Isolation, characterization and engineering of *Pseudomonas jinjuensis* biotype *acephalyticus* for degradation of wide range of organophosphates

A thesis submitted to the University of Hyderabad for the award of a Ph.D. degree in Department of Animal Sciences

By

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DECLARATION

I, **Pinjari Aleem Basha** hereby declare that this thesis entitled **"Isolation, characterization and engineering of *Pseudomonas jinjuensis* biotype *acephalyticus* for degradation of wide range of organophosphates"** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University for the award of any degree or diploma.

Date:

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CERTIFICATE

This is to certify that this thesis entitled **"Isolation, characterization and engineering of *Pseudomonas jinjuensis* biotype *acephalyticus* for degradation of wide range of organophosphates"** is a record of bonafide work done by **Pinjari Aleem Basha** a research scholar for Ph.D. programme in Department of Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Prof. S. Dayananda
Supervisor

//Countersigned//

Head
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Dean
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Introduction

It is well established that the dawn of human civilization is intricately associated with development of agricultural societies. The Neolithic Revolution in economic, political, and social organization began in the Middle East as early as 10,000 B.C. and gradually spread to other centers, including parts of India, North Africa, and Europe. With the rise of agricultural forms of economic production, humans were able to remain settled more permanently in a region. Additionally, the emergence of agriculturally based societies caused a massive increase in the sheer number of people in the world auguring newer agricultural practices such the use of better quality seeds and fertilizers to improve crop productivity as well as the use of pesticides, insecticides, herbicides etc., to protect crops and their produce.

In general, "Pesticide" is a substance or mixture of substances used to kill pests including insects, ticks, mites, plant pathogens, molluscs, nematodes, weeds and microbes which can have a devastating effect on crop yield by direct destruction of the crop or by competing for nutrients in the soil. It is estimated that nearly Rs. 12,000 crore worth of crops are consumed annually by pests (Shroff, 2000). Despite such huge losses, widespread use of pesticides is inevitable since they provide a sure cover for the farmer to protect his investment in seeds and fertilizers. Pesticides represent the direct control towards crop disease and pest management and thus help in increased yields of crops. Currently about 215 pesticides have been registered for use in India (NCIPM, National Centre for Integrated Pest Management) and the production and consumption of pesticides has increased several folds in the last three decades. In the United States alone, 50,000 tonnes of pesticides are used per year (Ballantyne and Marrs, 1992). As per Mathur et al (1999), pesticide utilization rate is much greater in India (76%) as against global utilization (44%). Due to inadequate

knowledge about pesticide practices, they have been used indiscriminately. Continuous and wide spread use of pesticides has added to environmental problems leading to contamination of air, water and terrestrial ecosystems, eliciting harmful effects on different biota, and disrupting biogeochemical cycling. However, pesticides will continue to be the indispensable tool for the farmer to manage insect pests in the absence of other viable alternative technologies.

On the basis of their chemical properties, pesticides are broadly classified into inorganic compounds, thiocyanates, organochlorines, organophosphates, carbamates, organosulfur, natural and synthetic pyrethroids. The first known pesticide was elemental sulfur dusting used by Sumerians about 4500 years ago. Since then, toxic chemicals like arsenic, lead and mercury were also applied to crops to control pests (Miller and Tyler, 2002). In the 17th century, natural products like nicotine sulfate (isolated from tobacco leaves), pyrethrum (derived from *chrysanthemum*) and rotenone (from roots of tropical vegetables) were used as insecticides. The first synthetic organic pesticides were organochlorine compounds such as dichlorodiphenyltrichloroethane (DDT), invented by Paul Muller in 1939 and considered as a brilliant invention at that time (Metcalf, 1995). The period between 1940's and 1950's ushered the 'pesticide era' experiencing 50-fold increase in pesticide usage. DDT and other organochlorine insecticides like aldrin, chlordane, endrin, dieldrin, diclofor, heptachlor, and lindane were used for many years to control mosquitoes along with broad spectrum insecticide to control insect pests that damaged foods and crops. Because of their persistency and susceptibility to bio-magnification these insecticides were subsequently banned in most of the countries (Munnecke, 1979; Alexander, 1981). Later these organochlorine pesticides were replaced by less

persistent organophosphates (Graetz et al., 1970; McEwens and Stephenson, 1979; Rosenberg and Alexander, 1979).

1.1 Organophosphate compounds

The term 'organophosphate' (OP) is the general term given to esters or thiols derived from phosphoric, phosphonic or phosphoramidic acid (Sogorb and Vilanova, 2002). The OP compounds were recognized as a diverse class of compounds as early as in 1854. However, the insecticidal properties of OP compounds were appreciated only in the 1930's mainly due to the pioneering work of Gerhard Schrader, a German chemist working in Bayer Company (IG Farben). This discovery lead to the synthesis of the first OP insecticide tetraethyl pyrophosphate (TEPP) and that of an extensively used OP compound, parathion (*O*, *O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate). Since then, thousands of OP compounds have been synthesized for use as insecticides, nerve agents, solvents, plasticizers etc (Graetz et al., 1970; Rosenberg and Alexander, 1979; Marquis, 1986; Minton and Murray, 1988; Aspelin, 1994).

