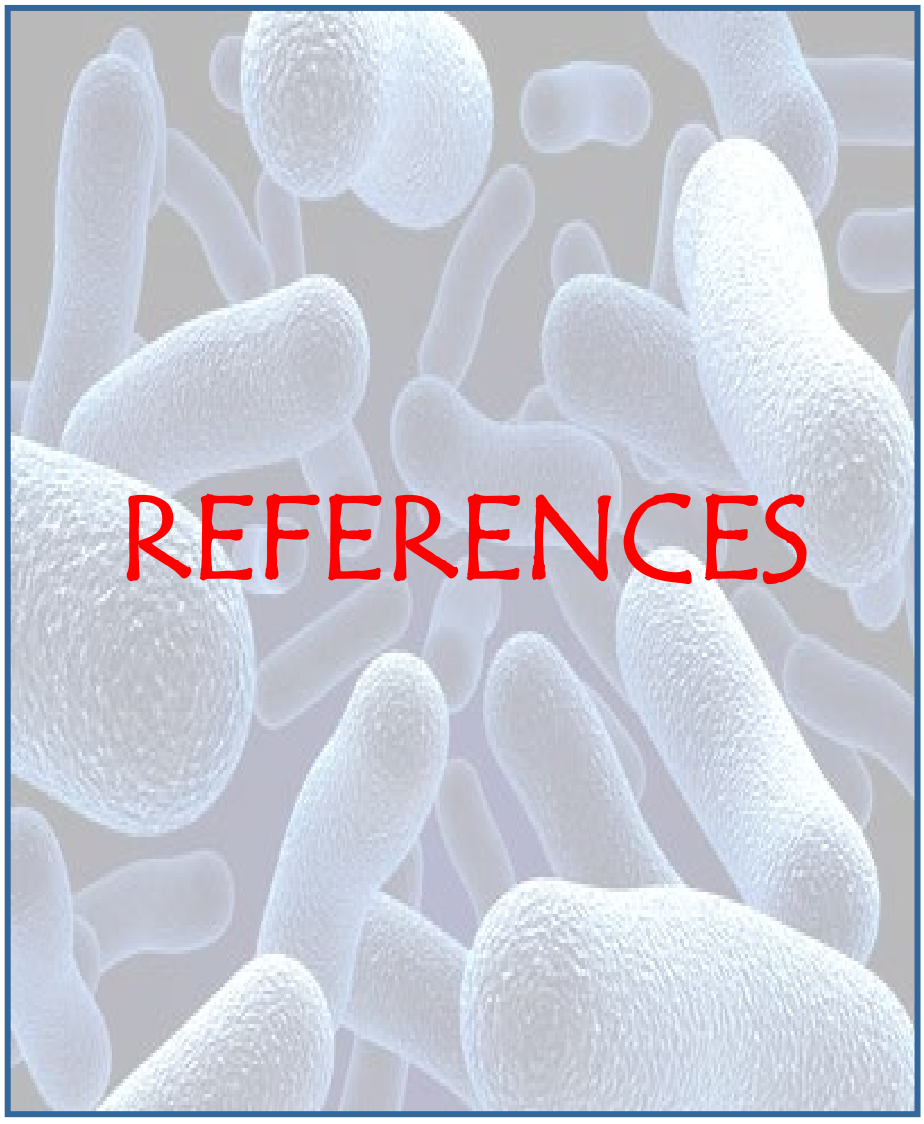
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INTRODUCTION

1.1 Introduction

Biodegradation is a viable bioremediation technology for organic pollutants. It has known that microorganisms degrade environmental pollutants such as insecticides, pesticides etc. in various matrices and environments. Bioremediation utilizes the metabolic versatility of microorganisms to degrade hazardous pollutants. A goal of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water (Alexander, 1999). Persistent organic pollutants (POPs) such as pesticides are among the most concerned environmental pollutants because they persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to the environment and human health.

The term "pesticide" covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscacides, nematocides, plant growth regulators and others. Insecticides represent the greatest proportion of pesticides used in developing countries, whereas herbicide sales have been greater than those of other pesticides in industrialized countries. About 1500 chemicals are registered for use in thousands of pesticide formulations in the world; however, among them less than 50 pesticides account for about 75% of the total uses. (Salem & Olajos, 1988). About 25% of all pesticides are presently used in developing countries, mainly on cash crops. (WHO/UNEP, 1990).

In Bangladesh pesticide was introduced as agricultural input in 1957. Till 1973, DDT and BHC was distributed by the Government to the farmers free of cost. At present 84 pesticides with 242 trade names have been registered in Bangladesh (PAB, 2000). The most commonly used pesticide under different trade names include Carbofuran, Diazinon, Fipronil, Carbosulfan, and Chloropyrifos among the granular form; Cypermethrin, DDVP, Diazinon, Dimethoate, Monocrotophos, Malathion,

Phosphamidon, Phenthoate, Fenitrothion, and Bidrin among the liquid form and Carbaryl, Cartap, and MIPC among the powder form (Islam, 2009).

The traditional varieties of rice such as Aman, Boro, Aus etc. are replaced in many areas by High Yielding Varieties (HYVs). At present, farmers are complaining regarding the declining yield of HYV rice despite increasing use of chemical fertilizers and pesticides. The general belief regarding this increased use is 'if little is good, a lot more will be better'. As a result, farmers have to compensate high cultivation costs due to rapidly increasing use of costly fertilizers and pesticides (Chinta, 1992). At the same time, these hazardous chemicals are playing havoc with human and others life form which these uneducated farmers are not aware of.

Synthetic Carbamate compounds have been widely used as pesticides and have played an important role in raising agricultural productivity such as food and fibre and controlling pests by the amelioration of vector-borne diseases throughout the world specially in the 3rd world countries. However, these compounds are often toxic in non-target organisms and their transformation products are frequently found in the environment (Van and Pletschke, 2010). There is now overwhelming evidence that some of these chemicals do pose a potential risk to humans and other life and unfortunately no part of population is completely safe from the adverse impact of these poisonous xenobiotics. Every year one million people have been suffered from chronic diseases and deaths due to pesticide poisoning and it would become more vulnerable if proper steps might not be taken in near future.

A study of Food and Agriculture Organization apprehended that in 21st century the pesticides use would increase in the developing countries (FAO, 1995). Bangladesh Rice Research Institute released a report on the use of pesticides in farmland during 1997 to 2008 and showed that the use of pesticides in Bangladesh , in 1997 was more than 8,000 tons; it doubled to 16,000 tons in 2000; in 2005-06, it increased to nearly 20,000 tons and in 2008 it rose up to 48, 690 tons (Rahman,1997; ESCAP,1987).Thus both the results

have been highlighting our agricultural practice based on huge amount of pesticide applications to increase production every year.

According to Ministry of Environment and Forests (People's Republic of Bangladesh under the project of hazardous waste management in Bangladesh: a country inventory 2010) yearly pesticides production rate was 12,756.25 metric tons and pesticide contaminated wastewater generation was projected to increase from 2007 figure of 10,920 to 14,070 cubic meters by 2012. Again according to BRRI, each year farmers are habituated to applying much more pesticides than previous year, that is 328% more every year and it poising a serious health hazards including residual effect and also economic impact that is 8 billion USD annually in developing countries.

Pesticides and other chemically contaminated rain waters enter low lying rice fields and inland fish producing waters (rivers, beels, haros). These 'haors' and 'beels' (wetlands) are a major wintering and resting site for many indigenous and migratory birds. About 330 species of plant, 270 species of bird and 120 species of commercially important fish are exposed to danger. A comprehensive study on the destruction of natural environments and ecosystems is not available. But the recent increase in fish diseases and human sufferings indicate an alarming situation. A study from the Department of Environment (DoE,1989), Ministry of Environment and Forest focused on the long term existence of organochlorine pesticides after it application in soils and 35% Aldrin, 37% Dieldrin, 43% Lindane (Ó-HCH), and 30% Hepatchlor were still available after 336 days of execution. The organo-phosphate pesticides after 80 days of spray show soil contains 15% Diazinon, 15% Parathion, 8% Dichlorvos (NGOS) and 5% Phosdrin (Mevinphos).

The wide spread use of these pesticides over the year has resulted in problem caused by their interaction with the biological system in the environmental pollution to such an extent that human health is adversely affected and ecosystems are endangered. Carbamate pesticides, such as: Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate) is one of the pesticides belonging to the N-methylcarbamate class used

extensively in the agricultural business (In Seon Kim *et al.*, 2004). Each year a massive quantity of pesticides are manufactured and used where carbofuran comprise a major proportion and its toxicity remains a major environmental problem associated with its usage (Eisler, 1985).

The World Health Organization (WHO) recommend a convenient method based on the LD_{50} measurement to estimate risk-weighted pesticides. According to this method , risk weighted pesticides are classified into 4 major hazard groups: Category Ia & Ib (extremely hazardous), Category II (moderately hazardous), Category III (slightly hazardous), and Category U (least hazardous or unlikely to present acute hazard under normal use). Field evidence suggests that human poisonings correlate reasonably well with these toxicity ratings (Levine and Davies, 1982).

1.2 Carbofuran

Carbamate pesticides have been developed as a biode-gradable and short-lived alternative to highly stable organochlorines (Baron, 1971). Among the carbamate pesticides, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-methyl-carbamate) is widely used in rice culture for controlling the brown plant hopper (*Nilaparvata lugens*) and other insect pests of rice. It is also used to control soil-dwelling and foliar-feeding insects such as corn rootworm, wireworms, boll weevils, mosquitoes, alfalfa weevil, aphids, and white grubs (Trotter *et al.,* 1991)

Table 1.1 An Analysis of Pesticide Use for Rice Pest Management in Bangladesh (Adopted from: S. Parveen *et al.,* 2001)

Common name	Trade name	WHO category	Type	% of farmers used	kg or l/ha used	Targetted pests
Insecticide						
carbofuran	Agrifuran5G Biesteren 5G Sunfuran 5G Furadan 5G Carbofuran	Class Ib Class Ib Class Ib Class Ib Class Ib	C C C C C	1 16 9 9 2	3.8kg 3.04kg 6.89kg 4.89kg 0.93kg	Steam borer Leaf hooper Steam borer,defoliator Steam borer,grasshooper Rice bug
Dioxathion	Bashudin10G	O	OP	44	8.27litre	Steam borer,gall midge
Cyhalothrin	Karate2.5EC	Class II	PY	3	5.00litre	Plant hooper,green leaf hooper
Cypermethrin	Cymbush10EC Ripcord10Ec	Class II Class II	PY PY	3 2	3.74litre 0.96litre	Defoliator,green leaf hooper Rice hispa
Diazinon	Diazinon60EC	Class II	OP	3	5.97litre	Greenleaf hooper,Steam borer

DDVP	Nogoz100EC	Class Ib	OP	3	9.23litre	Thrips
Fenitrothion	Sumithion50EC	Class II	OP	15	5.83litre	Leaf roller, Rice hispa
Monocrotophos	Monotaf40WSC	Class Ib	OP	14	8.76litre	Steam borer
Malathion	Malathion57EC Faifanon57EC	Class III Class III	OP OP	2 24	4.38litre 2.69litre	Green leaf hooper, thrips,rice bugs Steamborer,brown grass hoopers
Phosphamidon	Dimecron 100 SCW	Class Ia	C	34	4.23litre	Rice hispa
Cartap	Padan 50 SP	Class II	C	2	0.86kg	Plant hooper,green leaf hooper
BPMC	Baycarb500EC	Class II	C	26	5.24litre	Defoliators,Green leaf hooper, thrips
Fungicide						
Edifenphos	Hinosan 50 EC	Class Ib	OP	7	3.8litre	Blast
Carbendazim	Knowin 50WP	Class U	C	44	5.48kg	Sheath blight
Propiconazol	Tilt 250 EC	Class II	OP	9	7.96litre	Blast

1.3 Chemical Profile of Carbofuran

Chemical Name	:	2, 3-dihydro -2, 2 dimethyl-7-benzofuranyl methylcarbamate
Pesticide Name	:	Carbofuran
Trade Names	:	Furadan
Molecular formula	:	$C_{12}H_{15}NO_3$
Molecular Weight	:	221.25 g/mole
Solubility in water	:	300 mg/L at 25°c
Oral LD_{50} (rat)	:	8-14 mg/kg.
Derivatives	:	Carbofuran phenol; 3-Ketocarbofuran; 3-hydrooxycarbofuran
Common physical appearance	:	Odourless, White crystalline solid
Activity	:	Insecticides and nematicides
Mode of action	:	Cholinesterase inhibitor
Vapor Pressure[b]	:	8.3 x 10-6 mmHg (25°C)
Octanol-Water Partition Coeff- icient (Kow)[a]	:	17-26
Henry's Law Constant[b]	:	3.9 x 10-9 atm m^3/mol
Hydrolysis Half-Lives(days)[b]	:	27.7(pH7,25°C), 2.73(pH8,25°C), 0.54(pH9, 25°C)
Aqueous Photolysis Half-Life (days)[a]	:	7.95 x 103 (pH 7, 28°C)
Soil Photolysis Half-Life(days)[a]	:	138 (27°C, pH 5.7, sandy-loam, 2.1% organic carbon, 21% moisture)
Aerobic Degradation Half-Life (days) a	:	22 (25°C, pH 5.7, sandy- loam, 2.1% organic carbon, 21% moisture)
Anaerobic Degradation Half-Life (in days)[a]	:	30.0 (25°C, pH 5.7, sandy- loam, 2.1% orga- nic carbon, 21% moisture)
Field Dissipation Half-Life (in days) a	:	13.0 (pH 7.3, sandy- loam, .38% organic carbon)
Adsorption coefficient (Koc)[c]	:	22

a. DPR Pesticide Chemistry Database (2002) b. Handbook of Environmental Fate and Exposure Data, Vol. 3 (1991) c. EXTOXNET **(**Extension Toxicology Network**,**2001: http://ace.ace.orst.edu/info /extoxnet/pips/carbofur.htm)

Structure of Carbofuran:

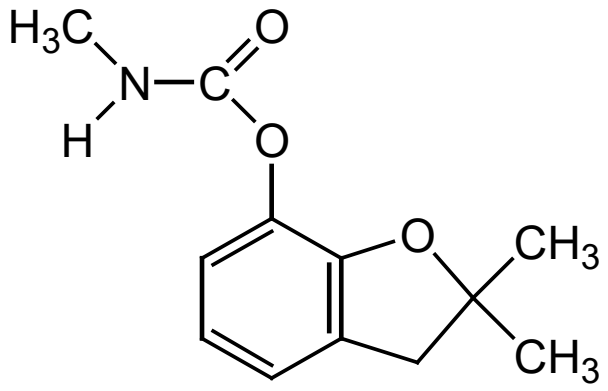


Fig. 1.1: Structure of Carbofuran

Carbofuran is formulated as a flowable or wettable powder, while pure carbofuran is an odorless to mildly aromatic white crystalline solid (Farm Chemicals Handbook, 2001). It is stable in acidic and neutral media, but hydrolyzes under alkaline conditions. Thermal breakdown may release toxic fumes (WHO, 1996a; 1996 b).

1.4 Environmental Fate of Carbofuran in Air, Water and Soil

Air

Carbofuran's low vapor pressure and low Henry's Law constant indicates it has a low tendency to volatilize from water or moist soils (Duel *et al.,* 1979). A number of works has been carried out to show the fate of carbofuran on environmental factors. Low concentrations of carbofuran were found in air when samplers were placed 20 yards from the edge of an agricultural field in Imperial County, California. Reported concentrations ranging from 0.03 to 0.66 ug/m^3 were observed after a 44-hour sampling period following an application of 44% active ingredient carbofuran (Shibamoto *et al.,* 1993).

Once in the air, carbofuran is subject to vapor phase photo oxidation by reacting with hydroxyl radicals. The half-life under this reaction is estimated to be 4.6 hours in a typical atmosphere (Handbook of Environmental Fate and Exposure Data, 1991). Therefore, it is evident that high rate of carbofuran dispersal in air makes it a dangerous candidate for the environment and human health.

Water

Base-catalyzed hydrolysis to carbofuran phenol is the major degradation pathway of carbofuran in both water and sediment (Yu *et al.,* 1974; Seiber *et al.,* 1978; Brahmaprakash *et al.,* 1987; Talebi and Walker, 1993). Other degradation products can include 3-hydroxy-7-phenolcarbofuran, carbofuranphenol, and N-methylcarbamic acid via hydroxylation of the benzofuranyl moiety (Yu *et al.,* 1974). The aqueous hydrolysis rate of carbofuran increases dramatically with increasing pH. Laboratory study reported 80-95% recoveries of initial carbofuran spikes at pH 3 after 1, 3, and 6 hours (25ºC). In contrast, at pH 10 only 65% of the original amount was recovered after 1 hour, 35% remained after 3 hours, and 10% remained after 6 hours (Bailey *et al.,* 1996). Seiber *et al.,* (1978) found that the hydrolysis of carbofuran was more than 700 times faster at pH 10 that at pH 7; the reported half-lives were 1.2 hours and 864 hours, respectively. Hydrolysis was also observed to be much more rapid in natural paddy water than in deionized (DI) water. Half-lives at pH 7 were 240 hours in paddy water and 864 hours in DI water. Overall, the mean laboratory half-life in paddy water was 40 hours at pH 8. This agreed well with the 57-hour average observed in the field at the same pH, considering factors such as slight variations in sunlight, pH, temperature, and microbial degradation (Seiber *et al.,* 1978).

Soil

Because of its high water solubility (351 ppm at 25° C) and low adsorption coefficient (Koc= 22), carbofuran is relatively mobile in soil and in surface runoff. Consequently

carbofuran has the potential to contaminate lakes, streams, and groundwater. Carbofuran has been detected in the Sacramento River (Nicosia et al., 1991), although concentrations were below U.S. Health Advisory Levels (HALs) and Maximum Contaminant Levels (MCLs) (Cohen, 1996). A study done by Kumari, Singh, and Saxena (1987) on the movement of carbofuran found a slight difference between adsorption coefficients in two soil types. In clay loam (OC content 0.53%, pH 8.6), carbofuran was found to have a Kd value of 22.4, while in silt loam (OC content .18%, pH 8.4) it had a Kd value of 19.9. Apparently less movement, and therefore higher adsorption, was seen in the clay loam, because of the presence of a greater organic matter and/or clay content. Leaching studies similarly demonstrated increased absorption and lesser mobility in the clay soil (Kumari *et al.,* 1987). Sharom *et al.,* (1980) compared leach ability of carbofuran from sand (OC content .7%, pH 7.0) and organic soil (OC content 75.3%, pH 6.1) with 10 successive rinses with 200 ml of distilled water. Carbofuran was almost completely leached out of the sand within the first two rinses (1st rinse recovered 94.8%, 2nd rinse recovered 4.1%). Amounts recovered from organic soil were less, with 73.8% from the 1st rinse, and 16.3% from the 2nd (Sharom *et al.,* 1980).

According to Johnson and Lavy (1995), carbofuran is expected to partition into the water from soil. Immediately following granular application to paddy soil, 54% of the carbofuran was found in the water while 46% was found remaining in soil. The study done by Nicosia *et al.,* (1991) disagreed with this data. They found the mass recovered from paddy soil was 5 times greater than mass recovered in paddy water immediately after application. 70 days after flooding, the mass recovered in soil was 98 times greater than the mass recovered in water. They noted low pH levels in soil compared to the water pH may have contributed to persistence in the soil (organic carbon content in this study ranged from 2.2% to 2.8%).

Similar to pH effects on aqueous hydrolysis rates, carbofuran degradation is much quicker in alkaline soils than in acidic soils. Getzin (1973) reported a 10-fold difference

in DT_{50} (time required for 50% breakdown) between soils at pHs 4.3 and 7.8. He concluded that while hydrolysis was the major route of degradation in alkaline soils, the slower degradation in acidic and neutral soils was dominated by microbial and chemical mechanisms. Breakdown products in soil include carbofuran phenol (Getzin, 1973), 3-hydroxycarbofuran, and 3-ketocarbofuran (Johnson and Lavy, 1995). Other studies indicate that microbial degradation is an important route of carbofuran degradation in neutral soils**.** Miles *et al.,* (1981) compared dissipation rates in two soils, a sandy loam (organic matter 3.3%, pH 7.3) and muck (organic matter 36%, pH 7.3) under sterile and nonsterile conditions. Carbofuran was persistent in sterile soils, with 77% remaining in sterile muck and 50% in sterile sandy loam after 8 weeks. In contrast, 25% remained in the nonsterile muck while carbofuran was undetectable in the nonsterile sandy loam after 8 weeks (Miles *et al.,* 1981). Carbofuran's metabolites also dissipated relatively rapidly in the nonsterile soils as well, and so are also suspected of degradation by microbes in the soil. Miles et al. (1981) observed disappearance of 90% of 3-hydroxycarbofuran within 1 week in both the nonsterile soils, while 3-ketocarbofuran disappeared from nonsterile loam in 1 week, and from nonsterile muck in 2 weeks.

Soil previously treated with carbofuran granules was found to have a dissipation half-life of 58 days in one field (2.4% organic carbon), and 43 in another (2.2% organic carbon) (Nicosia *et al.,*1991). These studies show the long persistence of carbofuran in soil has also been.

Szeto and Price (1991) found 78 µg/g of carbofuran in Canadian silt loam soils nearly a year after the application of granular material. The pH of similar soils from this area in the Fraser Delta of British Columbia, Canada, has been reported as between 5.0 and 5.9 (Mineau, 1993). Caro *et al.,* (1973) reported a soil dissipation half-life of 117 days in a cornfield. Low soil pH (5.3) and lower soil moisture content may explain the relatively slow rate of dissipation.

Singh and Sethunathan (1999) found that when recoveries of applied carbofuran were compared between soils that are previously treated with carbofuran and soils untreated,

the previously treated soils had a much lower recovery rate. The authors examined this theory using standing water from previously treated Azolla plots. After 2 days, 89.4% of the applied carbofuran was recovered in the uninoculated plot, while only 55.5% was recovered in the previously treated plot. After 5 days, 87.3% was recovered in the uninoculated plot, while only 29.8% was recovered from previously treated plot (Singh and Sethunathan, 1999). Getzin and Shanks (1990) found that enhanced degradation could develop with as little as one or two applications of carbofuran. Volatilization is not as important as microbial degradation to carbofuran dissipation, but is a contributing dissipation process. In a controlled laboratory study examining volatilization, amounts of carbofuran evolved after 20, 40, and 60 days in sandy loam were, in µg, were 4.4, 10.3, and 14.4, respectively (initial concentration applied was 7.3 ppm, 5.8% water). In sand, amounts of carbofuran evolved (in µg) were 216, 466, and 842 (initial concentration applied was 5.8 ppm, .7% water) (Caro *et al.,* 1976). It has been reported that carbofuran volatilization rates are much more rapid under flooded soil conditions than under non-flooded soil conditions. The authors state that this is most likely due to co-evaporation with the water on the surface of the soil (Lalah *et al.,* 1996). Photo degradation is generally considered a minor route of carbofuran degradation. One study found that carbofuran adsorbed onto silt loam had a half-life of 13.6 days when exposed to 2400 µW/cm2 and 26°C (National Research Council of Canada, 1979). The rate of dissipation in soil is also strongly affected by temperature (Yen *et al.,* 1997). One laboratory study found that carbofuran's half-life in silty clay loam (pH 6.7, organic matter 2.9%) was 105 days and 35 days at 15ºC and 35ºC, respectively (Yen *et al.,* 1997)

1.5 Effects of Carbofuran on Human Health

Working on contact or ingestion, carbofuran is a cholinesterase inhibitor, but the short-term effects on the nervous system are reversible (EXTOXNET, 2001). It is highly toxic to mammals, having an oral 50% lethal dose of 2 mg/kg in mice (Fahmy *et al.,* 1970)

The widespread use of pesticides in public health and agriculture has caused severe environmental pollution and health hazards including cases of severe, sub-chronic and chronic human poisoning (Abdollahi *et al.,* 1995; Abdollahi *et al.,* 1996; Jalali *et al.,* 2000 ; Pajoumand *et al.,* 2002). Symptoms of carbofuran poisoning include: nausea, vomiting, abdominal cramps, sweating, diarrhoea, excessive salivation, weakness, imblance, breathing difficulty, increased blood pressure or hypertension and lack of control of urine of feces release, referred to as "incontinence". Death may result from respiratory system failure associated with carbofuran exposure (Tucker and Richard, 1970).

People, who do not even directly work with pesticides, the dangers of exposure to carbofuran remain high with residues on foods and contamination of drinking water from field runoff. Carbofuran has high potential for groundwater contamination and has been detected in aquifers and surface waters (Polednikova *et al.*, 2010). The pesticides from domestic, industrial and agricultural effluents contaminate ground and surface water. The contaminated water is taken up by plants and animals and ultimately enters into the food chain. Finally all living beings are consuming their diet with poisonous pesticides. First report on pesticide poisoning was at kerala in India, where over 100 people died after consuming wheat flour contaminated with parathion. Recently, Twenty-two school children were dead so far in the village of Dharmasati Gandaman, in India's Bihar state exposed to organophosphorus (OP) pesticides in their daily free school Tiffin on Aug 16, 2013. These reports demonstrate the danger of carbofuran in our food chain.

1.6 Effect of Carbofuran on Aquatic and Terrestrial Species:

In USA Granular carbofuran products are no longer registered for use because carbofuran generally displays high avian toxicity. The formulated granules look very similar to seeds certain birds incorporate into their diet. Carbofuran is highly toxic to fish and birds particularly in granular form, which birds often eat seeds, or through contaminated drinking water. One granule of carbofuran is enough to kill a small songbird. Mistaking

the granules for food, they seize and die upon ingestion, leading to bird-kill incidents. Secondary poisoning of raptors feeding on contaminated birds has also been reported (Erwin, 1991).

Carbofuran has not been observed to accumulate significantly in aquatic systems. While carbofuran disrupts enzyme and lipid metabolism in fish, the effects are reversible with no observable permanent damage (Eisler, 1985). LC_{50} values ranged from 130 parts per bilion to 1,420 parts per billion in tests of 72 to 96 hours, with yellow perch (Perca flavescens), green sunfish (Lepomis cyanellus), and lake trout (Salvelinus namaycush) being among the most sensitive, and channel catfish (Ictalurus punctatus) and fathead minnow (Pimephales promelas) among the most resistant (Eisler, 1985). When compared with the toxicities of other aquatic species, marine worms seemed to be the most resistant to the pesticide, while fish were the most sensitive (National Research Council of Canada, 1979). For terrestrial species, honeybees are extremely sensitive to carbofuran (LD_{50} is 0.16µg/bee, Eisler, 1985). Earthworms (Lumbricus herculeus) are also particularly susceptible, with an LC_{50} value in soil of 0.5 ppm at 5 hours. Earthworm mortality could result in an increased likelihood of secondary poisoning in many species (Eisler, 1985).

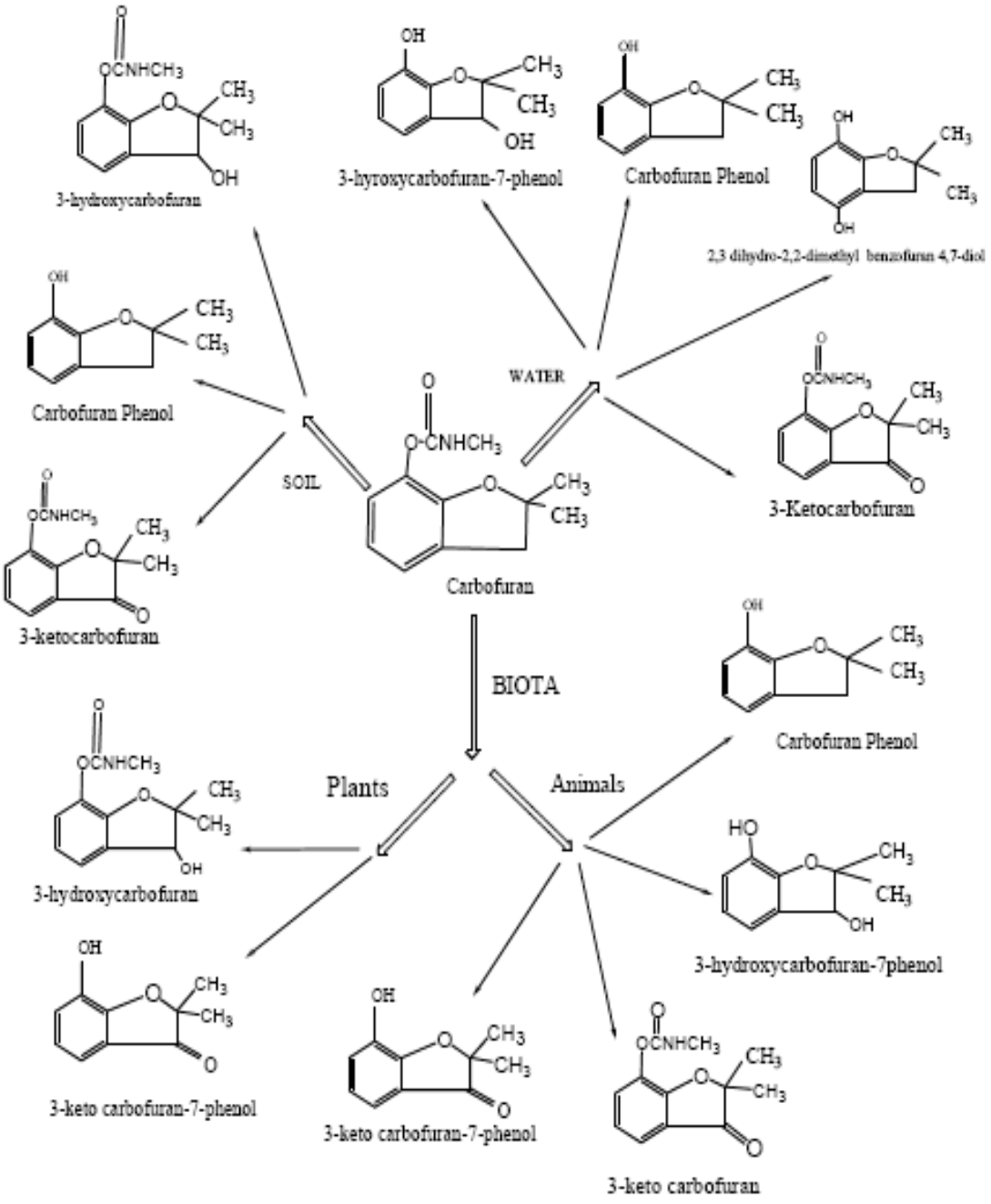


Fig. 1.2: Environmental fate of carbofuran

Adopted from: S. Evert: http://www.cdpr.ca.gov/docs/emon/pubs/fatememo/carbofuran.pdf

1.7 Microbial Degradation of Carbofuran

Carbofuran is chemically hydrolyzed under alkaline conditions, but microorganisms have been implicated in its degradation in near-neutral soil and water environments (Rajgopal *et.al.,* 1984). Microorganisms play an important role to degrade the toxic pollutants. The vast majority of natural and synthetic organic compounds are metabolized by microorganisms (Gibson, 1984). It is imperative that mechanisms exist by which the elements trapped in organic materials, either naturally occurring or man-made, are returned to the global elemental cycle. The role of microorganisms in this process is paramount and when one considers the large number of chemicals, which must potentially mineralized, it is not surprising that microorganisms are among the most metabolically diverse group on earth. One reason that such metabolic potency has arisen is the plasmid-borne nature of many degradative genes, which allows the evolution of novel catabolic enzymes. The genes that specify the enzymes responsible for degradation have frequently been shown to be borne by plasmids (Often termed as catabolic or degradative plasmids).

Interest has developed in recent years the ability of microorganisms to degrade and detoxify the large amount of toxic pollutants, which are introduced in to the environment through agricultural activities of man. One of the most important aspects concerning the environmental impact of xenobiotics is biological toxicity, which can be established by following the fate of the particular xenobiotics. Before studying the pathways, which the microorganisms use to degrade toxic pollutants, it is necessary to understand how these organisms have developed this ability. It is possible that during the evolutionary process microorganisms have been exposed to a wide variety of synthetic organic and inorganic compounds, which have chemical structures analogous to naturally occurring compounds. Thus, the microorganisms have genetically modified, or acquired *de novo* from other microorganisms, genes coding for enzyme systems capable of degrading them.

A number of isolates capable of degrading carbofuran have been isolated from soils and several bacterial taxa are reported, including *Pseudomonous*, *Flavobacterium***,** (Chaudhry and Ali, 1998) *Acromobacter* (Karns *et al*., 1996) *Arthrobacter s*p. (Ramanand *et al*., 1988) and *Sphingomonas* sp. (Feng *et al*., 1997). The degradation of carbofuran by microorganism is depend on the enzymes secreted by the substrate utilizing bacteria. The following pathways have been utilized by various bacteria. An organism which can initiate the pathway is given, but other organisms may also carry out later steps. However, till now there is no report of the rate of degradation of carbofuran by any of these microorganisms.

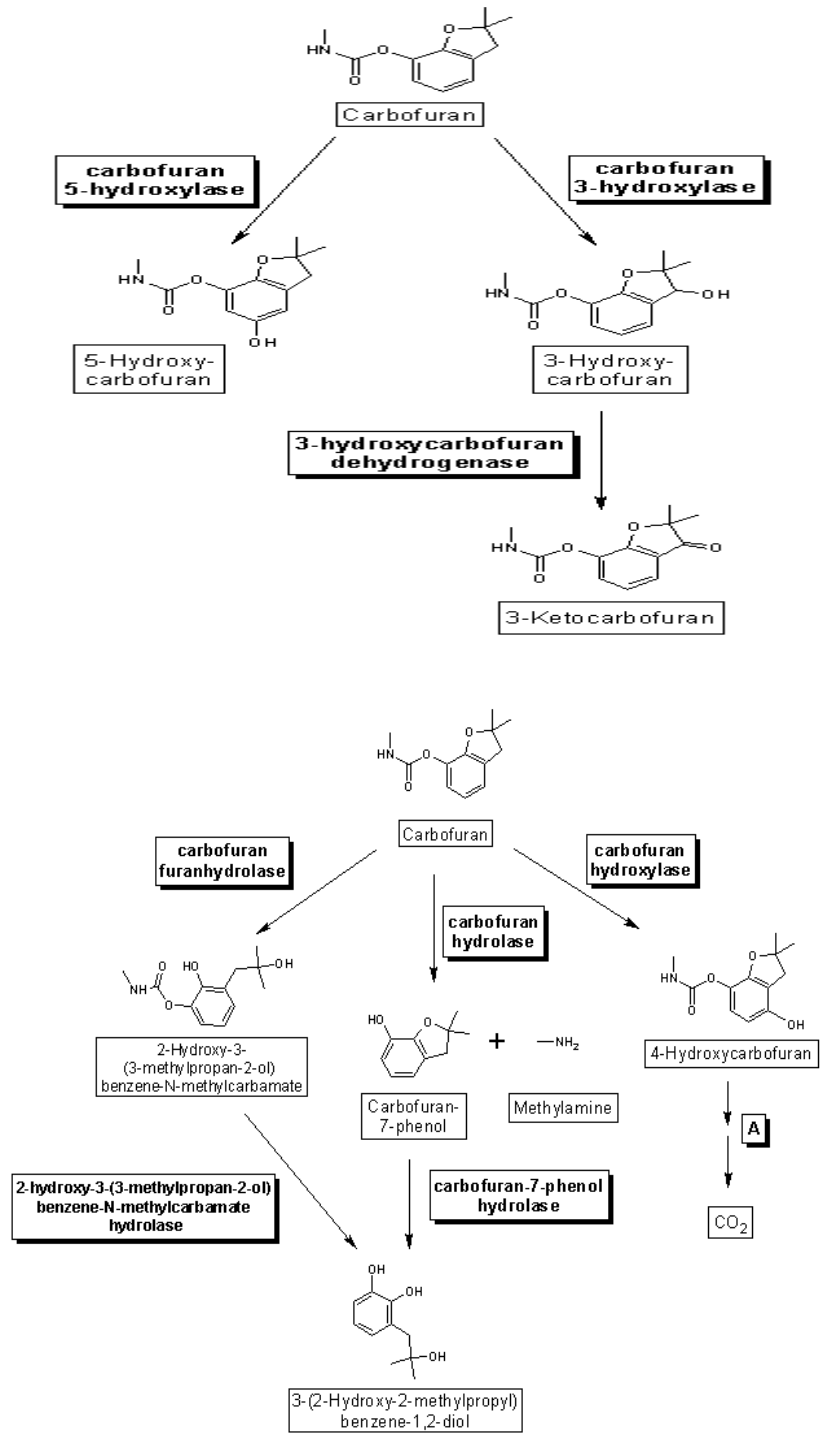


Fig. 1.3: Degradative pathway of carbofuran

Reference: This pathway was contributed by Junfeng Gao, University of Minnesota, BioC/MicE 5309, Luke Kane and Michael Turnbull, University of Minnesota.

Sphingomonas sp. strain SB5, can degrade carbofuran into carbofuran-7-phenol and methylamine, hydrolyze carbofuran-7-phenol to 3-(2-hydroxy-2-methylpropyl) benzene-1,2-diol (DP-1), and then degrade DP-1 into a red metabolite (DP-2). More study is needed to identify this red metabolite (Kim *et al.,* 2004). *Pseudomonas sp.* 50432 is capable of hydroxylating carbofuran at the 4-position; further metabolites are unknown (Chaudhry *et al.,* 2002). Carbofuran may also be hydroxylated at the 5 position by *Novosphingobium* sp. FND-3 and *Rhodococcus* TEI (Yan *et al.,* 2006). Fungal degradation of carbofuran may occur via hydroxylation at the three position and oxidation to 3-ketocarbofuran. However, finding a better strain for more efficient degradation and with known degradation rate for proper estimation of quantity needed to detoxify a particular amount of carbofuran used in the soil is always a better option.

1.8 Involvement of Plasmids in Bacterial Degradation

Plasmids are extra chromosomal elements, which are wide spread in bacteria. Recently, it has become a tool in genetic engineering to use as a vehicle for cloning foreign DNA due to remarkable features.

They mediate the transfer of properties, such as drug resistance, between individuals belonging to different genera. This may be an important factor for bacterial evolution (Broda, 1979). According to Lederberg (1952), the behaviors and the properties of some bacteria suggest that their genetic control is not governed by the chromosome but the extra chromosomal structures known as plasmids. Plasmid have been further defined as being non essential for normal growth of the host species and in many conditions may be gained or lost without lethal effects (Clowes, 1972).

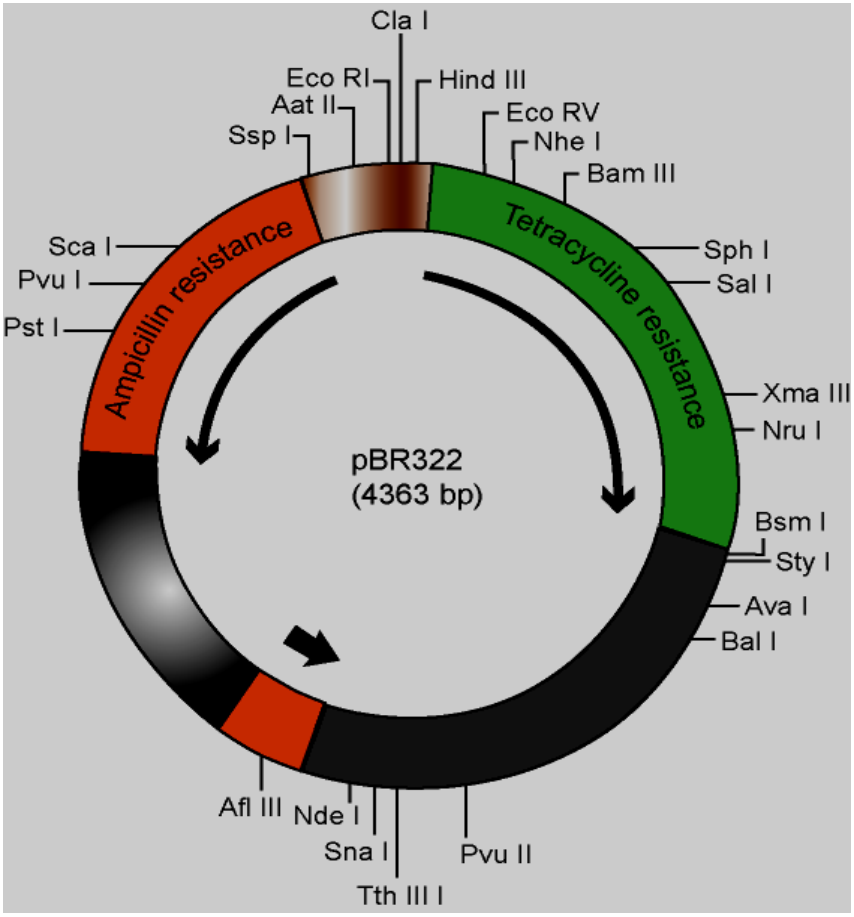


Plate 1.1: A typical structure of plasmid pBR322

Table 1.2 Naturally occurring catabolic plasmids for the degradation of xenobiotic compounds

(Adopted from: Sayler *et. al*., 1990)

Primary substrate	**Plasmid**	**Size (kb)**	**Bacterial strain**	**Trans-missible**	**Reference**
Acyclic isoprenoid (citronellol, geraniol)	pSRQ50	75	*Pseudomouas putida*	+	Vandenbergh & Wright (1983)
Alkylbenzene sulfonates	ASL	91.5	*p. testasteroni*	+	Cain (1981)
Aniline	pCIT1	ND	*Pseudomonas sp.*	+	Anson & Mackinnon (1984)
6-Amino hexanoic acid. (nylon oligomer)	pOAD2	28.8	*Flavobacterium sp.* K172	+	Negoro *et al.* (1982) Negoro *et al.* (1984)
Benzoate	pCB1	17.4	*Alcaligenes sp.*	+	Blake & Hegeman (1987)
Biphenyl	pBS241	195	*P. putida BS 893*	+	Kochetkov and Boronin (1984)
Bromoxynil (3, 5 dibromo-4 hydroxy benzonitrile)	pBXI	81	*Klebsiella ozaenae*		Stalker & Mcbride (1987)

Camphore	pPGI (CAM)	~500	*Pseudomonas sp.*	+	Rheinwald *et al.* (1973)
2-4 dichloro phenoxy acetate	pJP1	87	*Alcaligenes paradoxus* Jmp 116	+	Fisher *et al.* (1978); Pemberton *et al.* (1979)
	pJP2	55	*A. paradoxus* Jam 130	+	Don and Pemberton (1981) Ghosal *et al.* (1985)
	pJP3	78.1	*A. paradoxus* Jmp 133	+	Don and Pemberton (1981). Ghosal *et al.* (1985)
	pJP4	78.1	*A. eutrophus* Jmp 134	+	Don and Pemberton (1981)
Naphthalene	pBS4	173	*P. fluorescence*	+	Skryabin *et al.* (1980).
Naphthalene	Nah7	83	*P. putida*	+	Dunn & Gunsalus (1973); White & Dunn (1978)
Naphthalene	pWW60	87	*Pseudomonas* sp.		Cane and Williams (1982).

Naphthalene	pBS211	165	*Pseudomonas* sp.	+	Kochetkov & Boronin (1984)
2-Chloroacetate,	pBS212	165	*P. fluorescens*	+	Kochetkov & Boronin (1984)
2-Fluoroacetate	pBS213	150	*P. putida*	+	Kochetkov & Boronin (1984)
2-Chloroacetic acid,	pUO1	65.5	*Morexella* sp.	+	Kawasaki *et al.* (1981)
2-Chloropropionic acid	pU202	230	*Pseudomonas* sp E2	-	Hardman *et al.* (1986)
	pU204	287	*Pseudomonas* sp E4	-	Hardman *et al.* (1986)
	pU206	156	*Pseudomonas* sp E3	-	Hardman *et al.* (1986)
	pU220	287	*Alcaligenes* E20	-	Hardman *et al.* (1986)
	pU222	264	*Alcaligenes* E22	-	Hardman *et al.* (1986)
3-Chlorobenzoate	pAC25	117	*P. putida*	+	Chattergee & Chakrabarty (1983), Chattergee *et al.* (1981).

Phenol	pPGHI	200	*P. putida*	+	Herrmann *et al.* (1987)
Octone	OCT	~500	*P. oleovorans*	+	Chakrabarty *et al.* (1973)
Toluene	pWWO(TOL)	117	*P. putida*	+	Duggleby *et al.* (1977), White & Dunn (1978), Williams & Murray (1974)
p-cresol	pND50	-	*P. putida* NCIB 9866	+	Hewetson *et al.* (1978)

1.9 Molecular Methods of Bacterial Identification

Upon isolation of a carbofuran degrading bacteria, the immediate step is to identify the bacteria. The most reliable molecular method for identifying unknown bacteria is the sequencing of 16S ribosomal RNA gene (16S rDNA) and compare the sequence with the known sequence available in the gene bank. The rRNA gene is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms. This work was pioneered by Carl Woese, who proposed the three Domain system of classification - Archaea, Bacteria, and Eucarya - based on such sequence information (Woese and George 1977).

The Ribosomal RNAs

In Bacteria, Archaea, Mitochondria, and Chloroplasts the small ribosomal subunit contains the 16S rRNA (where the S in 16S represents Svedberg units). The large ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. There may be one or more copies of the operon dispersed in the genome (for example, *E coli* has seven). The Archaea contains either a single rDNA operon or multiple copies of the operon. To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category are those that definethe ribosomal RNAs (rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA. The 5S has been extensively studied, but it is usually too small for reliable phylogenetic inference. The 16S and 23S rRNAs are sufficiently large to be useful. The 16s rDNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (www.cme.msu.edu/RDP/html/index.html). These sites also provide search algorithms to compare new sequences to their database.

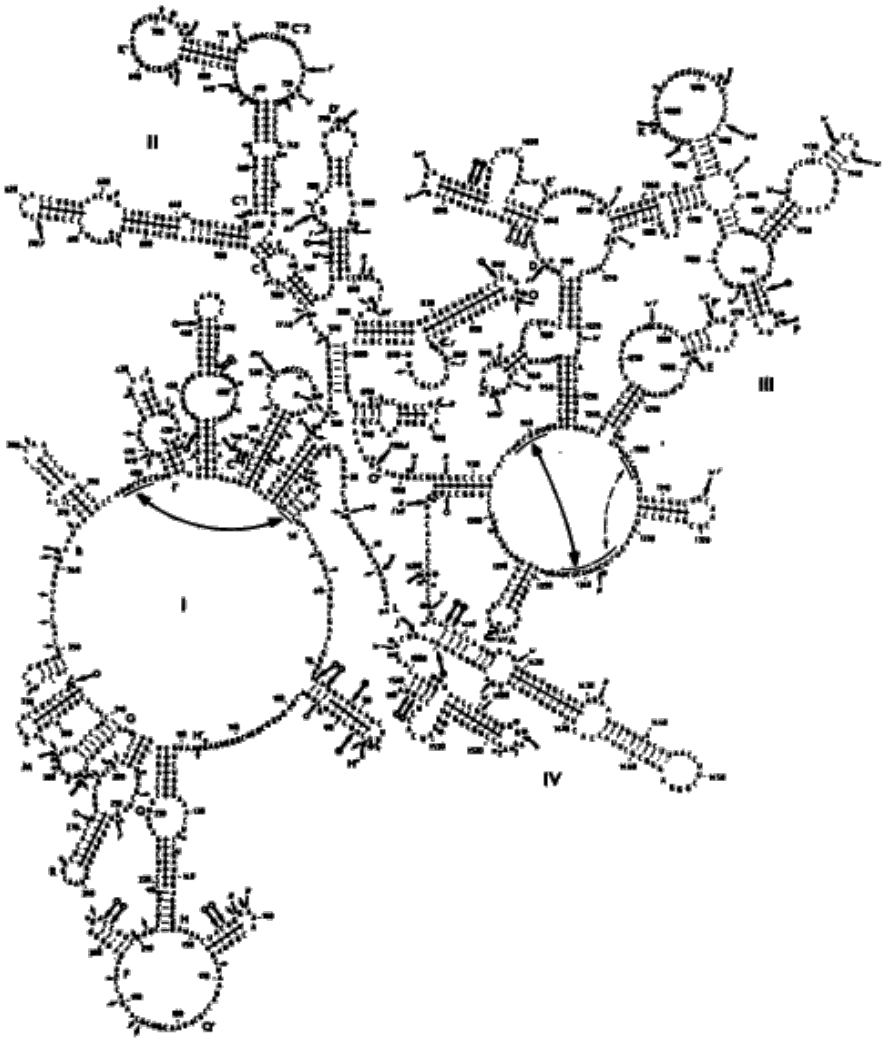


Fig. 1.4: Structural organization of the 16S ribosomal RNA from *E. coli*. Topography and secondary structure. Adopted from: P Stiegler, P Carbon, M Zuker, J P Ebel, and C Ehresmann. Nucleic Acids Res. 1981 May 11; 9(9):2153–2172

Carl Woese recognized the full potential of rRNA sequences as a measure of identification of bacterial species and deciphering phylogenetic relatedness. He initially used an RNA sequencing method that determined about 1/4 of the nucleotides in the 16S rRNA (the best technology available at the time). This amount of data greatly exceeded anything else then available. Using newer methods, it is now routine to determine the sequence of the entire 16S rRNA molecule. Today, the accumulated 16S rRNA

sequences (about 10,000) constitute the largest body of data available for inferring relationships among organisms.

Principle of 16S rDNA sequencing

DNA sequencing is achieved by utilizing labeled nucleotides for incorporation into a copy of a piece of DNA. The DNA sequence can then be derived by the positions of the labeled nucleotides. Sequencing methods have evolved from relatively laborious gel-based procedures to modern automated protocols based on dye labelling and detection in capillary electrophoresis that permit rapid large-scale sequencing of genomes and transcriptomes. Cycle sequencing is a simple automated method in which successsive rounds denaturation, annealing and extension in thermal cycler result in amplification of extension products. This high-throughput method is based on the chain termination sequencing method developed by Sanger *et al.,* in 1977.

The classical chain-termination method requires a single-stranded DNA template, a oligonucleotide primer, a DNA polymerase, all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) but only one dideoxynucleotides that terminate DNA strand elongation. Four separate sequencing reaction is needed to add each of the four types of dideoxynucleotides to selectively stop the chain elongation and resulting in DNA fragments of varying length. The newly synthesized strand is heat denatured, electrophoased and sequence is determined by the comparison of bands in gel image.

Dye-terminator sequencing utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission. All the purified cycle sequenced products are separated by capillary electrophoresis and a laser within an automated sequencing machine is used to analyze the DNA fragments produced. Owing to its greater expediency, and speed dye-

terminator sequencing is now the mainstay in automated sequencing. Its limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis

1.10 Aims and Objectives of the Research Work

There is a general consensus among researchers that repeated application of pesticide to soils can result in enhanced rates of microbial degradation. Enhanced degradation of a soil-applied pesticide such as carbofuran may occur when a population of soil microorganisms is repeatedly exposed to a chemical and adapts by developing the ability to catabolize carbofuran. Our present research was undertaken to satisfy the following objectives

- Isolation of bacteria, which are able to metabolize the carbofuran as a sole source of carbon
- Characterization of carbofuran degrading bacteria by morphological, physiological, biochemical tests
- Identification of the bacteria by sequencing 16S rRNA gene
- Investigation of the plasmid involvement of the isolated bacterium
- Detection of degradation rate by isolated bacteria through Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC)
- Study of the antibiotic resistance pattern and the utilization ability of different carbohydrates
- Detection of MIC of gentamycine against the isolated bacterium

Antagonistic effect of the isolated bacteria against good bacteria growth promoting (PGPR) Rhizobacteria.

MATERIALS AND METHODS

2.1 Sources of Inoculums

Samples of carbofuran (furadan) treated soil were collected from agricultural fields (Shibgonj, Bogra), which had 10 years of histories of carbofuran uses. Collected soil samples were used as a source of inoculum for enrichment culture and isolation of bacteria capable of degrading carbofuran.

2.2 Culture Media for Bacteria Isolation

Bacterial culture enrichments were carried out in either in minimal salts media.

Minimal Salts (MS) Media Composition:

Di-potassium hydrogen phosphate (K_2HPO_4)	2.0 gm/L
Ammonium sulphate $(NH_4)_2\ SO_4$	0.5 gm/L
Potassium dihydrogen phosphate (KH_2PO_4)	0.02 gm/L
Magnesium sulphate ($MgSO_4$)	0.05 gm/L
Trace elements	(1ml/L)

Trace elements comprised the following:

$FeSO_4$, $4H_2O$	400 mg/L
$MnSO_4$, $4H_2O$	400 mg/L
$ZnSO_4$, $7H_2O$	200 mg/L
$CuSO_4$, $7H_2O$	40 mg/L
CoCl, $6H_2O$	40 mg/L
KI	300 mg/L
Na_2MoO_4, $2H_2O$	50 mg/L

p^H of the media was adjusted to 6.8 with 2M HCl. The minimal salts media was prepared using distilled water and sterilized at 121°C and 15 lbs pressure for 20 min. 3% bacteriological agar was added to the above mentioned composition and poured into petridish to prepare MS agar plates after sterilization.

For selection of carbofuran degrading bacteria, carbofuran was added to the media after the steam sterilization of the bulk liquid media.

Nutrient Culture Media for Culture of Isolated Bacterial

Isolated bacteria from enrichment culture were further cultured in nutrient Luria-Bertani (LB) Broth or LB agar media

Composition of LB broth:

Yeast extracts	5 gm/L
Peptone	10 gm/L
NaCl	5 gm/L

P^H for nutrient broth was adjusted at 7.2

Nutrient medium was prepared using distilled water and sterilized at 121°C and 15 lbs pressure for 20 min. 3% bacteriological agar was added to the above mentioned composition and poured into petridish to prepare nutrient agar plates after sterilization.

2.3 Enrichment of Carbofuran Degrading Bacteria

Carbofuran treated soil was collected from agricultural fields with 10 year history of carbofuran application. One gm carbofuran treated soil sample was suspended to individual 250 ml Erlenmeyer flasks each containing 100 ml of minimal salts (MS) medium supplemented with 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml concentration of carbofuran(99% pure). Control flasks without an inoculum were also prepared to take account of any abiotic disappearance of the carbofuran. The primary enrichment was

incubated for seven days at 37°C with shaking at 120 rpm (revolution per minute) on an orbital shaker. When the cultures reached adequate turbidity they were used as a source of inocula in subsequent experiments.

2.4 Isolation of Carbofuran Degrading Bacteria

Bacteria from soil sample grown in the MS media supplemented with carbofuran was considered to be capable of degrading carbofuran Individual bacterial population was isolated from the above mentioned enrichment cultures by plating out on minimal salts agar containing different concentrations of carbofuran. The single colonies from these plates were sub cultured on to replicate plates and colonies from these eventually transferred into minimal salts (MS) medium supplemented with viable cell containing carbofuran(4µg/ml). Pure strains were maintained by weekly passage in liquid mineral salts (MS) medium containing carbofuran and also by weekly subculture on to the same medium solidified with 3% agar.

The single colonies were picked and streaked on MS agar plates under sterile conditions. This isolate bacterium was used as carbofuran degrading bacteria for the further experiments.

2.5 Morphological and Biochemical Tests

2.5.1 Morphological Tests

Following morphological tests were done to evaluate morphological characteristics of the isolated bacterium:

1. Microscopic Examination of Bacterial Cells

Gram staining

For gram staining of the bacteria the following stains were prepared using standard procedure.

i) 0.5% crystal violet solution.

ii) Lugol's iodine solution (1% iodine solution).

iii) 1% safranin solution.

Procedure:

i. A drop of sterilized distilled water was taken on the middle of the clean slide. Then a loopful bacterial suspension was transferred to the sterilized drop of water and very thin film was prepared on the slide by spreading uniformly

ii. The slide with fixed bacterial film was flooded with crystal violet solution for 30 second and washed thoroughly with gentle stream of tap water

iii. The slide was flooded with iodine solution for 1 minute and washed thoroughly with gentle stream of tap water.

iv. The slide were then washed with alcohol (95%) for 10-15 second

v. Alcohol was washed thoroughly with gentle stream of tap water

vi. The slide was flushed with safranin for 1 minute

vii. Excess safranin was washed with tap water and bloated dry

viii. Slides were examined under inverted microscope (OPTIKA, ITALY, MODEL NO XDS-2ERG0) at 400 X (40X X 10) magnification.

2. Motility Test

Motility test of the bacteria was carried out using Motility test agar Media.

Media composition:Peptone	10g/L
Sodium chloride	5.0g/L
Agar	4g/L

Procedure:

i. Fresh broth cultures of bacteria were prepared for the motility test.

ii. Motility test agar medium was prepared using distilled water and sterilized at 121°C and 15 lbs pressure for 20 min.

iii. Then the sterilized medium was poured into a test tube to make stab on the surface of the test tube.

iv. The inoculated sterilized loop from the fresh bacterial culture was streaked on to the agar surface of the testtube.

v. The test tubes were preserved into incubator at 37°C for 18 hr.

vi. The test tubes were observed after 18 hr, whether the inoculated bacteria were spread in the whole media or not.

2.5.2 Biochemical Tests

Following biochemical tests were carried out to understand biochemical properties of the isolated bacteria.

1. Carbohydrate Utilization Test

In order to find out the ability of the isolates to utilize different carbohydrates, the cultures were inoculated in minimal salt medium containing different carbohydrates *viz.* glucose, mannose, arabinose, sucrose, lactose, and cellulose. The final concentration of the carbohydrates was maintained 1%. The tubes were incubated at 37°C for 18 hr and observed for any growth.

2. Indole Test

Generally indole test is done to determine the ability of the organism to convert tryptophan into the indole.

For the Indole, one loopful fresh bacterial culture was inoculated in SIM (Sulfide Indole Media) broth medium and incubated at 37^0C for 18 hr, after incubation, Kovac's solution

was added and shaken vigorously for one minute. Color on the top of the surface were observed.

Media composition:

Enzymatic Digest of Casein	20 g /L
Enzymatic Digest of Animal Tissue	6.1 g/L
Ferric Ammonium Citrate	0.2 g/L
Sodium Thiosulfate	0.2 g/L
Agar	3.5 g/L
Final pH	7.3 ± 0.2 at 25°C

3. Methyl Red Test (MR Test)

Usually, methyl red test is done to evaluate whether the microbe performs mixed acids fermentation or not. Basal salt media was used for methyl red test. Components of the basal salt medium were mixed with distilled water in conical flask and the pH was adjusted to 7.2 with NaOH or HCl. The trace salts solutions were prepared distinctly in distilled water and was added in the basal salt medium at a concentration of 1ml/L.

Media composition:

Basal salt medium:		Trace element:	
$K_2Cr_2O_7$	2.34g/L	$CaCl_2$. $2H_2O$	4.77g/L
KH_2PO_4	1.33g/L	$FeSO_4$. $7H_2O$	0.37g/L
$MgSO_4$, $7H_2O$	0.02g/L	$CoCl_2.6H_2O$	0.37g/L
(NH_4), $2SO_4$	1.00g/L	$MnCl_2.4H_2O$	0.10g/L
Yeast extract	0.10g/L	$Na_2MoO_4.2H_2O$	0.02g/L
NaCl	0.50g/L		
Yeast extract	0.10g/L		
Glucose	1.00g/L		

After inoculation of bacteria into the MR broth medium in test tubes, cultures were were incubated at 37^0C for 16-18 hours for observation. Then, 2-3 drops of methyl red reagent was added and observed yellow or red color of the media .

4. Lactose Fermentation Test

MacConkey agar is the most widely used media for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacteria. MacConkey agar plates were prepared using peptone (Difco, USA) or gelysate (BBL) 17.0 gm/l, proteose peptone (Difco, USA) or polypeptone (BBL,USA) 3.0 gm/l, lactose 10.0 gm/l, sodium chloride 5.0 gm/l, crystal violet 1.0 mgl/, neutral red 30.0 mg/l, bile salts (Carl Roth, Germany) 1.5 gm/l and agar 13.5 gm/l in distilled water. p^H of the media was adjusted to 7.1 and heated to dissolve the agar. Then the medium was sterilized at 121^0C for 20 minutes. The medium was poured into the Petri dishes and cooled to solidify at room temperature in the laminar airflow. Then streaking was done on the plates with desired pure or mixed culture. Good colonies will ensure the best differentiation of lactose fermenting and non-fermenting colonies of bacteria. Then the plates were incubated at 37^0C for 18 hours. Then the plates were observed for the colony and color of the colony. The colony with pink color can ferment lactose and transparent colony cannot ferment.

5. Citrate Test

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source. The citrate test is based on the generation of alkaline by-products of citrate metabolism and subsequent increase in the pH of the medium which is demonstrated by the color change of a p^H indicator. The citrate test is often part of a battery of tests used to identify gram-negative pathogens and environmental isolates.

Citrate medium was prepared in deionized water. The pH of the medium was adjusted to 6.9 and heated to dissolve the agar and was dispensed as 4.0 to 5.0 ml into 16 mm tubes.

Sterilization was done at 121^0C under 15 psi pressure for 20 minutes. Then the medium was cooled in slanted position (long slant, shallow butt) to give a forest green color due to the p^H of the media and the bromothymol blue.

A single pure isolated colony of the sample was picked with a needle and the slant surface was lightly streaked. The incubation was done at 37^0C for 18 hours. Citrate-positive result was interpreted on the basis of intense prussian blue and in case of citrate-negative no color change occurred.

Media composition:

Magnesium sulfate (heptahydrate)	0.2 gm/L
Ammonium dihydrogen phosphate	1.0 gm/L
Dipotassium phosphate	1.0 gm/L
Sodium citrate (dehydrate)	2.0 gm/L
Sodium chloride	5.0 gm/L
Agar	15.0 gm/L
Bromothymol blue	0.08 gm/L

6. Catalase Test

Some bacteria contain flavoproteins that reduce oxygen (O_2), resulting in the production of hydrogen peroxide (H_2O_2) and in some cases, an extremely toxic superoxide (O_2^-). Accumulation of these substances will result in death of the organism as they are powerful oxidizing agents and destroy cellular constituents very rapidly unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles bacteria use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. A bacterium must be able to protect itself against

such O_2 products or it will be killed. Many bacteria possess enzymes that afford protection against toxic O_2 products. Facultative anaerobes and obligate aerobes usually contain the enzymes superoxide dismutase, which has the ability to catalyze the destruction of superoxide, and either catalase or peroxidase, which catalyze the destruction of hydrogen peroxide as follows:

Organisms capable of producing catalase rapidly degrade hydrogen peroxide which is a tetramer containing four polypeptide chains, which are usually 500 amino acids long. It also contains four porphyrin heme groups (ie. iron groups) that will allow the enzyme to react with the hydrogenperoxide. The enzyme catalase is present in most cytochrome-containing aerobic and facultative anaerobic bacteria. Catalase is the enzyme which has one of the highest turnover numbers compared to all other enzymes; one molecule of catalase has the ability to convert millions of molecules of hydrogen peroxide to water and oxygen in each second.

Catalase production and activity can be detected by adding the substrate H_2O_2 to an appropriately incubated (18 to 24hour) tryptic soy agar slant culture. Organisms which produce the enzyme break down the hydrogen peroxide, and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to break down hydrogen peroxide, into O_2 and water and are catalase negative. Catalase activity is very useful in differentiating between groups of bacteria. For example, the morphologically similar *Enterococcus* (catalase negative) and *Staphylococcus* (catalase positive) can be differentiated using the catalase test.

For catalase test first LB agar medium was prepared then for preparing culture plates. 15-20 ml of sterile medium was was poured in petridish and left the airflow cabinet for solidification.Streaking loop was used to streak bacterial colony on LB agar plate then kept in incubation for 18 hours. Bubble formation at the bottom of the test tube was observed.

7. Urease Test

Urease broth is a differential medium that tests the ability of an organism to produce an exoenzyme, called urease, which hydrolyzes urea to ammonia and carbon dioxide. According to the standard protocol of urease test we dissolved 20.30 gm of Urease broth in 1000 ml distilled water, mixed well, sterilized by filtration and finally dispensed in sterile tubes. Color change of the medium was observed after incubation of the isolated bacteria at 35-37°C for 18 hours.

Media composition (gm / L):

Yeast extract	0.10
Urea	20.00
Monopotassium phosphate	0.091
Disodium phosphate	0.095
Phenol red	0.010

8. Lysine Test

Lysine decarboxylase (LDC) broth is used to determine whether the microbe can use the amino acid lysine for carbon and energy. The decarboxylase test is useful for differentiating the *Enterobacteriaceae*. We Suspended 14 grams of the LDC medium in one liter of distilled water; mixed well and dissolve by heating with frequent agitation. Finally we dispensed quantities of 5 ml into screw-capped tubes and sterilized in autoclave at 121°C for 15 minutes. After 18hr of incubation at 37°C, color cahnged was observed to find out the result.

Media composition (gm / L):

Gelatin Peptone	5.00
Dextrose	1.00
L-Lysine	5.00
Bromocresol Purple	0.02
Yeast Extract	3.00

9. Triple Sugar Iron (TSI) Test

Triple Sugar Iron Agar is used for the identification of gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulphide production. 64.52 grams of triple sugar iron agar was dissolved in 1000 ml distilled water. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and allowed the medium to set in sloped form with a butt about 1 inch long. Cultural characteristics observed after incubation at 37°C for 18 hr.

Media composition (gm / L):

Peptic digest of animal tissue	20.0
Yeast extract	3.0
Meat extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate, heptahydrate	0.20
Sodium thiosulphate, pentahydrate	0.30
Phenol red	0.024
Agar	12.0

10. Starch Hydrolysis Test

Isolated bacteria were screened for starch hydrolysis test in starch agar media comprising the following in gm/L: yeast extract 1.5 gm, peptone 0.5 gm, sodium chloride 1.5 gm, starch 10 gm, agar 15gm. p^H of the medium was adjusted to 5.6. Bacteria were streaked centrally on sterile solidified starch agar plates. A negative control without inoculation

was also maintained for comparison. Plates were incubated at 28°C for 48 hours. Subsequently, all the plates along with the negative control were flooded with iodine and observed for zone of hydrolysis. Identification of the isolate showing zone of hydrolysis during screening was identified based on its physical and staining parameters.

2.6 Effect of Temperature and pH on Bacterial Growth

Temperature and p^H influenced on the bacterial growth. For the effect of p^H, culture medium was adjusted to p^H 5.0, 6.0, 7.0 and 8.0. Incubation temperature was varied at, 25°C, 30°C and 35°C. Bacterial cell density of liquid cultures was determined by measuring optical density at 600 nm using double beam spectrophotometer (ANALYTK JENA AG, SPOKOL 1500/1, GERMANY).

2.7 Determination of Toxic Effect of Carbofuran against Artemia salina

Brine shrimp lethality bioassay is an important development in the bioassay which indicates cytotoxicity as well as a wide range of pharmacological activities of the tested samples. Xenobiotics compounds are almost always toxic in high doses. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, AIDS, anti-microbial and pharmacological activities of any samples. Here *in vivo* lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening toxicity of compounds at cellular level. Generally, the median effective dose (ED_{50}) values for cyto-toxicity are one tenth (1/10) of median lethal dose (LC_{50}) values in the brine shrimp test.

2.7.1 Procedure

Brine shrimp eggs are hatched in simulated sea-water to get nauplii. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea-water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are left for 24 hours and then the live and dead nauplii are

counted again to find out the cytotoxicity of the test agents and compared to the results with positive control.

Test materials:

(i) *Artemia salina* Leach (brine shrimp eggs)

(ii) Sea salt (non ionized NaCl)

(iii) Beaker with aeration facility to hatch the shrimp

(iv) Light to control temperature of the simulated sea water.

(v) Pipette (1 ml and 5 ml)

(vi) Micropipette (10-200 µl adjustable)

(vii) Glass vials (5 ml)

(viii) Magnifying glass

2.7.2 Preparation of Simulated Sea water (brine water)

Since the lethality test involves the culture of brine shrimp nauplii which should be grown in the sea water, therefore, simulated sea water was prepared. Sea water contains 3.8% of sodium chloride. Accordingly 38 gm sodium chloride was dissolved in 1000 ml distilled water and was filtered off. The p^H of the brine water thus prepared was maintained between 8 and 9 using $NaHCO_3$.

2.7.3 Hatching of Brine Shrimp

Brine water was taken in a small tank and shrimp eggs (1.5 gm/l) were added to one side of the perforated divided tank with constant oxygen supply. Constant temperature 37°C and sufficient light were maintained to provide sufficient aeration. After 48 hours, matured shrimp as nauplii (larvae) was collected and used for the experiment.

2.7.4 Preparation of sample solution

The test sample carbofuran (99.00% pure) was used as treatment and the following doses were prepared;

a) 20.0 µg

b) 10.0 µg

c) 5.0 µg

d) 2.5µg

e) 1.25µg

2.7.5 Preparation of Negative Control

For each concentration, one vial containing 10 ml simulated sea-water and 10 shrimp nauplii was used as negative control group. It was used to verify the validity of the test. When the nauplii in the control showed a rapid mortality, then the test is considered to be invalid as the nauplii might die due to reasons other than the cytotoxicity of the compounds.

2.7.6 Application of Test Sample and Brine Shrimp Nauplii to the Vials

In each of the five vials, 10 ml simulated sea-water was taken, containing 10 brine shrimp nauplii Specific volumes of each sample were transferred from the stock solution of carbofuran with the help of a micropipette to the respective vials to get, final concentrations of 20.0 µg, 10.0 µg, 5.0 µg 2.5 µg 1.25µg of carbofuran in the culture.

2.7.7 Counting of Nauplii

After 24 hours, the vials were observed. The number of survived nauplii in each vial was counted and the results were noted. From this, percentage of mortality of brine shrimp nauplii was calculated at each concentration for each sample

2.7.8 Analysis of the Data

The dose mortality data were analyzed statistically by Probit analysis. The effectiveness or the dose mortality relationship (concentration-mortality relationship) of any product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

2.8 Antagonistic Test of the Isolated Bacteria

The aim of this experiment is to evaluate whether the isolated bacteria can stimulate or inhibit the growth of plant pathogenic fungi or bacteria as well as plant growth promoting Rhizobacteria (PGPR).

2.8.1 Materials

- Distilled water
- Aluminums foil paper
- Filter paper
- Micropipette
- Eppendorf tubes
- Micro tips
- Petri dish
- Forceps
- 70% alcohol,
- Conical flask,
- Measure cylinder

2.8.2 Test Organisms

The antagonistic effect of the isolated bacteria was tested against some pure culture strain of bacteria and fungi. The fungal strains were obtained from Pathology laboratory, Department of Botany and bacterial strains were obtained from the Microbiology laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi.

Table 2.1: Pure culture strains of bacteria and fungi

Fungi	**Bacteria**
Sclerotinia sclerotiorum	*Rhizobium RLC* 107
Penicillium chrysogenum	*Rhizobium RVM* 307
Fusarium oxysporum	*Rhizobium RCA* 220
Aspergillus niger	*Pseudomonas putida*
Rhizopus oryzae	*Xanthomonas campestris*

2.8.3 Fungal Culture Media Preparation

Luria broth (LB) and Luria agar (LA) Medium were used as culture medium for the growth of bacteria which has been described earlier in the chapter. Potato dextrose agar (PDA) media was used to perform the antifungal activity and for subculture of the test organisms. The composition o f the PDA media is as follows:

Potato dextrose agar (PDA)media

Potato Infusion = 200 g/L

Dextrose = 20 g/L

Agar = 15 g/L

pH = 5.6 ± 0.2

2.8.4 Methods

- **Preparation of Luria broth (LB) and Luria agar (LA) Medium:**

For broth (liquid) media all the ingredients except agar was suspended in 1 L of double distilled water and was mixed thoroughly. For agar plates 15g/l of agar was added to the desired medium for solidification. The pH of the medium was adjusted at 7.0 using 1N NaOH and autoclaved at 121ºC for 20 minutes.

- **Preparation of PDA Medium:**

The constituents of the media were accurately weighed and dispersed in a conical flask with distilled water. It was heated in water bath to dissolve the ingredients until a transparent solution was obtained. The pH of the medium was adjusted to 5.6. The volume was adjusted by adding distilled water and sterilized in an autoclave at 121°C and 15 Ibs/sq. inch pressure for 20 minutes.

- **Plate Preparation:**

For preparing of culture plates, sterilized Petri-dishes and medium were taken under running laminar airflow as soon as possible. The medium was cooled to 50-55 deg. C. Then the sterilized medium was poured in equal (about 20ml) in each Petri dish and allowed to cool and solidify. In this time the cover of Petri dishes were opened partially to reduce condensation. When the medium became solid the Petri dishes marked with a glass marker pen according to their medium name and date. Then the plates were sealed by parafilm and stored at 4^0C until use.

- **Culture of Collected Strains and Plating:**

The stock of collected bacterial and fungal strains were taken out of -20^0 C and revived in either LB medium or onto PDA medium subsequently. Bacterial cultures were incubated at 37°C for overnight and fungi were at 30°C. These suspensions were used as inoculums.

- **Sample Disc Preparation of The Isolated Bacteria:**

Before disc preparation, isolated bacterial subculture was centrifuged to get culture medium without bacteria. So we had following three sample:

- Subculture of bacteria
- LB medium
- Centrifuged subculture medium
- Disk diffusion method (Barry *et al.,* 1976) was used in the current experiment to evaluate the antagonistic activity of selected bacterium. The discs (6 diameters) were made by punching the Whatman No.1 filter paper with the help of punch machine. These discs were taken into the screw capped tube and sterilized in an autoclave machine at 121 deg. C for 20 minutes to ensure sterilization. Three different samples were userials as test material for disk diffusion assay. (i) Subcultured bacteria, (ii) Centrifuged subcultured medium and (iii) blank LB media

The paper discs were soaked with 20µl of above three types of test materials with the help of micropipette and kept them at laminar air flow hood for dryness (5-10 minutes). The disks containing the test materials were introduced on the upper layer of the seeded agar plate using sterile forceps. Subsequently, the plates were incubated overnight at 37^0 C.

2.8.5 Antagonism Testing

When a filter paper disc saturated with a chemical is placed on the agar medium the chemicals will diffuse from the paper disc into the agar medium. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar medium, it will not grow in the area around the disc if it is susceptible to the chemical. Here we spread collected bacterial and fungal strain on to Luria agar plate and PDA plates respectively. This area of no growth is known as ‘zone of inhibition’. Then microbial growth was determined by measuring the diameter of zone of inhibition.

2.9 Antibiotic Sensitivity Test

The isolated bacterial strains were grown overnight in nutrient broths through shaker at 30°C temperature and 120 rpm. for the antibiotic sensitivity test. Nutrient agar plates were dried at 30°C. The overnight grown LB culture (O.D. = 0.5) was pour onto nutrient plate and dried. Antibiotics disks were placed centrally on the respective plates and incubated overnight at 30°C (Table 2.2).After overnight incubation than the zone was observed on the plate and measured the zone with the help of mm scale.

Table 2.2: List of antibiotic disks containing different concentrations of antibiotic that were used in the antibiotic sensitivity test

Antibiotics	**Symbol**	**Concentration**
Cefuroxime sodium	CXM	30 µg
Nitrofurantoin	F	300µg
Azithromycin	AZM	15µg
Penicillin	P	10 µg
Ceftazidime	CAZ	30µg
Vancomycin	VA	30 µg
Gentamicin	CN	10µg
Ciprofloxacin	CIP	5µg
Nalidixic acid	NA	30µg
Mecillinam	MEL	25µg

2.10 Determination of Minimum Inhibitory Concentration (MIC) of Gentamycin (Broth Tube Dilution Method)

The tube dilution test is the standard method for determining levels of resistance to an antibiotic. Serial dilutions of the antibiotics are made in a liquid medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the antibiotic is bacteriostatic**.**

Additionally, the minimal bactericidal concentration (MBC) can be determined by subculturing the contents of the tubes onto antibiotic-free solid medium and examining for bacterial growth.

Materials:

1. Antibiotic Gentamycin
2. Test tubes.
3. Pipette.
4. LB broth and nutrient agar plates.
5. Petriplates.
6. Incubator.

Procedure:

1. The sterile capped test tubes were marked from 1 to 9. All of the following steps were carried out in aseptic condition.
2. 2.0 ml of Gentamycin solution (100 µg/ml) were added to the first tube containing 1.0 ml of sterile broth. All other tubes were also filled with 1 ml sterile broth.

3. 1.0 ml solution from the first tube was transferred to the second tube.
4. Subsequently, the contents were mixed and again 1 ml of the solution was taken to the third tube.
5. The dilutions were continued in this manner to tube number 8, being certain to change the pipette tips tach time to prevent carryover of antibiotic on the external surface of the pipette.
6. 1.0 ml solution from tube 8 was removed and was discarded. The ninth tube, which serves as a negative control, received no Gentamycin.
7. The fresh bacterial culture were prepared and used for the experiment. 1.0 ml of the culture suspension was added to each of the tubes. Finally, all test tubes were incubated at 30^{o}C for overnight.
8. These tubes were examined for visible signs of bacterial growth. The highest dilution without growth was the minimal inhibitory concentration (MIC).

2.11 Viable Cell Count through Serial Dilutions Technique

A primary characteristic of a culture of bacteria cells is its population density, *C*: the number of living cells per ml of solution. A culture would commonly have between a million (10^6) and a billion (10^9) "colony forming units" (cfu: i.e., viable cells) per ml, Furthermore it is hard to distinguish a living cell from a dead cell even in a microscope. The usual procedure is to dilute the solution by a huge factor so that in the end only a few (say 20-300) viable cells are sampled. The easiest way to count those *viable* cells is to let each one multiply producing a visible "colony" of millions of cells founded by just one cell from the culture. Thus, one visible colony represents one founding cell from the culture. The serial dilutions is 6 dilutions in sequence each reducing the concentration by a factor of 10 (say by diluting 1 mL of the concentrate to 10 mL of solution).

Once we count the *N* colonies from the 0.1 mL of the most diluted tube, we know the concentration in that most diluted tube is:

concentration = (*N* cfu)/(0.1 mL) = 10 *N* cfu/mL

Before the dilution those *N* bacteria were in a volume of only: (0.1 mL) × 10^{-6}

Thus the original concentration *C* is given by:

C = (*N* cfu)/(0.1 mL × 10^{-6}) = 10^{7} × *N* cfu/mL

e.g., 10^{8} cfu/mL = 10^{2}× 10^{6} cfu/mL = 100 Mcfu/mL ,where, Mega=M=million=10^{6}

Materials:

a) Six tubes each containing 9.0 ml sterile saline,

b) Plates of Nutrient agar containing carbofuran,

c) 1.0 ml micropipette and sterile pipette tips,

d) Pipette,

e) Bent glass rod,

f) 70% Alcohol

Procedure:

1. Six dilution tubes with 9.0 ml of sterile saline were taken. 1.0 ml of a sample of isolated bacterium culture and were aseptically diluted to the O.D. 0.5. The dilution was done as follows: 1 ml of sample were dispensed aseptically at the first dilution tube and the bacterial suspension were mixed thoroughly by either holding the tube in one hand or vigorously tapping the bottom with the other hand or by using a vortex mixer to assure an evenly distribution of the sample throughout the liquid. Using the same procedure, 1.0 ml from the first dilution tube and dispensed into the second dilution tube. Continue doing this from tube to tube until the dilution was completed.

2. 0.1 ml diluted sample were transferred from each of the last three dilution tubes onto the surface of the corresponding nutrient plates supplement with various carbofuran concentration.

3. Using a turntable and sterile bent glass rod the solution over the surface of the plates was immediately spread as follows:

a. Place the plate containing the 0.1 ml of dilution on a turntable.

b. Sterilize the glass rod by dipping the bent portion in a dish of alcohol and igniting the alcohol with the flame from burner. Let the flame burn out.

c. Place the bent portion of the glass rod on the agar surface and spin the turntable for about 30 seconds to distribute the 0.1 ml of dilution evenly over the entire agar surface.

d. Replace the lid and re-sterilize the glass rod with alcohol and flaming.

e. Repeat the technique for various carbofuran concentrations after 0h, 2h, 4h, 6h, 12h, 24 h.

f. Maintain a control of nutrient agar plates without carbofuran for each concentration.

g. Discard the pipette in the bio-waste disposal containers at the front of the room and under the hood.

4. The agar plates were incubated at 30°C in incubator for 24 hour and the single colony were counted in every plate and the equation was applied separately.

2.12 Identification of the Isolated Bacteria by 16S rRNA Gene Sequencing

2.12.1 Extraction of chromosomal DNA

Chemicals: RNase A

TE buffer (0.5M EDTA-pH 8.0+1M Tris HCl pH 8.0)
Chloroform
Isoamylalcohol
Ethanol (70%)
Phenol
Isopropanol
SDS
Proteinase K

Chromosomal DNA was extracted by according to Sambrook *et al*. (1989). The procedures are described below:

- Bacterial colony was inoculated on LB agar plate and incubated at 37°C.
- Subsequently, single bacterial colony from the agar plate was inoculated into 3 ml LB Broth and incubated at shaking water bath at 37°C for 16 hours.
- 1.5 ml culture broth was taken into a micro centrifuge tubeand harvested cell by centrifugation at 8,000 rpm for 8 minutes. Supernatant was discarded
- Bacterial cells were resuspended into 455 µl 1X TE buffer. Then 30 µl of 10% SDS was added & mixed properly by inverting the 1.5 ml microcentrifuge tube.
- 3 µl Proteinase K (800U/ml) was added and mixed well.
- Then it was incubated at 37°C for 1 hour (use water bath or Incubator).
- 100 µl 5M NaCl was added and mixed well by inverting the micro centrifuge tube.
- Equal volume (650 µl) of Chloroform: Isoamylalcohol (24:1) was added and mixed thoroughly by inverting the microcentrifuge tube for 15 minutes until milky white appearance observed.
- Then it was centrifuged at 13,000 rpm for 8 minutes and 500 µl clear supernatant were collected carefully into a fresh microcentrifuge tube. The inter-phase was not disturbed during supernatant collection.
- Equal volume (500 µl) of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed thoroughly by inverting the microcentrifuge tube for 15 minutes until milky white appearance observed.
- Then it was centrifuged at 13,000 rpm for 8 minutes and 500 µl clear supernatant were carefully collected into a fresh microcentrifuge tube. The inter-phase was not disturbed during supernatant collection.
- 5 µl RNase A (10mg/ml) was added to make a final concentration of 100 µg/ml.

- Then it was incubated at 37°C for 1 hour (using water bath or Incubator).
- Equal volume (500 µl) of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed thoroughly by inverting the microcentrifuge tubefor 15 minutes until milky white appearance observed.
- Then it was centrifuged at 13,000 rpm for 10 minutes and 500 µl clear aquas layer (supernatant) was carefully transferred into a fresh microcentrifuge tube. The inter-phase was not disturbed during upper aquas layer collection.
- 0.6 (60%) volume (300 µl) of Isopropanol was added to precipitate genomic DNA. The microcentrifuge tube was shaken back and forth until a string white DNA becomes visible.
- Then it was centrifuged at 13,000 rpm for 10 minutes and supernatant were discarded very carefully with retaining those precipitated DNA.
- 300 µl of Ice cool Ethanol (70%) was added & Centrifuged at 13,000 rpm for 10 minutes. Supernatant was discarded very carefully with retaining those precipitated DNA. This step was repeated twice.
- Then the microcentrifuge tube was opened and wrapped the floating rack with a tissue paper. Then it was allowed for air drying overnight.
- When the residual Ethanol was completely evaporated from the eppendorf, 100 µl 1X TE buffer was added and DNA was dissolved carefully.
- Isolated DNA was stored at -20°C.

2.12.2 PCR Amplification of the 16s rRNA gene

Gene fragments specific for the highly variable region of the bacterial 16S rRNA gene was amplified by PCR. This reaction was carried out by the universal primer 16S F & 16S R (Sigma, USA). The primer sequences are as follows:

Table 2.3: Primer of the detection of 16s small subunit ribosomal RNA genes

Gene	Primer	Oligonucleotide sequence(5'-3')	Annealing Temperature	Size of amplifying Product(bp)
16S rRNA	Forward	GAGTTTGATCCTGGCTCAG	55 °C	1500
	Reverse	GAAAGGAGGTGATCCAGCC		

All PCR reagents are used from the invitrogen. PCR is performed in a 50 µl reaction mixture containing 5µl of 10X PCR Buffer, 4µl of 2.5mM dNTPS, 2µl of 50mM $MgCl_2$, 2.5µl of each primer (10µM), 0.2µl of 5U/µl Taq DNA polymerase, 1 µl genomic DNA and rest of the PCR water. PCR program was as follows: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, 1 min of primer extension at 72 °C and 10 min of final extension at 72 °C for one cycle. Amplicons were separated by agarose gel electrophoresis (1 %) in 0.5X Tris/borate/EDTA buffer. Products were stained with ethidium bromide, destained with distilled water, and visualized under UV light and photographed on a gel-documentation system. 1500 bp PCR products are obtained. 1 kb^+ DNA size markers (invitrogen, USA) is used to determine the size of the PCR product.

2.12.3 Purification of the PCR Products

The PCR product was cut from the gel and purified by using commercially available Microcon Centrifugal Filter device, USA according to the following way:

The desired band was excised from the gel and purified by the Freeze 'N Squeeze DNA Gel Extraction Spin Columns. Then partial purified sample reservoir was inserted into screw cap vial. The entire solution (PCR product) was pipetted into sample reservoir (0.5 ml maximum volume), without touching the membrane with the pipette tip. The attached

cap of screw cap vial was then sealed. The assembly (screw cap vial with sample reservoir) was placed in a centrifuge machine. When placing the assembled device into the centrifuge rotor, the cap strap was aligned toward the centre of the rotor. The assembly was spin at 12000 rpm for 20 mins. The assembly was then removed from the machine and the vial was separated from the sample reservoir. The sample reservoirs were placed upside down in new vial, and then spun at 10000 rpm for 3 min to transfer the concentrate to vial. The sample reservoir was then separated from vial and discarded. 100µL Tris EDTA (TE) buffer is added to vial containing the purified PCR product. Concentration of PCR product was measured using UV spectrophotometer and diluted to 10ng/µl with TE buffer.

2.12.4 Cycle Sequencing

Both forward and reverse primers were used for sequencing. For sequencing PCR the primers were diluted to 3.2 µM using PCR water. The reagents added to a separate tube for each reaction are shown in Table 2.3.

Table 2.4: The reagents used in cycle sequencing

Reagent	**Quantity (µl)**
Big dye (BDV3.1) reaction mix	4
Template 10 ng/µl	1
Primer 3.2 µM	1
PCR water	14
Total volume	20

The contents of the tubes were mixed well and spun briefly. The tubes were placed in the thermal cycler (Applied Biosystem 9700, USA) and the following cycle sequencing program employed is summarized in the following table. The sample was then stored at 4°C and hold at this temperature until ready to purify.

Table 2.5: Cycle Sequencing Program

1. Place the tubes in a thermal cycler and set to the correct volume.
2. Perform an initial denaturation. a. Rapid thermal ramp to 96 ° C b. 96 °C for 4 min
3. Repeat the following for 25 cycles: Rapid thermal ramp to 96 °C 96 °C for 10 seconds. Rapid thermal ramp to 50 °C 50° C for 5 seconds. Rapid thermal ramp to 60°C 60 °C for 4 minutes
4. Rapid thermal ramp to 4 °C and hold ready to purify.
5. Spin down the contents of the tubes low spin in a microcentrifuge.
6. Next step was purification of extension products.

*Rapid thermal ramp is 1 °C /second.

- Use Applied BioSystem 9700 Series or Similar machine

2.12.5 Purification of the Cycle Sequence Product and Detection of Nucleotides by ABI- PRISM 310

2 µl of 3M sodium acetate (pH 4.6) and 50 µl of 95% ethanol were placed in a 1.5 ml eppendorf tube. The entire content of each reaction was transferred to a tube where sodium acetate and ethanol mixture was placed. Then they are mixed thoroughly. The tubes were then incubated at room temperature for more than 15 min to precipitate the extension products. The tubes were then centrifuged at 13,000 rpm for 20 min. The supernatant was discarded carefully using a micropipette. The pellet was washed with 250 µl of 70% ethanol by washing the walls of the tubes. The tubes were spin at 13,000 rpm for 10 min. The supernatant was discarded very carefully using a micropipette and tips were used to remove the supernatant from the bottom of the tubes. The washing steps were repeated twice. The pellet was dried by keeping the caps of the tubes opened for 2-3 hours at room temperature. The opened tubes were covered by parafilm. The pellet was then resuspended into formamaide and mixed well. Then it was heated at 95°C for 2 min & immediately transferred into ice & kept for 5 min. Then the products are transferred to septal tube.

2.12.6 Detection of the Nucleotides by ABI- PRISM 310

The purified cycle sequence products were analyzed by electrophoresis in the genetic analyzer (Prism 310, USA). DNA was separated through the POP6 contained in the capillary and detected by the laser beam. When the nucleotides reach a detector window in capillary electrophoresis, the fluorescent labeled fragments were excited by the laser beam of the machine. The laser excites the fluorescent dye labels and emited fluorescence was collected by CCD camera. The fluorescence intensity data was interpreted into sequence data by specific bioinformatics software.

2.12.7 Sequence Analysis

The sequences were then edited by bioinformatics software Chromas. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other

organisms that had already been submitted to GenBank database using the BLASTN (http://www.ncbi.nih.gov/BLAST).

2.13 Extraction of Plasmid DNA

Plasmid extraction through TIANprep Mini Plasmid Kit :

TIANprep Mini Plasmid Kit (Cat.no. DP103) was used for plasmid extraction, which is based on alkaline lysis technology followed by adsorption of DNA onto silica membrane in the presence of high salt.

Kit contents following enzyme, buffer, column and collection tubes:

Contents	DP103-02 50 preps	DP103-03 200 preps
RNaseA (10 mg/ml)	150 µl	600 µl
Buffer BL	30 ml	120 ml
Buffer P1	15 ml	60 ml
Buffer P2	15 ml	60 ml
Buffer P3	20 ml	80 ml
Buffer PD	30ml	120ml
Buffer PW	15 ml	50 ml
Buffer EB	15 ml	30 ml
Spin Columns CP3	50	200
Collection Tubes (2 ml)	50	200

At first we did following steps:

1. RNase A solution was added to Buffer P1, mixed, and stored at 2-8°C.

2. (96-100%) Ethanol was added to buffer PW . At the same time we also checked buffer BL, P2 and P3 for avoid any salt precipitation.

3. 18hr old cultured bacteria were used for this plasmid extraction experiment.

Protocol:

1. Column equilibration: 500 µl buffer BL was added to Spin Column CP3, centrifuged for 1 min at 12,000 rpm (~13,400 × g) in a table-top micro centrifuge and discarded the flow-throw, and placed Spin Column CP3 into the collection tube.
2. 2. 1-5 ml bacterial cells were harvested in a microcentrifuge tube by centrifugation at 12,000 rpm (~13,400 × g) in a conventional, table-top microcentrifuge for 1 min at room temperature (15-25°C), then all traces of supernatant was removed by inverting the open centrifuge tube until all medium has been drained.
3. 250 µl resuspension buffer P1 was added .
4. 250 µl lysis buffer P2 was added and mix thoroughly by inverting the tube 6-8 times for 5 min.
5. 350 µl nutrilization buffer P3 was added and mix immediately and thoroughly by inverting the tube 6-8 times. The solution became cloudy.
6. 6. Centrifuged for 10 min at 12,000 rpm (~13,400 × g) in a table-top microcentrifuge. A compact white pellet was formed.
7. The supernatants from step 6 was taken in to the Spin Column CP3.
8. The Spin Column CP3 was washed by adding 500ul buffer PD and centrifuged for 1 min at 12,000 rpm (~13,400 × g).
9. 700 µl buffer PW (96%-100% ethanol has been added to Buffer PW) was added into Spin Column CP3 and centrifuging for 1 min at 12,000 rpm (~13,400 × g). Discarded the flow-through.
10. Again 500 µl Buffer PW was added to wash Spin Column CP3 through centrifuged for 1 min at 12,000 rpm (~13,400 × g).
11. The flow-through was discarded, and centrifuged for an additional 2 min at 12,000 rpm (~13,400 × g) to remove residual wash buffer PW.

12. Finally, the Spin Column CP3 was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 100-300 µl Buffer EB was added to the center of each TIANprep spin column CP3, wait for 2-5 min, and then centrifuged at 12,000 rpm (~13,400 × g). for 2 min to complete plasmid extraction.

Agarose Gel Electrophoresis

Horizontal submerged agarose gel electrophoresis was carried out to check the existence of plasmid. % agarose was used for this purpose. 0.5 gm Agarose was added to 50 ml TBE buffer and it was heated until it dissolved. The solution was cooled at 50°C and 5µl ethidium bromide was added to stain the gel.

Thus the gel was prepared and was submerged in TBE buffer. TBE was prepared as a 5 fold (5 X) concentrated stock solution.

Extracted plasmid DNA samples were mixed with the loading dye and loaded into the well of agarose gel. Gels were run submerged in TBE buffer at 80 volt (DC) for 50 minute.

The dye could be observed visually but the DNA was only visualized with a U.V. transilluminator.

2.14 Determination of carbofuran degradation rate by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

High pressure liquid chromatography is a powerful analytical tool in proteomics as well as for analytical chemistry. It is also called high performance liquid chromatography and is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through the column under high pressures of up to 400 atmospheres which makes it much faster. It also uses a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

The other major improvement over column chromatography concerns the detection methods which can be used (figure 2.1). These methods are highly automated and extremely sensitive.

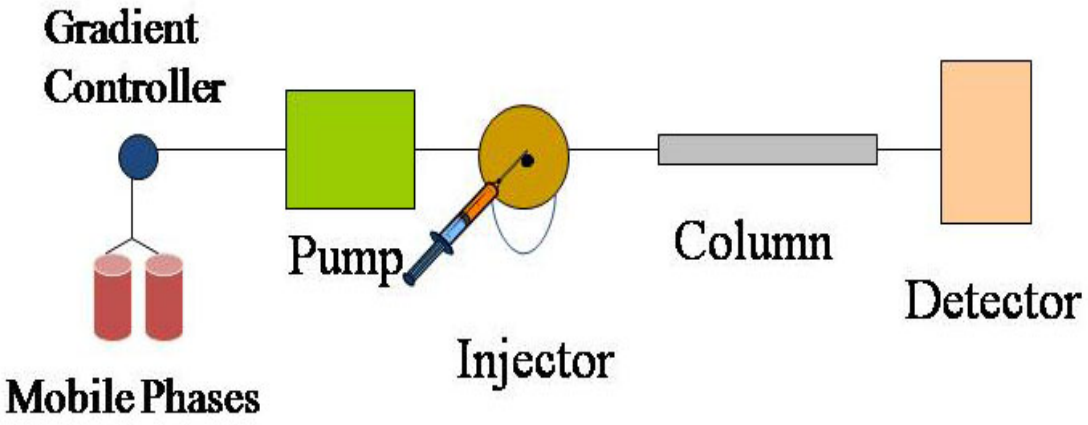


Fig. 2.1: HPLC System**:** The sample are injected into the injection loop and passed through the column. Using pump the flow rate is controlled. The eluted protein is detected by a detector and elution peak is observed on monitor

2.14.1 Normal phase HPLC

This is essentially just the same as thin layer chromatography or column chromatography. Normal-phase HPLC (NP-HPLC) is also known as, adsorption chromatography and this method separate analytes based on adsorption to a stationary surface chemistry and by polarity. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte molecule, but also on steric factors. The use of more polar solvents in the mobile phase will decrease the retention time of the analytes,

whereas more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. This behavior is somewhat peculiar to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

2.14.2 Reversed phase HPLC (RP-HPLC)

The mobile phase is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electro-chromatography (CEC)), a gas (GC), the mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. Stationary phase is a layer or coating on the supporting medium that interacts with the analytes and is fixed in a place either in column or a planar surface. It can be solid, liquid, gel or solid-liquid mixture.

In this case, the column size is the same, but the matrix inside the column (silica) is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, Acetonitrile or a mixture of water and an alcohol such as methanol.

Reversed phase HPLC is the most commonly used form of HPLC. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the

solvent and this will slow them down on their way through the column. Two different solvents are used in this chromatography one is 99% water (polar) and the other is acetonitrile (80% acetonitrile and 19% water) or non-polar solvent. In both the solvent 1% TFA is added as ion pairing agent. These two solvents are placed in two different bottles and connected to two different pumps (pump A and pump B) of HPLC machine. In the process first the mixture of protein is injected and bound to the column. Then solvent A (water) is passed though the column and subsequently, slowly the amount of A is decreased and amount of B is increased by gradient mixing of the two solvent in a mixing chamber of HPLC machine to elute the proteins bound to the column. So, the proteins are eluted based on their hydrophobicity profile. Less hydrophobic (more polar) proteins are eluted is less amount (or %0) of B and more hydrophobic phobic proteins that are more tightly bound to the column needs higher concentration of solvent B to elute out from the column.

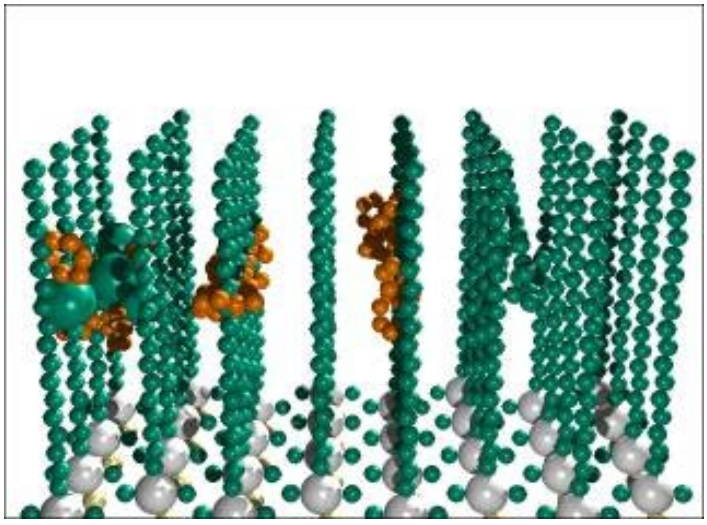


Fig. 2.2: The Surface of Silica Bonded with C18; Non-polar (hydrophobic) molecules are bound to the hydrocarbon chains of the column and polar molecular are passed through the column matrix. Strength of attachment of the proteins to the hydrocarbon chains depends on their degree of hydrophobicity. Proteins are eluted by breaking the bond between the matrix and protein. The proteins with less hydrophobicity will elute easily with polar solvent whereas highly hydrophobic solvent will require more non-polar solvent like acetonitrile.

2.14.3 Preparation of Buffer A and Buffer B for reverse phase HPLC

One Liter of Buffer A was prepared using 1000 ml of de-ionized water. 1 ml of TFA was added to it and mixed by swirling. It was then filtered through a 0.45µ PTFE membrane using a Sartorius vacuum buffer filtration system. Buffer was stored at room temperature until use. 80% acetonitrile was used as buffer B for the current experiment. To make one liter of buffer B 800 ml was acetonitrile was mixed with 200 ml of water and 1 ml of TFA was added to it. Buffer B was also filtered and stored in the same way as buffer A.

2.14.4. Equilibration of the HPLC system and Colum

Binary gradient HPLC of Waters Corporation was used for the current experiments. Both the pumps of the HPLC were initially purged with respective buffer: Pump A with water and pump B with acetonitrile. Then the whole system including the pump, tubing, and sample loop was washed with 100% buffer A. The C18 column was connected to the HPLC and first it was washed with 100% buffer B to remove any residual protein attached to it (figure 2.2). The column was washed with 100% buffer B (80% acetonitrile) until the base line monitored at 280nm was stable. Two Column volumes (4ml X 2 = 8ml) of buffer B was needed to pass through the Column for complete washing. Then the column was equilibrated with three CV (4ml X 3 = 12 ml) of 100% buffer A. The flow rate was set as 1ml/min during equilibration. The equilibration was done until the base line became linear.

2.14.5 Sample Preparation and Injection

For the HPLC experiment, isolated bacteria were inoculated into MS media supplemented with different concentration of 99.9% carbofuran and incubated into rotary shaker at 160 rpm for seven days. Samples of the different carbofuran concentrated media were collected after every 24hr and supernatant of each samples were preserved after centrifugations at 8000 rpm for HPLC experiment. Finally, cell free supernatants were sterilized through 0.22 µ syringe filter and for each samples 20 µl were injected

separately for every run into the C18 column through the Rheodyne manual injector. Elution was monitored at UV absorption 280nm.

2.14.6. Gradient Elution

For elution of samples the concentration of buffer B was increased gradually. As the binding affinity or retention time of the proteins depends on the hydrophobicity and that is universal for a specific compound or proteins. It is expected that the proteins which are loosely bound to the column (less hydrophobic and more polar) will elute in less amount of buffer B from the column whereas the more hydrophobic proteins will need more concentration of buffer B to release from the column. Initially 100% buffer is passed through the system and then gradually percentage of buffer A is decreased and buffer B is increased. Gradual increase of buffer B with the decrease of buffer A is maintained in a mixing chamber of HPLC system and pumped in the system through the column.

Table 2.6: Gradient profile program used for all the HPLC experiments are as follows

Time	%A	%B
-	100	0.0
6 min.	100	0.0
20 min.	80	20
35 min.	45	55
36 min.	0	100
44 min.	0	100
45 min.	100	0.0
50 min.	100	0.0

Plate 2.1: HPLC apparatus used to detect carbofuran concentration

2.14.7 Running of Standard

99.9% carbofuran was used as HPLC standard in the current experiment. 1 mg of carbofuran was dissolved in 1ml of MS broth, filter sterilized using 0.22 µ syringe filter and 20 µl was injected into the column through the Rheodyne manual injector. Upon completion of the run (run time 65 minutes) the chromatogram was obtained using the software Empower-2and retention time was recorded from thechromatogram.

2.14.8 Running of Samples

Samples of 2µg/ml, 4 µg/ml, 8µg/ml carbofuran concentrated MS liquid media with bacteria were collected at a regular basis after 24 hr up to 7 days and centrifuged at 8000 rpm. Finally, cell free supernatants were collected separately, filtered through 0.22 µ syringe filter and samples of day 0, day 3, day 5, day 7 were run through C18 column to gain the chromatograms.

2.15 Preservation of Bacterial Isolates

Glycerol stocks (25% Glycerol) were also prepared to preserve the bacteria at -20^{o}C.

RESULTS

3.1 Isolation of Carbofuran Degrading Bacteria

Soil samples from carbofuran treated rice field were collected within a plastic container and brought it into Microbiology Laboratory for the isolation of bacteria, which can detoxify carbofuran. Initially, 1 gm soil was dissolved into 10 ml distilled water and 1 ml filtered inoculums was poured into minimal salt liquid media which was supplemented with 2µg/ml (99%) carbofuran. Then the inoculated ms media was incubated at 37ºC and 160 rpm in orbital shaker for seven days. A control without inoculums was also maintained at the same condition.

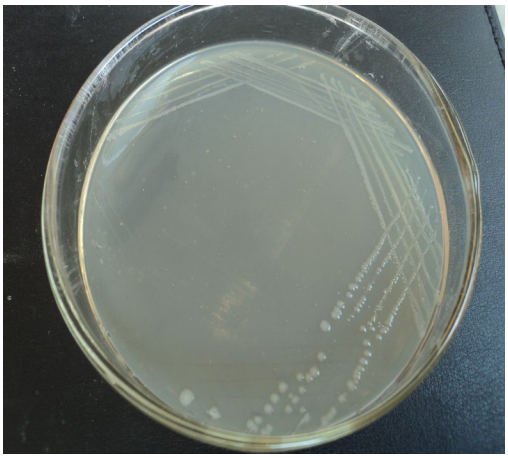
Finally, a carbofuran-degrading bacterium was isolated by plating from the old bacterial suspension of the liquid medium onto an agar solidified MS medium supplemented with 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml concentration of (99% pure) carbofuran. The plates were incubated at 30°C for 24 hr and bacterial colonies were found to grow on the medium supplemented with 2µg/ml, 4µg/ml concentration of carbofuran and no growth were found at 8 µg/ml and more than that concentration of the substrate.

3.2 Microscopic Observation of the Bacterial Strain

Microscopic examination of bacterial cells was done after gram staining and the results are given in the Table 3.1. Additional morphological, Physiological tests were conducted (Table 3.1 & Plates 3.1, 3.2).

Table 3.1 Culture media dependent characteristics and microscopic observation of the isolated bacteria

Agar plate	**Characters**	**Results**
Mineral salts agar plate	Colony Size	(1-2) mm
	Colony shape	Round
	Color	Creamy white
	Consistency	Sticky
	Opacity	Translucent
	Elevation	Raised
	Margin	Entire
Nutrient agar slant	Abundance of growth	Moderate
	Color	Creamy White
Nutrient broth culture	Uniform	fine turbidity
Microscopic observation	Gram staining	Gram-negative
	Motility	Motile



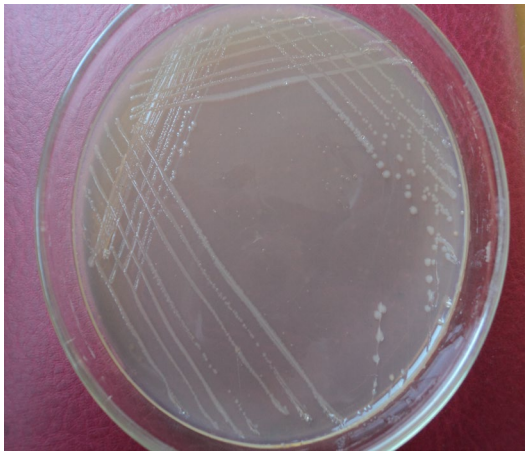


Plate 3.1: Isolated bacterial colonies were obtained from carbofuran treated soil. The plate contained minimal salt agar medium, which was supplemented with (99%) carbofuran at a concentration of 4µg/ml

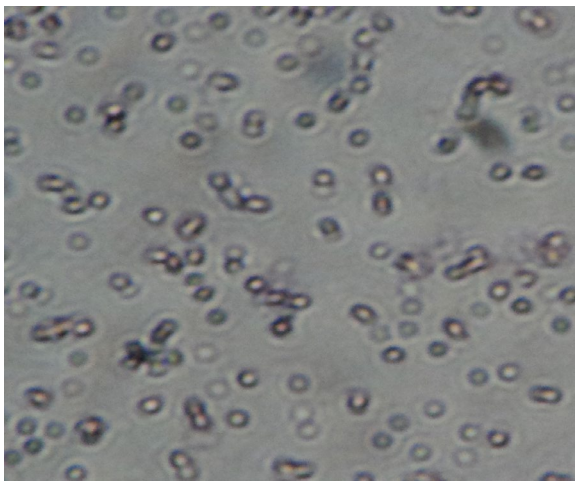


Plate 3.2: Microscopic view of isolated bacterium at 400X (40X X 10) magnification under inverted Optika microscope after gram staining

3.3 Effect of pH and Temperature on Bacterial Growth

Different bacteria grow well in different pH and temperature. For example; the optimum temperature and pH for growth of *E. coli* is 37°C and pH is 7.4, whereas in case of *Lactococcus lactis* it is 30°C and pH 5.0 respectively. Therefore, it is essential to determine the optimum culture condition of the isolated carbofuran degrading bacteria to

ensure highest possible yield of the bacteria for commercial use in agricultural field. In the current experiment, different temperature ranging from 25°C to 35°C and different pH ranging from 5 to 7 was tried. Our experiments show that the optimum pH for the growth of our bacterial isolate was between 7.0 and 8.0 (Fig.3.1). To further fine tune the best pH for the isolated bacteria 7.0, 7.2, 7.4, 7.6, 7.8 and 8 was used and the best bacteria growth was observed in pH 7 (Fig . 3.2)

To determine the optimum growth temperature of the isolated bacteria four different temperatures was tried viz. 25°C, 30°C and 35°C. Our results show that 30°C is the perfect temperature for the optimum growth of the isolated bacteria. Results are plotted in figure 3.3.

Thus, the optimum pH for the growth of the isolate was 7.0 and extreme pH 5.0 restricted for the bacterial growth (Appendix 1 & 2). The optimum temperature 30°C for the growth of bacteria and extreme temperature 35°C restricted for the bacterial growth (Appendix 3).

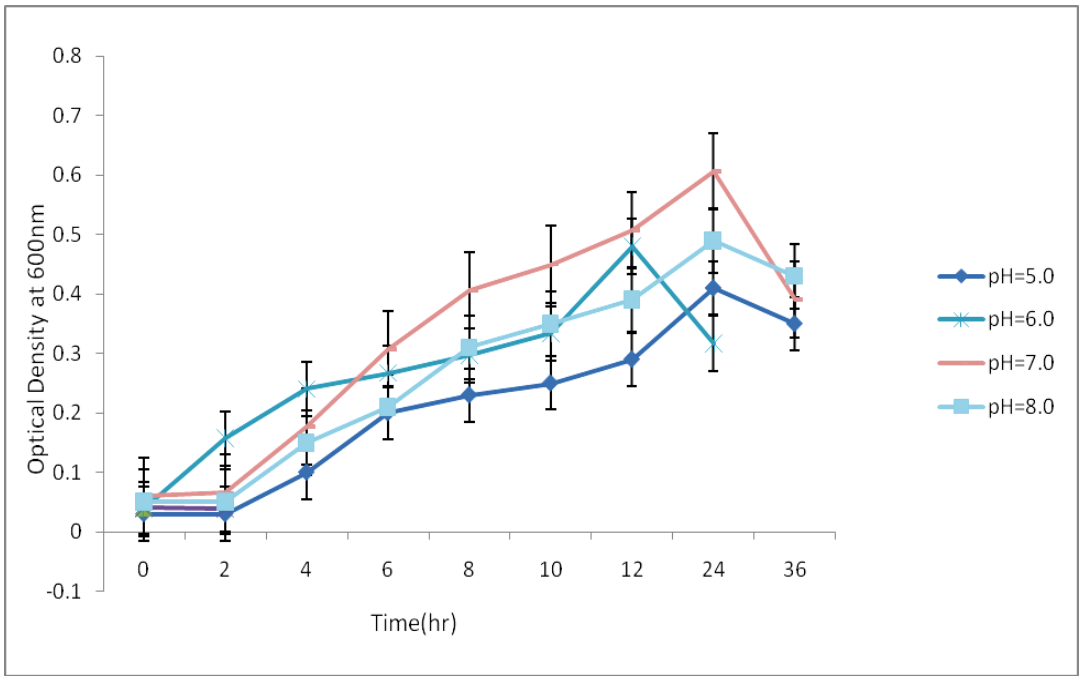


Fig. 3.1: Effect of pH 5, 6, 7 and 8 on bacterial growth (Optical Density) measured at 600 nm wavelengths within 36 hr of inoculation. Error Bars represent the standard deviation of the mean.

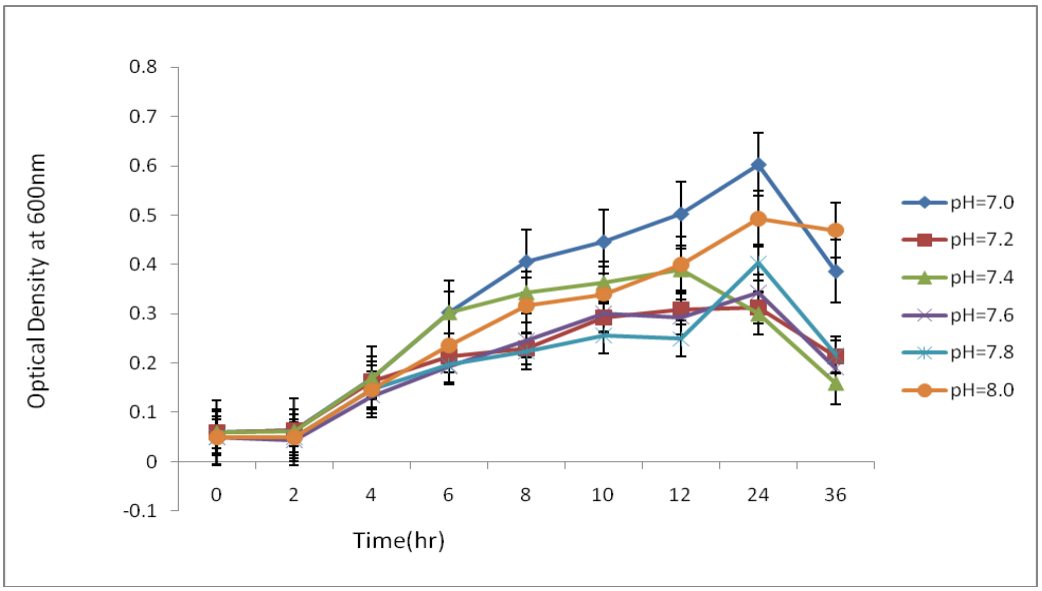


Fig. 3.2: Bacterial growth (Optical Density) comparison at different pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0 measured through 600 nm wavelength through Spectrophotometer (Anylytk Jena, Germany). Standard deviation mark as Error Bars

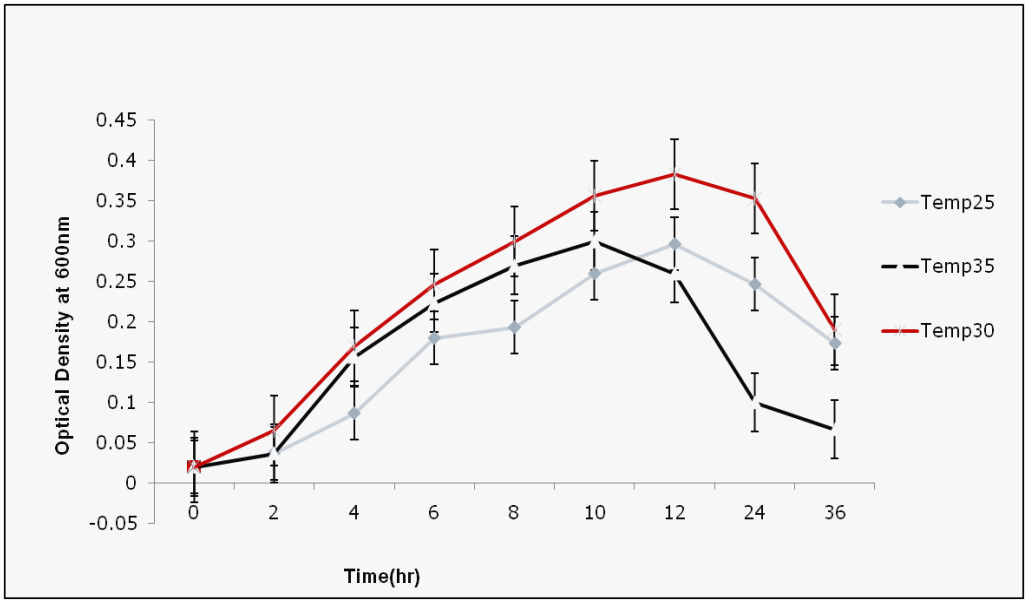


Fig. 3.3: Effect of temperature 25, 30, 35 on bacterial growth (Optical Density) at 600 nm wavelength within 36 hr of inoculation. Error Bars indicate standard deviations

3.4 Biochemical Tests

Utilization observed in MS medium with respective sugar, at 30°C after 48 hours. The overall results of the biochemical tests of the isolated bacterial strains are summarized in the Table 3.2 and Plates. 3.3 & 3.4.

Table 3.2 Biochemical tests used for the characterization of the isolated bacteria.

Tests	**Result**
Lactose Fermentation	+
Methyl Red	-
Indole	-
Catalase	+
Oxidase	-
Citrate	+
TSI	+
Lysine	+
Sugar Utilization tests	**Results**
Glucose, Mannon, Arabinose, Sucrose, Lactose	All positive
Cellulose	+
Control	-

The '+' signs indicate the growth of the microorganisms while '-' signs indicate no growth

Plate 3.3: Biochemical tests of the isolated bacteria showing Methyl red (-); Catalase test (+); Lysine test (+); Urea hydrolysis test (+); Lactose fermentation test (+); Citrate test (+).Control for each experiment also maintained at the same time

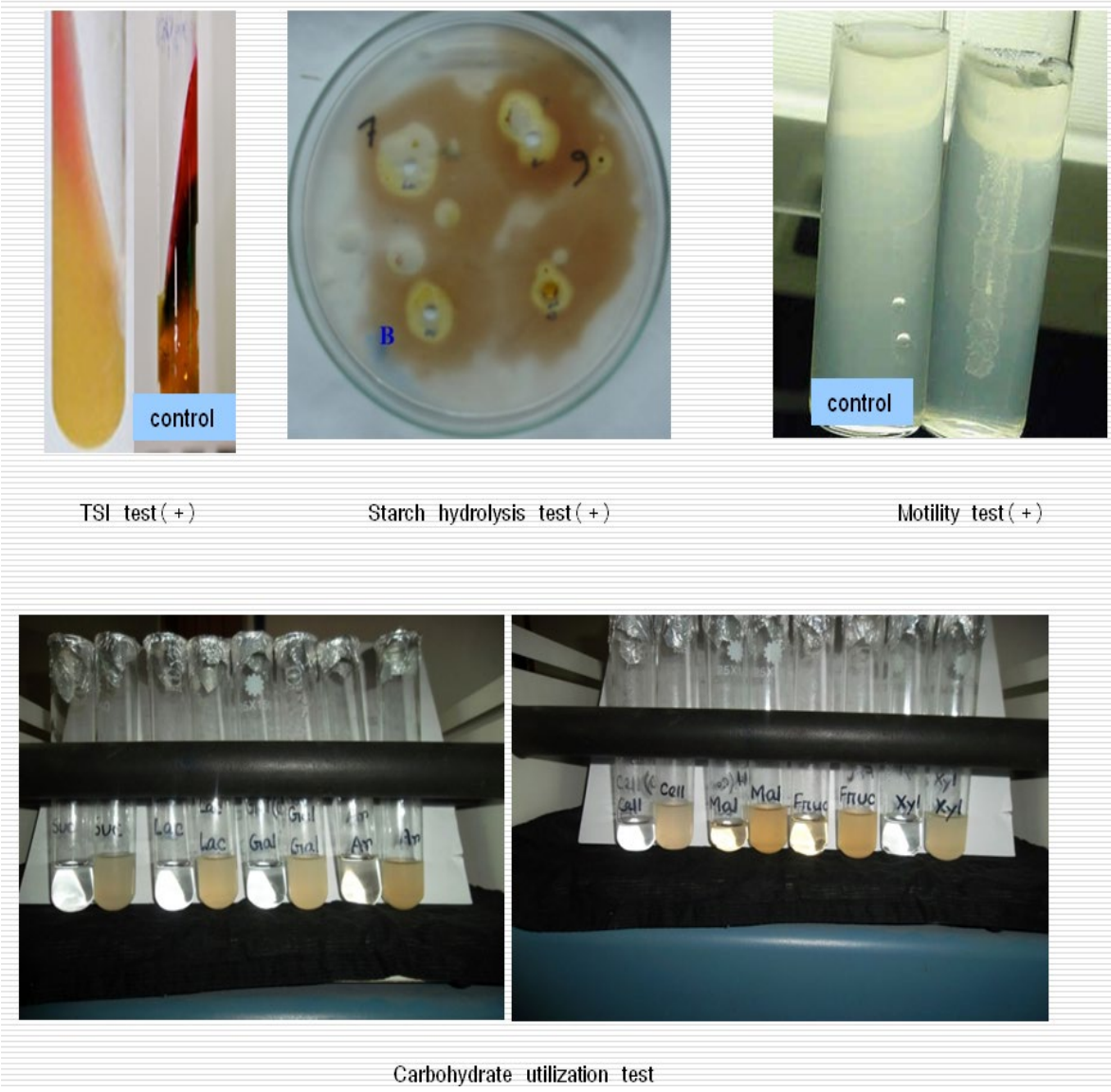


Plate 3.4: Biochemical and morphological tests of the isolated bacteria showing TSI test (+), Strach hydrolysis test (+). The isolate can utilize a wide range of carbohydrate. Motility test showed bacterial movement in the semisolid medium

3.5 Antibiotic Sensitivity Test

The patterns of sensitivity and resistance of isolated bacterial cultures to 10 different antibiotics were tested by the disc diffusion method on nutrient ager medium. The fresh bacterial culture was spread on the nutrient agar plate and antibiotic discs were placed on the surface of ager plate. The diameter of the inhibition zone, if any was measured, after incubation overnight at 30°C. Antibiotic sensitivity is presented in the (Table 3.3). From the table it is evident that the isolated bacteria is resistant to 5 antibiotics out of ten antibiotics tested (Plate 3.5).

Table 3.3: Antibiotic sensitivity test used for the detection of the resistance pattern of the isolated bacteria

Antibiotics	Range of antibiotics	R	S and I
Cefuroxime sodium	-	R	-
Nitrofurantoin	13mm	-	I
Azithromycin	29mm	-	S
Penicillin	18mm	-	S
Ceftazidime	22mm	-	S
Vancomycin	13mm	-	I
Gentamycin	22mm	-	S
Ciprofloxacin	18mm	-	S
Nalidixic acid	-	R	-
Mecillinam	13mm	-	I

(5-10mm) = Resistance to antibiotic(R), (15-20mm) = Sensitive to antibiotic(S), (10-15mm) = intermediate resistance (I).

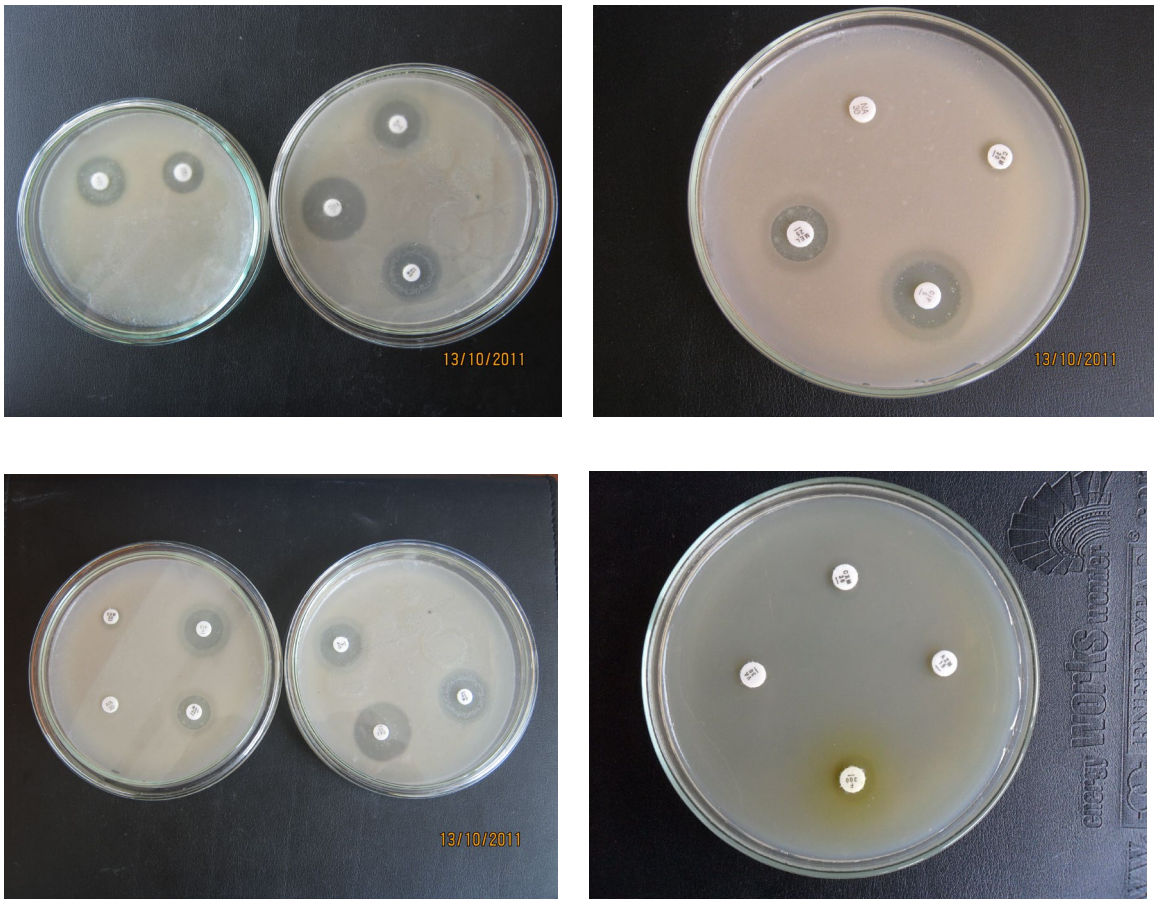


Plate 3.5: Antibiotic sensitivity tests through disc diffusion method. The tested bacteria showed resistance against cefuroxime sodium and nalidixic acid on LB ager plates after 18 hr of incubation at 30ºC

3.6 Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of a drug at which inhibited the visible growth of bacteria. The MIC of antibiotics, Gentamysin was determined by turbidimetric method against isolated bacteria through broth tube dilution method. The antibiotics, Gentamysin in various concentrations were applied to the LB broth media in each test tube and incubated at 30°C for 48 hours.

The result of MIC value of the Gentamysin against isolated bacteria are presented in the Table 3.4.

Table 3.4: Minimum Inhibitory Concentration of Gentamysin against the isolated bacteria

Test Organism	**Growth response in different concentration**					
Carbofuran degrading soil borne bacteria	Gentamysin (μg/ml)					
	50 ++	100 ++	200 ++	300++	400 ++	500 -

The '+' sign indicate the growth of the microorganisms while '-'sign indicate no growth.

The MIC of this antibiotic against isolated bacterium was 400 μg/ml, demonstrating that high concentration of this antibiotic was required to inhibit the growth of this microorganism.

3.7 Viable Cell Count

Viable cell count determinations Aliquots (2.5 ml) of 18hr old bacterial cultures grown in LB medium were inoculated into 100-ml Erlenmeyer flasks containing 25 ml of Luria-Bertani broth . A control was maintained with equal volume of broth without bacterial culture. Bacterial growth was followed by viable cell counts immediately after inoculation at 24 h of incubation. A bacterial inoculum (1 ml) was drawn at regular intervals from the test and control cultures and serial dilutions were performed using 9 ml of sterile saline (0.85% NaCl; pH8.5). Appropriate dilutions were plated in triplicate on nutrient agar and the plates incubated at 30^0C for 24 h.

Table 3.5 Detection of viable cells of the isolated bacteria on Luria-Bertani agar medium after 24 hr of incubation at 30ºC

Time (hour)	**OD at nm**	**Culture media**	**CFU/ml**	**Control**
24	600	LB	459×10^7	0

3.8 Determination of Toxic Effect of Carbofuran against Artemia salina

We measured carbofuran toxicity on the basis of LC_{50} value against *Artemia salina* through probit mortality software. We calculated dead number of *A. salina* at a regular basis after 12hr, 24hr, 36hr and 48hr. After 48hr, no live *A. salina* was found. Finally the data were transferred in probit mortality software to find out LC_{50} values.

The LC_{50} value for the tested carbofuran sample was 9.103558µg/ml and the regression equation was Y = 3.942777 + 1.102179 X, while the 95% confidence limits were 3.810799 to 21.74736 µg/ml for 12h of exposure. Thus, the carbofuran showed toxicity only after 12hr and the lethal dose was 9.103558µg/ml.

Table 3.6: Determination of LC_{50} value of the carbofuran against *A. salina* after 12,24,36 and 48 hours of exposure

Tested sample	**Exposure (hours)**	**LC_{50} value**	**95% confidence limits**		**Regression equation**	**χ^2 value (3df)**
			Upper	**Lower**		
Carbofuran	12	9.103558	21.74736	3.810799	Y = 3.942777 + 1.102179 X	1.105836
	24	5.586349	10.42564	2.993322	Y = 3.981846 + 1.362757 X	0.1559134
	36	2.768667	5.688329	1.347587	Y = 4.408405 + 1.337631 X	1.449404
	48	-	-	-	-	-

3.9 Evaluation of Antagonistic Effect of the Isolated Bacteria

The antagonistic effect of the isolated bacteria was tested against some pure culture strain of bacteria and fungi.

Table 3.7: Pure culture strain of the fungi and bacteria used in the antagonistic assay

Fungi	Bacteria
Sclerotinia sclerotiorum	*Rhizobium RLC* 107
Penicillium chrysogenum	*Rhizobium RVM* 307
Fusarium oxysporum	*Rhizobium RCA* 220
Aspergillus niger	*Pseudomonas putida*
Rhizopus oryzae	*Xanthomonas campestris*

In all cases no antagonistic effect was found. So, the bacteria would not be harmful for any Rhizobacteria at field level.

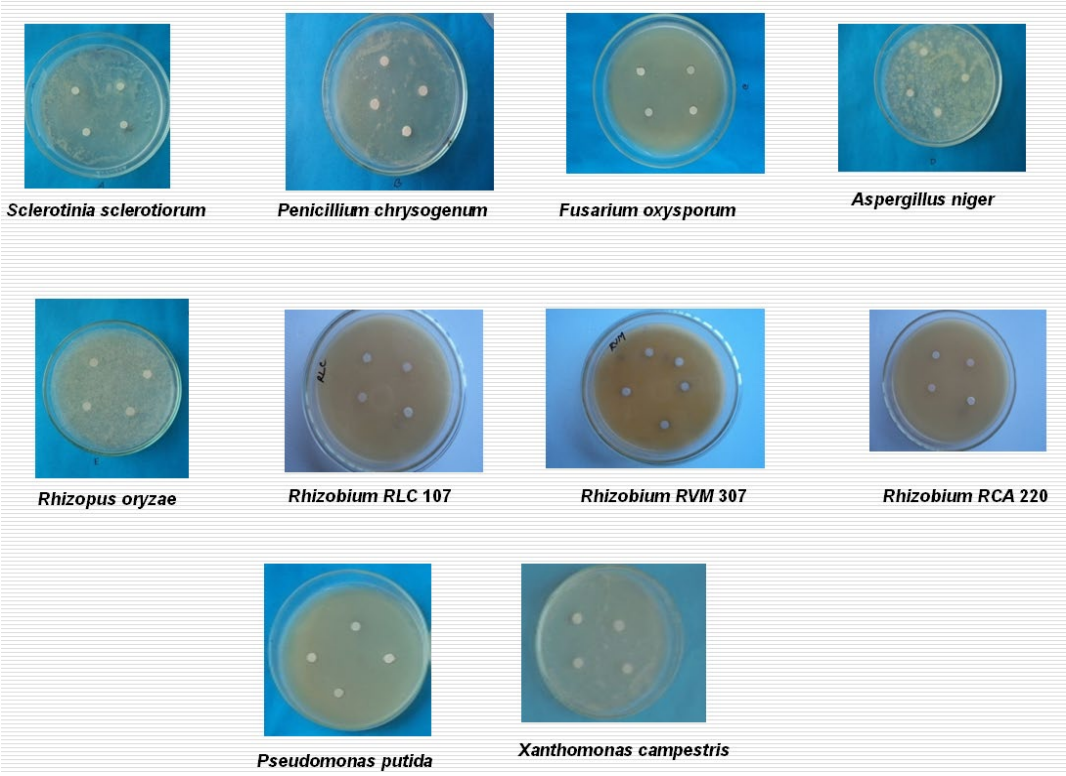


Plate 3.6: Antagonistic assay of the isolated bacterium against plant pathogenic bacteria on LB agar plate at 37ºC and fungi on PDA plate at 30ºC

3.10 Extraction of Chromosomal DNA

The extraction of chromosomal DNA was done according to Sambrook *et al* (1989). There was 1 band of Chromosomal DNA was found the agarose gel after illumination with the UV light. The use of 1 kb+ DNA marker for size determination. Lane one indicate the DNA marker and lane 2 indicate the Bacterial Chromosomal DNA. The isolated chromosomal DNA was 1500 bp which compared with 1 kb+ DNA marker (Plate 3.7).

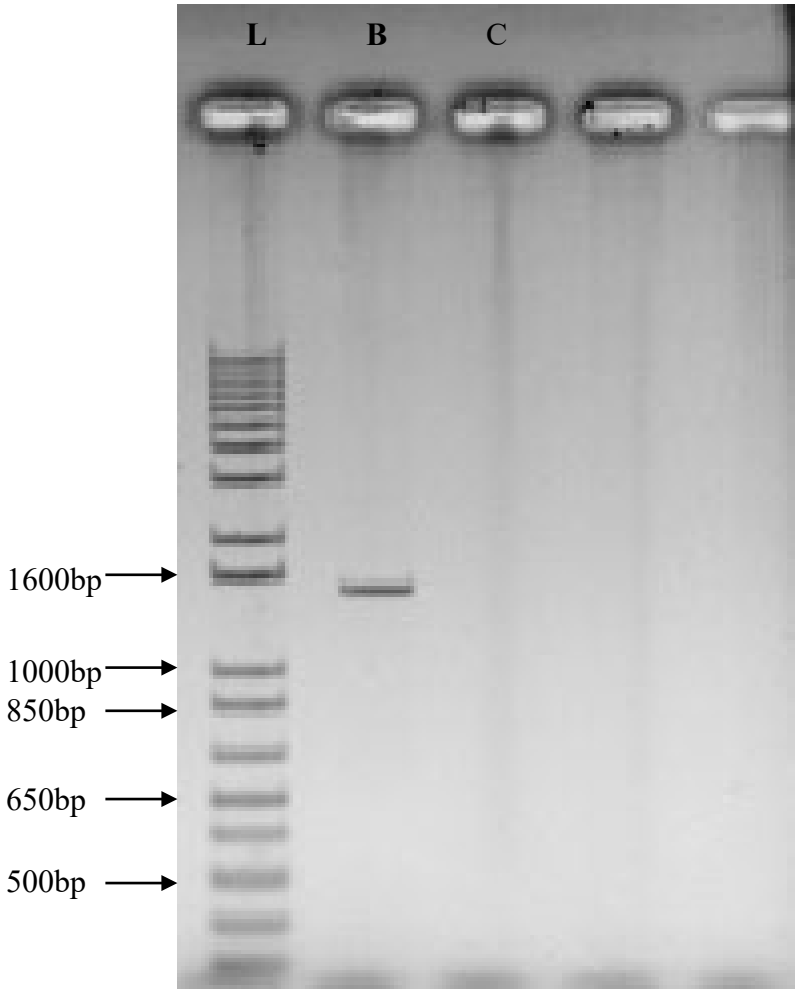


Plate 3.7: PCR Amplification of 16S rRNA gene yielded a 1500 bp PCR product, 1 kb+ DNA marker is used for size determination .Here **L** for ladder and **B** for bacteria

3.11 Identification of the Isolated Bacteria by 16S rRNA Gene Sequencing

DNA Sequencing Analysis of 16S rRNA gene

After the 16S rRNA gene sequencing and editing of sequences it was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to Genebank database. From the genebank, several sequences are found with significant identity.

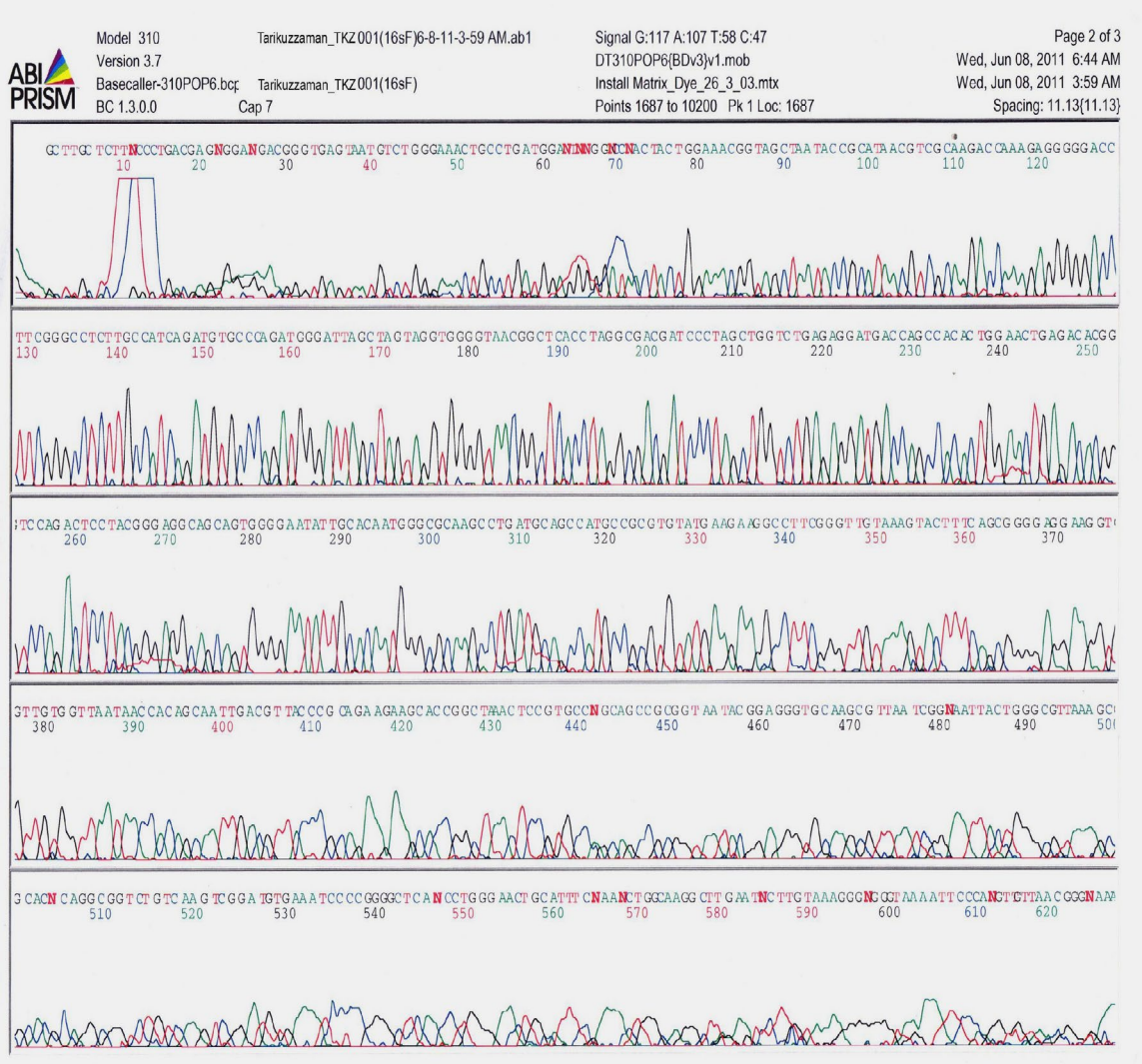


Fig. 3.4: Chromatogram of 16S rRNA gene sequencing of the isolated bacteria

Sequences producing significant alignments:

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured bacterium clone 3-42001 16S ribosomal RNA gene, partial sequence	977	977	78%	0.0	92%	JN033022.1
Uncultured bacterium clone BICP598 16S ribosomal RNA gene, partial sequence	977	977	80%	0.0	91%	HM557445.1
Enterobacter cloacae strain CAC02 16S ribosomal RNA gene, partial sequence	966	966	70%	0.0	95%	KF359992.1
Enterobacter cloacae strain A27CK 16S ribosomal RNA gene, partial sequence	961	961	70%	0.0	95%	KF220490.1
Enterobacter cloacae strain F2 16S ribosomal RNA gene, partial sequence	961	961	68%	0.0	95%	FJ608235.1
Uncultured bacterium clone 1M1 16S ribosomal RNA gene, partial sequence	961	961	68%	0.0	95%	DQ079818.1
Uncultured bacterium clone 2 16S ribosomal RNA gene, partial sequence	961	961	68%	0.0	95%	DQ011237.1
Uncultured gamma proteobacterium clone DH67 16S ribosomal RNA gene, partial sequence	959	959	70%	0.0	95%	JQ861287.1
Enterobacter sp. OVC27 16S ribosomal RNA gene, partial sequence	959	959	68%	0.0	95%	JQ660743.1
Enterobacter hormaechei strain AIMST 4TP2.1L 16S ribosomal RNA gene, partial sequence	959	959	70%	0.0	95%	JN835505.1
Enterobacter sp. G12 16S ribosomal RNA gene, partial sequence	959	959	69%	0.0	95%	JF783987.1
Uncultured bacterium clone UA001_CBR620_0022_G10 16S ribosomal RNA gene, partial sequer	959	959	68%	0.0	95%	GU003763.1
Enterobacter cloacae strain RU15 16S ribosomal RNA gene, partial sequence	957	957	68%	0.0	95%	KJ607596.1
Enterobacter sp. X40 partial 16S rRNA gene, strain X40	957	957	68%	0.0	95%	HE662680.1
Enterobacter sp. MW-D 2206 16S ribosomal RNA gene, partial sequence	955	955	68%	0.0	95%	KC835096.1
Enterobacter cloacae strain RU07 16S ribosomal RNA gene, partial sequence	955	955	68%	0.0	95%	KJ607606.1

Enterobacter produced significant 95% identity with the 16S rRNA gene sequencing of the isolated bacterium.

- **Highest 95% identity with *Enterobacter* Genus**

Enterobacter cloacae strain CO1 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|KF607094.1| Length: 1473 Number of Matches: 1

Range 1: 75 to 659 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
955 bits(517)	0.0	567/595(95%)	10/595(1%)	Plus/Plus

```
Query  15   TGACGAGNGGANGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGANTNNGGNCCNAC  74
            ||||||| ||  ||||||||||||||||||||||||||||||||||    ||    ||
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Query  75   TACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG  134
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Sbjct  133  TACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG  192

Query  135  CCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCT  194
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Sbjct  193  CCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCT  252

Query  195  AGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGT  254
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Sbjct  253  AGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGT  312

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            |||||||| | || |||||| |||| || | |||||| |||| ||||| ||||||
Sbjct  609  ACTGCATT-CGAAACTGGCA-GGCTAGAGT-CTTGTAGAGGG-GGGTAGAATTCC  659
```

From the biochemical test and 16S rRNA gene sequence analysis it can be indicated that the isolated bacterial strain is a member of the genera *Enterobacter*.

3.12 Investigation of plasmid involvement in carbofuran metabolism

After isolation, the bacterial strains were inoculated in the nutrient broth and incubated at 37°C in a shaker for overnight. The broth cultures were used for the isolation of plasmid DNA. The isolation of plasmid DNA was done according to alkaline lysis technology. The plasmid DNA was electrophoresis on the 0.8% and 1% high-melting agarose gel. Ethidium bromide was used to stain the gel.

No Plasmid DNA band of the bacteria was found on the agarose gel after illumination with the UV light.

3.13 Detection of Carbofuran Degradation Rate by RP-HPLC

The persistence and biodegradation of carbofuran was investigated under laboratory condition through the isolated bacteria in the MS media with various concentration of carbofuran at 30^{o}C. The samples from different concentration were run through HPLC and data were analyzed through empower-2 software. The chromatograms of the analyzed data were compared to find out the degradation results.

In the RP-HPLC experiment, we ran standard carbofuran (99% pure) and found the retention time of the pesticide after 37minute. So, that was the pick of the used carbofuran at standard level. Next we ran samples collected from day 0 to day 07 and observed carbofuran pick after 37 minute with specific absorbance for different samples. When we ran day 3 sample, pick absorbance was 0.0070 and day 0 sample was 0.0095 (Fig. 3.5**)**. So, the reduction of absorbance after 2 day was due to the reduction of carbofuran in the sample. These results clearly indicate that the bacteria consumed or utilized the carbofuran as a carbon source from the minimal salts (MS) liquid medium. In this consequence, we also ran day 05 sample (Fig. 3.6) and day 07 (Fig. 3.7) and found different absorbance.

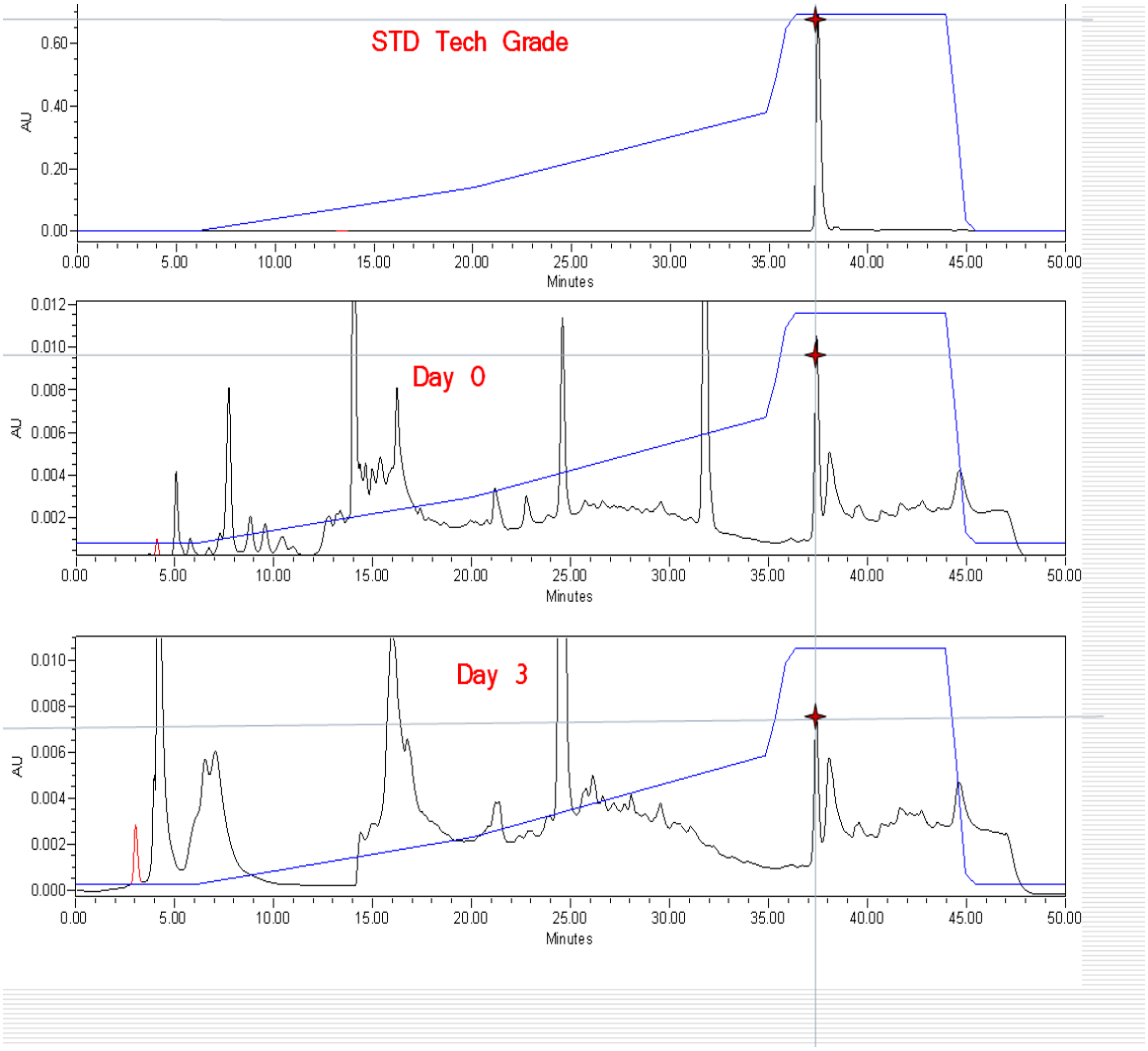

Fig. 3.5: Chromatogram of RP-HPLC for the detection of carbofuran absorbance at 0 day and 3 day compare to the STD Tech Grade. X axis shows the time and Y axis shows the absorbance intensity at 280nm

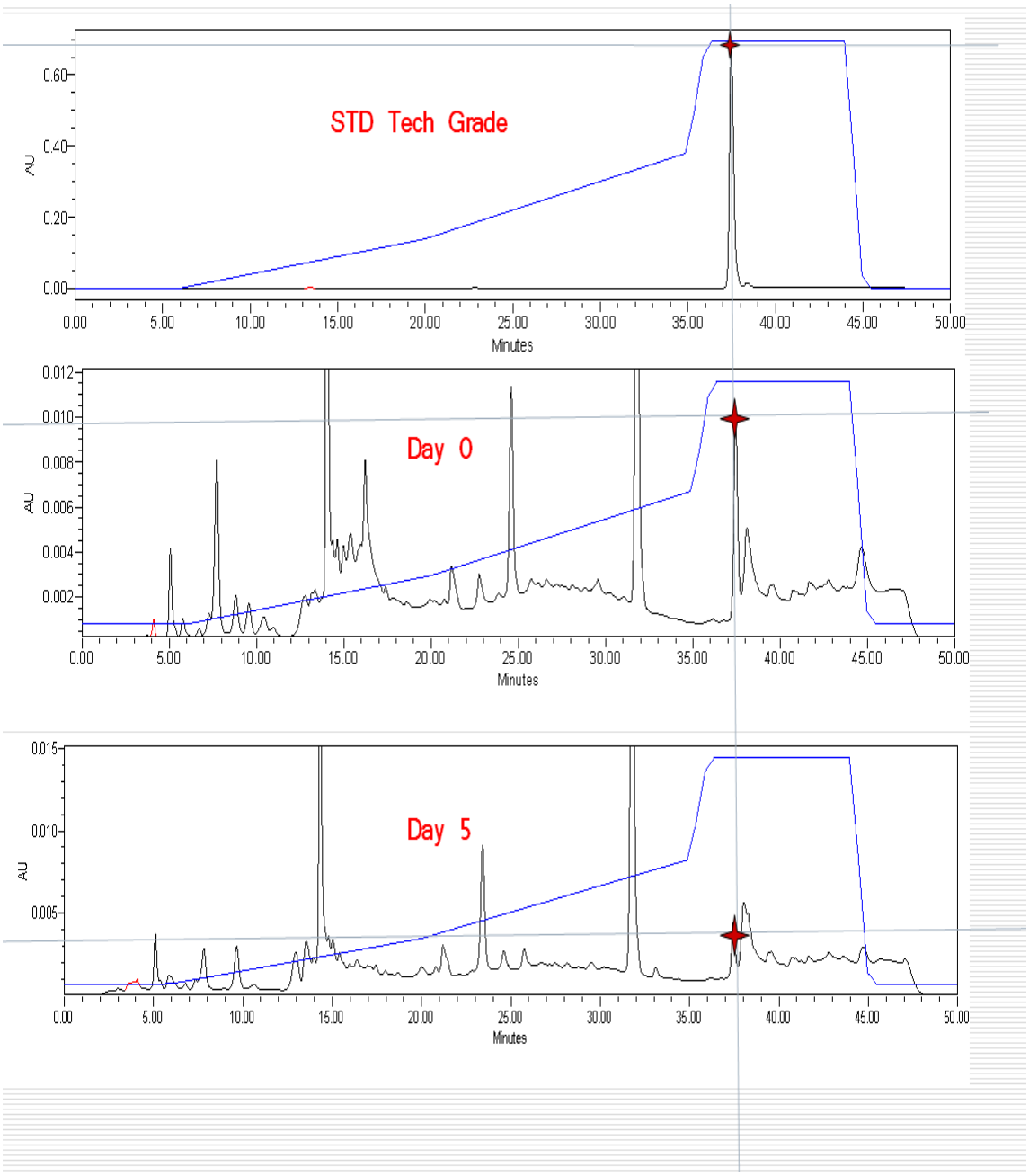

Fig. 3.6: Chromatogram of RP-HPLC for the detection of carbofuran absorvance at 0 day and 5 day compare to the STD Tech Grade. X axis shows the time and Y axis shows the absorbance intensity at 280nm

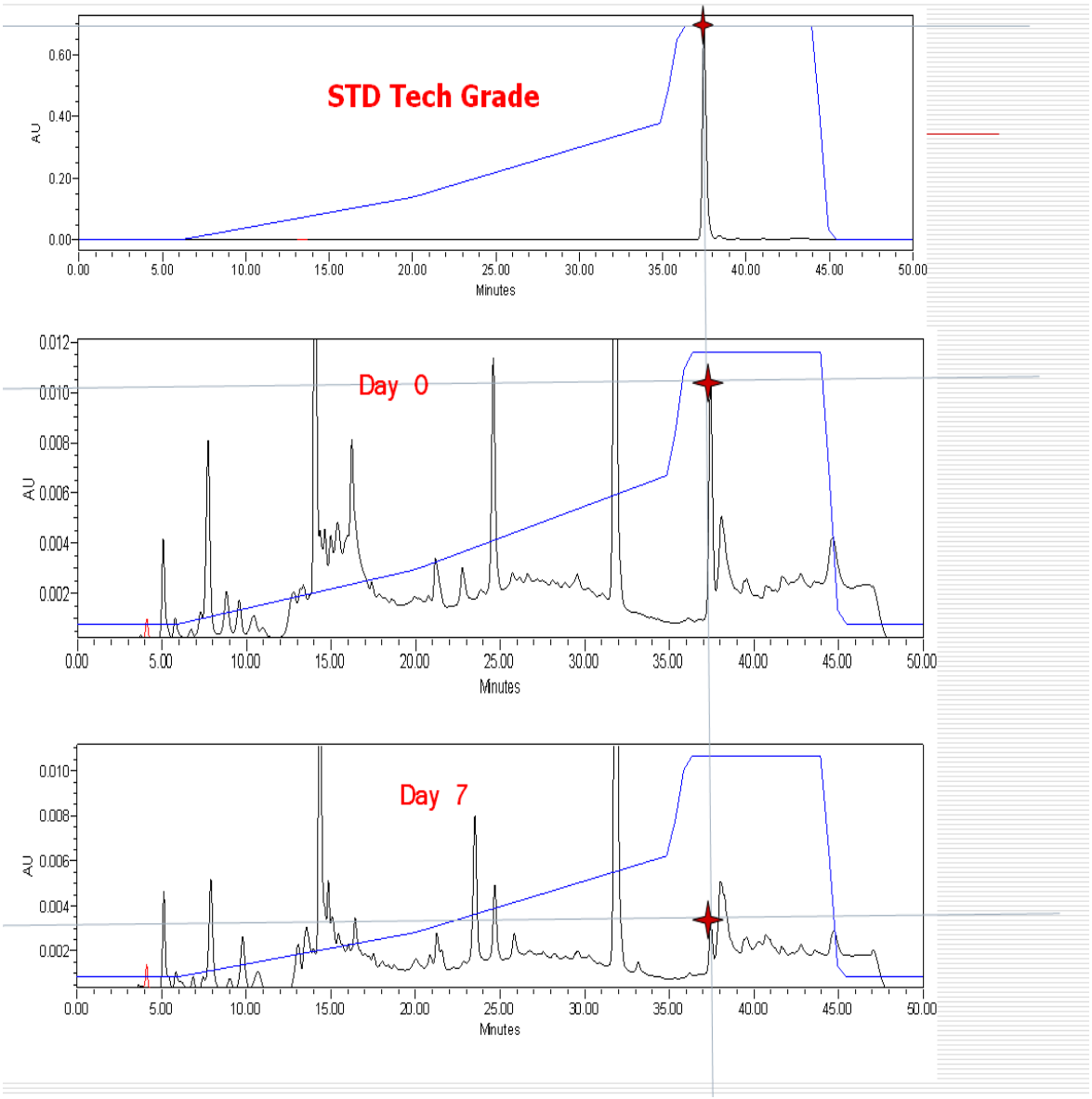

Fig. 3.7: Chromatogram of RP-HPLC for the detection of carbofuran absorbance at 0 day and 7 day compare to the STD Tech Grade. X axis shows the time and Y axis shows the absorbance intensity at 280nm

Finally, degradation rate was measured on the basis of absorbance and peak area through Empower-2 software (Table3.8).

Table 3.8: Degradation Efficacy Quantificatation on the basis of absorbance and peak area

Sample name	Absorbance(AU)	Peak Area	Degradation rate (%)
Day 0	0.0095	3.60	0
Day 3	0.0070	2.40	30
Day 5	0.004	1.10	56
Day7	0.0025	0.72	80

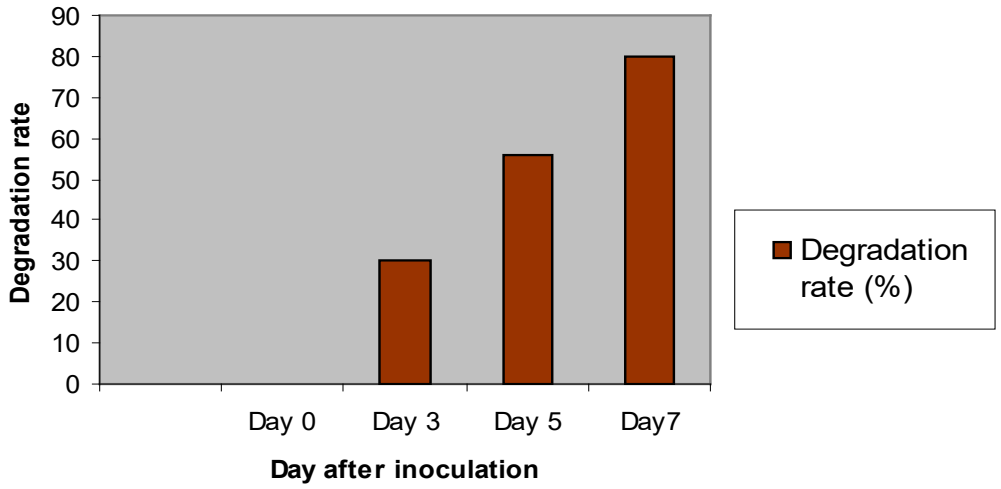

Fig.3.8: Degradation rate of carbofuran by the isolated bacteria *Enterobacter* sp. after day 3, 5 and 7

HPLC experiment confirmed that the bacteria can utilize up to 4 µg/ml of 99% carbofuran as a carbon source and showed 30% and 80% decreased carbofuran concentration within 3 and 7 days of inoculation respectively. Thus, the isolated bacteria showed remarkable degradation efficacy of the toxic pure carbofuran.

DISCUSSION

Now a days bioremediation became one of the most effective and eco-friendly means of degradation or scavenging of pollutants released, created or thrown away everyday in the environment by the human being (Chukwuma *et al.,* 2012). Recent industrial progresses in one hand have revolutionized the agricultural production but on the other hand have became a curse for our environment us as well mankind. However, by using microbiological and various other biotechnological approachs it can minimize the environmental pollution caused by the release of a wide range of compounds as a consequence of industrial and agricultural progress.

For the last few decades, thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotics in soil, water and air. Xenobiotics compounds are manmade chemicals that are excreted in the environment and pollute the environment when present in high concentrations. They pollute the environment as some of them are recalcitrant. As for example nitroaromatic compounds (NACs), polycyclic aromatics and other hydro-carbons (PAHs) that are constituents of crude oil and halogenated organic compounds together constitute a large and diverse group of chemicals that are responsible for causing widespread environmental pollution (Ball *et al.,*2013).The environmental crucial component includes all of the living and non-living things that surround us, including the air, water, plants, soil and wildlife. Pesticides are one of the toxic xenobiotics designed to be deliberately released into the environment at a regular basis. Although each pesticide is meant to kill a certain pest, a very large percentage of pesticides reach a destination other than their target. Instead, they enter the air, water, sediments, and even end up in our food. Pesticides easily contaminate the air, ground and water when they run off from fields, escape storage tanks, are not discarded properly and especially when they are sprayed aerially. The use of pesticides decreases the general biodiversity in the soil. If there is no chemicals in the

soil there is a higher soil quality, and this allows for higher water retention, necessary for plants to grow. As for example, nitrogen fixation, which is necessary for the growth of many large plants, is hindered by pesticides that can be found in soil. This can lead to a large decline of crop yields. Carol Potera (2007) reported that some organochlorine pesticides suppress nitrogen-fixing bacteria from replenishing natural nitrogen fertilizer in soil, resulting in lower crop yields, stunted growth, and an ever-greater need for additives to boost production. Application of pesticides to crops that are in bloom can kill honeybees, which act as pollinators. According to a UNEP report 2010, of the 100 crops which provide 90% of the world's food are mostly (> 70%) pollinated by bees. Pesticides used in farming are killing bumble bees and affecting their ability to forage. The USDA and USFWS estimate that US farmers lose at least $200 million a year from reduced crop pollination because pesticides applied to fields eliminate about a fifth of honeybee colonies in the US and harm an additional 15%. Certain pesticides, some fungicides, can be slightly toxic to birds and mammals, but fatal to earthworms, which in turn reduce populations of the birds and mammals that feed on them (George, 2004.). Other pesticides can cause growth abnormalities in embryos and reduce the number of chicks that hatch successfully, reduce the habitat of birds and some that come in granular form can be mistaken as grains of food by birds; a mistake which can be fatal for them (Bright *et al.,*2008) Pesticides are exacerbating the worldwide decline in amphibian populations. Pesticides are causing tadpoles to have behavioral and growth abnormalities, is turning male frogs into hermaphrodites, which decreases their ability to reproduce, is causing tadpoles to take longer to metamorphose into frogs and become smaller when they do, which decreases their ability to catch prey and avoid predators, can cause a sex reversal during the development of turtles, has been causing both reproductive and non reproductive effects in crocodiles, turtles and lizards, and has been causing disorders such as decreased hatching success, feminization, skin lesions, and other such developmental abnormalities. Fish and other aquatic biota may be harmed by pesticide-contaminated water. Application of herbicides to bodies of water can cause plants to die, diminishing the water's oxygen and suffocating the fish.

With the rapid changing environment insects, pests and especially microbial plant pathogens are evolving at a rapid rate. Therefore, to combat the situation and to protect our crop from these new enemies advanced chemicals are being invented and synthesized frequently or increased dose of prevailing pesticide chemicals are being used. However, although these chemicals are able to eradicate the plant pests or pathogens to some extent, they pose a serious problem by contaminating our environment and also create serious health threat. So, use of pesticides in our agricultural system is almost an inevitable hazard. But the question is how we can minimize this hazard or decontaminate the environment from these xenobiotics.

Pesticides are widely used to protect the crops from a variety of pests. The use of pesticides benefits in increasing agricultural production but the indiscriminate uses of pesticides have led to their accumulation in plants, animals, solid and sediments, thus effecting wide spread contamination of the environment. Such application of pesticides has the drawback of pesticide residues which remain on fruits and vegetables, constituting a potential risk to consumers.

Abundant use of pesticides in agricultural field is a great health hazard as well as environmental concern in Bangladesh. In recent time, use of pesticide almost reached an abusive stage in Bangladesh. There is no effective regulation on dose and uses of pesticide in our agricultural field. These chemicals either directly contaminates the surface of fruits and vegetables or they contaminate the soil first and afterwards plants absorb these chemicals which ultimately is accumulated in the fruits or vegetables. By consuming these foods, a large number of populations specially the children and the elderly are exposing themselves everyday to severe health risk. Severe illness like cancer, liver cirrhosis and kidney failure is increasing alarmingly among Bangladeshi population. Therefore, there is an urgent need to find an effective way to degrade these hazardous chemicals from the soil.

Conventional approaches for proving *in situ* biodegradation of organic pollutants such as pesticides have severe limitations. On the contrary, microbes are the most abundant and

versatile diverse organisms on earth which can consume various organic chemicals as a source of their nutrition and thereby can degrade them. Therefore, these diverse groups of organism and their physiological reactions have immense importance in managing global environmental pollution and finally prevent health hazards.

The physico-chemical remedial strategies to clean up sites contaminated by these compounds are expensive and safeer. Therefore, research is increasingly being focused on biological methods for the degradation and elimination of these pollutants. Sites contaminated by these compounds need urgent remedial solutions, the search for which has revealed a diverse range of bacteria that can utilize these xenobiotics as substrates, often mineralizing them or converting them into harmless products, and in the process helping to clean up the environment. A huge number of bacterial and fungal genera possess the capability to degrade organic pollutants (Thakur, 2006).

Carbofuran is a one of the most widely used pesticide of carbamate group. Due to its severe adverse effect on human, wild life and eco-system it is banned in USA. However, the constant use of it in third world countries like Bangladesh without any rules and regulation is causing severe health hazards to human being along with many other problems to the environment. The current research work was carried out to find out an effective and fruitful way to minimize these probable problems caused by carbofuran through bioremediation using microbiological approach. Bioremediation is now considered as an important mechanism of organic chemical removal in natural system. Microorganisms play an important role to degrade or detoxify the toxic chemicals. The microorganisms that contain a variety of mono and dioxygenase enzymes are capable of degrading xenobiotic compounds into smaller compounds that are again taken up by other series of microbes and degraded wholly. Thus, microorganisms can be employed for treating and degrading these compounds to make our environment free of any solid pollutants if handled carefully. Bioremediation is one such process that exploits the catabolic abilities of microorganisms to degrade harmful and toxic xenobiotics (Leahy and Colwell, 1990).

In this study, we isolated a bacterial strain from such soil which has a long history of carbofuran uses, with the hypothesis that the bacteria present there will be able to uptake carbofuran and degrade it. In our experiment used 99.00% pure carbofuran as a carbon source for the isolated bacteria to test degradation efficacy. So first, we measured Lethal concentration (LC_{50}) of the used carbofuran (99%) against *Artemia salina* and the value was 9.103558µg/ml with the regression equation was Y = 3.942777 + 1.102179 X, while the 95% confidence limits were 3.810799 to 21.74736 µg/ml for 12h of exposure only. U.S. EPA, 1990 reported about carbofuran toxicity and oral LD_{50s} were 6.4 to 14.1 mg/kg for rats, 18.5 mg/kg for dogs, and 25 to 38.9 mg/kg for chickens. Our results suggest that, the pesticide showed much more cytoxicity on aquatic organisms compared to vertibrates.

The optimal growth condition of isolated bacterium was found to be at optimum p^H and temperatures were 7 and 37ºC respectively. The goal of the optimization was to identify the most favorable growth condition for the isolated bacterium so that we can obtain enough bacterial growth with minimal cost for use in agricultural field. When morphological and biochemical tests were done, the isolated bacteria was found as gram-negative, motile, lactose fermenting and able to detoxify hydrogen peroxide. The bacteria has the ability to utilize lysine and citrate as the carbon sources. Sugar utilization test also confirmed that it can use a wide range of carbohydrates such as glucose, mannitol, arabinose, sucrose, lactose and cellulose as the source of nutrient. Carbohydrate utilization is important in distinguishing among the members of the family *Enterobacteriaceae*. The *Enterobacteriaceae* is a family of organisms that are facultatively anaerobic, oxidase negative, gram negative rods that ferment glucose (Bopp *et al.,* 2003). According to Bergey's Manual of Determinative Bacteriology, the members of this family lives in the mesophilic environment with its optimal temperature at 37°C and uses its peritrichous flagella for movement. Again observation from Bergey's Mannual of Systematic Bacteriology (Second Edition, Volume Two, The Proteobacteria Part B) *Enterobacter* are found in soil, water, fruits, meats, eggs, vegetables, grains, flowering plants and trees, and in animals from insects to man. Thus, our biochemical

and physiological out puts from the present investigation have a similarity with our subsequent molecular identification data.

Molecular identification has been used as a worldwide acceptable technique to identify any bacterium up to genus level across all major phyla. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique (Clarridge *et al.,* 2004). Our sequencing result of the 16S rRNA gene revealed that the bacterial isolate is *Enterobacter.*

A number of earlier studies showed the degrading ability of carbofuran by several bacterial strain of *Pseudomonas* sp., *Aspergillus, Fusarium*, *Flavobacterium*, *Achromobacterium*, *Sphingomonas*, *Arthrobacter*, *Bacillus pumilis, Pseudomonas stulzeri, Rhodococcus* sp. (Mohaputra and Awasthi 1996) and. In a recent study Feng *et al.,* (1997) and Kim *et al.,* (2004) showed that *Sphingomonas* sp.strain CF06 (U52146) could also degrade carbofuran. A similar study achieved by Yan *et al.,* (2007) showed that *Novosphingobium* sp. is also involved in carbofuran degradation and it hydrolysis products 2-hydroxy-3(3-methypropan-2-ol) phenol via hydrolysis in laboratory condition.

In our laboratory earlier we also have reported the degradation of carbofuran by the same bacterial strain. However, in our earlier experiments we used commercial grade carbofuran, whereas, in our current studies we have used technical grade carbofuran. More importantly, we have quantified the degradation rate of carbofuran using analytical method by RP-HPLC.

The genus *Enterobacter* was first proposed by Hormaeche and Edwards (1960). Approximately, 50 per cent of clinically significant bacteria isolated in a clinical bacteriology laboratory are *Enterobacteriaceae* and 20 species account for most *Enterobacteriaceae* isolated from clinical specimens. *Enterobacteriaceae* are part of the normal gastrointestinal flora; however, several species can cause gastrointestinal disease (Barie B. Coyle 2005). As the 16S rRNA gene sequencing and biochemical tests of the isolated bacterium was found to be the member of *Enterobacteriaceae* family and which

are predominantly a family of pathogenic bacteria. Therefore, it created a concern for us to control it in case of accidental human pathogenicity. So, Antibiotic sensitivity of this isolate bacterium against ten different antibiotics was checked. is The bacteria was resistant against cefuroxime sodium and niladixic acid which are second generation antibiotics. However, it is susceptible to azithromycin, penicillin, ceftazidime, ciprofloxacin and gentamycin which implies that it can be controlled by these antibiiotics. The MIC of gentamycin was also detected and value was 400 µg/ml against the isolated bacterium.

Several chromatographic methods have been used worldwide so far to detect pesticide degradation efficiency of bacteria. For example HPLC has been used for detection of sulfadoxine degradation by *Pseudomonas* sp. DX7 (Zhang *et al.,* 2011), organophosphorus by *Enterobacter* sp.(Chino-Flores *et al.,* 2012), Differential Pulse Voltammetry (DPV) has been used for phenol biodegradation by *Bacillus amyloliquefaciens* WJDB1 (Lu *et al.,*2012), GC-MS method was used for TBP(Tributyl phosphate) degradation by *Bacillus* (Ahire *et al.,* 2012), LC-MS method was employed for Chlorpyrifos degradation by *Enterococcus faecalis* (Harishankar *et al.,* 2013) and radiorespirometric analyses was utilized for atrazine mineralization by soil bacteria (Ce´cile *et al.,* 2012). Among all these techniques High-performance liquid chromatography (HPLC) is one of the most precise, reliable and convenient technique for quantification of biodegradation of compounds using proper standard which is also used in analytical chemistry to separate the components in a mixture, to identify component, and to quantify each component. However, in this investigation we performed RP-HPLC analysis to confirm efficacy of the pesticide degradation by the isolated bacteria in minimal salt media. We confirmed that the isolated *Enterobacter* sp. can degrade 99.00% pure carbofuran at 4µg/ml as a sole source of carbon and energy , which is much more toxic than commercially available (5% pure) carbofuran. Previous report from Mohanta *et al.,* 2012 showed that *Enterobacter* sp. can degrade 20µg/ml (5% pure) carbofuran and they found plasmid involvement. However, even after using two different techniques we could not identify

the existence of any plasmid in the isolated bacteria. Chino-Flores *et al.,* 2012 also got similar result with no plasmids in this species. Therefore, it may be concluded that the bacterial system itself has the inborne mechanism of carbofuran metabolism.

Many bacteria that metabolize carbamate insecticides have been isolated from soils. However, only a few of these bacteria were found to harbor plasmid which encodes an insecticide- hydrolyzing enzyme. Four bacterial strains from the closely related genera *Pseudomonas, Achromobacter, Sphingomonas* and *Flavobacterium* found to encode carbamate insecticide by its plasmid genes (Feng *et al.*, 1997 and Serdar *et a.l*, 1982). A few other microorganisms are found to be capable of not only hydrolyzing carbamate insecticides by its plasmid genes but also can utilize one or more hydrolysis products as carbon or nutrient sources (Rani *et al.*, 1994).

As a developing country like Bangladesh, pesticides are being used by the farmers at a large volume to mitigate increasing demands of foods and deliberately discharged it into the environment at a regular basis without considering it's adverse impacts. So, we have to enrich our soil with biodegrader such as microorganisms, only they can protect all living beings. From our present investigation, we have successfully isolated a potential carbofuran degrader from natural soil. The isolated bacteria *Enterobacter* sp. showed degradation efficiency of (99% pure) carbofuran up to 4µg/ml and reduced 80% carbofuran within 7 days. Again, the bacteria did not show any antagonistic effect against any Rhizobacteria and *Enterobacter* sp. is a soil borne bacteria. So, they have especial adaptability in soil environment. We also confirmed through plasmid extraction experiment that the genes responsible for degradative enzyme might be reside in it's chromosomal DNA. This property of the isolated bacteria would inherit accurately. Thus we can recommend this *Enterobacter* sp. in future application at field level to minimize environmental pollution from the toxic carbofuran.

APPENDIX

Appendix 1: Effect of pH on bacterial growth

Time (hr)	Optical Density (OD) at 600 nm wavelength			
	pH 5.0	**pH 6.0**	**pH 7.0**	**pH 8.0**
0	0.03±0.0	0.04±0.00	0.06±0.0	0.05±0.0001
2	0.03±0.0033	0.04±0.0044	0.07±0.0	0.05±0.0033
4	0.1±0.0022	0.16±0.0033	0.18±0.0033	0.15±0.0067
6	0.2±0.01	0.24±0.0058	0.31±0.0033	0.21±0.0115
8	0.23±0.0058	0.27±0.0033	0.41±0.0067	0.31±0.0033
10	0.25±0.0057	0.3±0.0033	0.45±0.0	0.35±0.0033
12	0.29±0.0033	0.33±0.0022	0.51±0.0033	0.39±0.0058
24	0.41±0.0058	0.42±0.01	0.61±0.0033	0.49±0.0058
36	0.35±0.0120	0.31±0.0033	0.39±0.0001	0.43±0.0058

Appendix 2: Effect of pH on bacterial growth

Time (hr)	Optical Density (OD) at 600 nm wavelength					
	pH 7.0	**pH 7.2**	**pH 7.4**	**pH 7.6**	**pH 7.8**	**pH 8.0**
0	0.06±0.00	0.06±0.00	0.06±0.00	0.05±0.0001	0.05±0.0001	0.05±0.0001
2	0.07±0.00	0.06±0.0033	0.07±0.0003	0.04±0.0033	0.05±0.0001	0.05±0.0001
4	0.17±0.00	0.16±0.0033	0.17±0.00	0.13±0.0033	0.15±0.0033	0.15±0.0145
6	0.3±0.0033	0.21±0.0033	0.3±0.0033	0.19±0.0033	0.2±0.0033	0.24±0.0067
8	0.41±0.0033	0.23±0.00	0.34±0.0067	0.25±0.0033	0.22±0.0033	0.32±0.0033
10	0.45±0.0033	0.29±0.0033	0.36±0.0067	0.3±0.00	0.26±0.0318	0.34±0.0057
12	0.5±0.0033	0.31±0.0058	0.39±0.0058	0.29±0.0033	0.25±0.0351	0.4±0.0001
24	0.6±0.0033	0.31±0.0067	0.3±0.00	0.34±0.0033	0.4±0.0033	0.49±0.0067
36	0.39±0.0033	0.21±0.0033	0.16±0.0058	0.19±0.0001	0.21±0.0067	0.47±0.01

Appendix 3: Effect of Temperature on Bacterial growth

Time in hour	Optical Density (OD) at 600 nm wavelength		
	Temperature 25°C	**Temperature 30°C**	**Temperature 35°C**
0	0.02±0.000	0.02±0.0	0.02±0.0
2	0.04±0.0058	0.07±0.0	0.04±0.0033
4	0.09±0.0058	0.17±0.0	0.16±0.0033
6	0.18±0.0003	0.25±0.0033	0.22±0.0033
8	0.19±0.0115	0.3±0.0	0.27±0.0033
10	0.26±0.0100	0.36±0.0066	0.3±0.0033
12	0.27±0.0058	0.38±0.0033	0.28±0.0033
24	0.29±0.0000	0.39±0.0033	0.31±0.0033
36	0.17±0.0058	0.19±0.0001	0.06±0.0033

Appendix4: Probit analysis of carbofuran effect on *Artemia salina* for 12h exposure

Dose(µg/ml)	logdose	number	kill	% kill	corr%	emp probit	expt probit	wrk probit	weight	final probit
20	1.301017	10	6	60	60	5.25	5.406001	5.24	6.01	5.376731
10	.9999897	10	5	50	50	5	5.048001	**5**	6.370001	5.044945
5	.6989628	10	5	50	50	5	4.69	5.01	6.01	4.71316
2.5	.3979359	10	3	30	30	4.48	4.331999	4.49	5.32	4.381374
1.25	9.690899E-02	10	1	10	10	3.72	3.973998	3.74	4.05	4.049588

Appendix5: Probit mortality analysis of carbofuran effect on *Artemia salina* for 24h exposure

Dose(µg/ml)	logdose	number	kill	% kill	corr%	emp probit	expt probit	wrk probit	weight	final probit
20	1.301017	10	8	80	80	5.85	5.777999	5.83	5.32	5.754816
10	.9999897	10	6	60	60	5.25	5.363	5.24	6.16	5.344589
5	.6989628	10	5	50	50	5	4.948	4.99	6.34	4.934363
2.5	.3979359	10	3	30	30	4.48	4.533001	4.46	5.81	4.524136
1.25	9.690899E-02	10	2	20	20	4.16	4.118001	4.17	4.71	4.113909

Appendix 6:.Probit analysis of carbofuran effect on *Artemia salina* for 36h exposure

Dose(µg/ml)	logdose	number	kill	% kill	corr%	emp probit	expt probit	wrk probit	weight	final probit
20	1.301017	10	8	80	80	5.85	6.175999	5.810001	4.05	6.148686
10	.9999897	10	9	90	90	6.28	5.774	6.15	5.32	5.746022
5	.6989628	10	6	60	60	5.25	5.372	5.24	6.16	5.343359
2.5	.3979359	10	5	50	50	5	4.970001	4.99	6.34	4.940696
1.25	9.690899E-02	10	3	30	30	4.48	4.568002	4.46	5.81	4.538034