a. Chemistry and Toxicology of Organophosphate Compounds

Most organophosphorus compounds are ester or thiol derivatives of phosphoric, phosphonic or phosphoramidic acid. Their general formula is presented in Fig. 1.1 R_1 and R_2 are mainly the aryl or alkyl groups, which can be directly attached to a phosphorus atom (phosphinates) or via oxygen (phosphates) or a sulphur atom (phosphothioates). In some cases, R_1 is directly bonded with phosphorus and R_2 with an oxygen or sulfur atom (phosphonates or thion phosphonates, respectively). At least one of these two groups is attached with uni-, mono- or di-substituted amino groups in phosphoramidates. The X group can be diverse and may belong to a wide range of

aliphatic, aromatic, heterocyclic or halide groups. The X group is also known as a leaving group because it is released from phosphorus on hydrolysis of the ester bond (Sogorb & Vilanova, 2002).

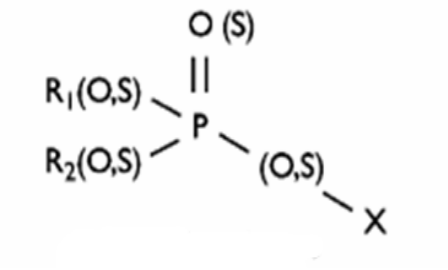
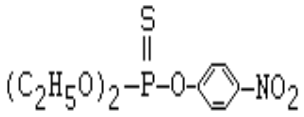
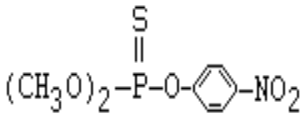


Fig.1.1 General formula of organophosphorus compounds where R_1, R_2 - is alkyl or aryl group, X- is the complex group, often referred to as the leaving group.

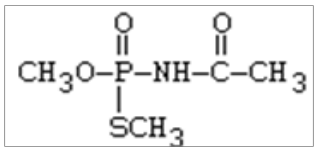
Globally, organophosphate pesticide usage accounts for approximately 38% of total pesticides. In the USA alone over 40 million kilos of OP's are applied annually (EPA, 2004). Some of the widely used pesticides around the world are parathion, methyl parathion, chlorpyrifos, acephate, coumaphos, monocrotophos etc., and all of them are used for the control of various insect pests (Fig.1.2).



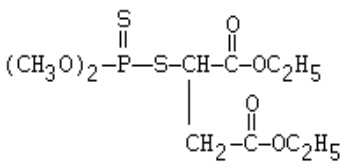
Parathion



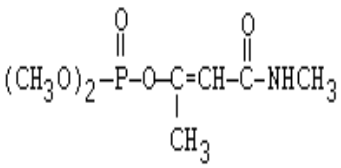
Methyl parathion



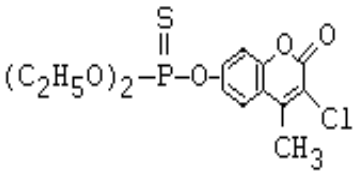
Acephate



Malathion



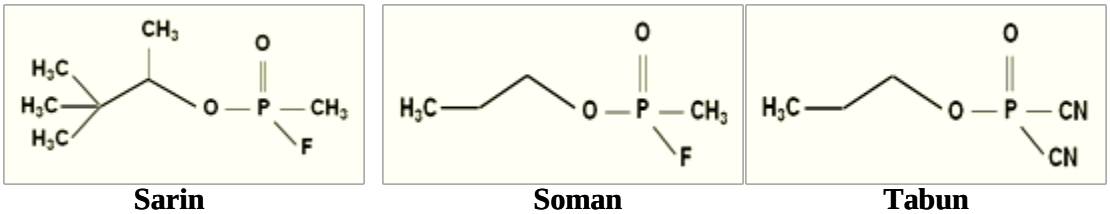
Monocrotophos



Coumaphos

Fig.1.2. Chemical structures of commonly used organophosphorus pesticides.

These pesticides are highly neurotoxic and exclusively inhibit the acetylcholinesterase activity causing serious problems to non-target organisms including the humans (Gaines, 1969; Eto, 1974; Green et al., 1977; Cremlyn et al., 1978; Ashani et al., 1991). It is noteworthy that OP compounds, due to their anticholinesterase activity are also used as chemical warfare agents. Sarin, Soman and Tabun are few examples of nerve agents which were produced on large scale during the Second World War (Fig.1.3).



Sarin **Soman** **Tabun**

Fig.1.3. Chemical Structures of Nerve gases that belongs to organophosphate group.

The mode of action of organophosphorus compounds includes inhibition of ester-splitting enzymes in living organisms. They are particularly effective against acetylcholine esterase, which hydrolyzes acetylcholine generated in myoneural junctions during the transmission of motor commands. Acetylcholine is required for the transmission of nerve impulses in the brain, skeletal muscles and other areas (Toole & Toole, 1995). However, after the transmission of the impulse, acetylcholine must be hydrolyzed to avoid over-stimulation of the nervous system. The breakdown of acetylcholine is catalyzed by an enzyme called acetylcholine esterase which converts acetylcholine into choline and acetyl CoA by binding the substrate at its active site at serine 203 to form an enzyme substrate complex (Somani, 1992). Further reactions involve release of choline from the complex and subsequent, rapid reaction of acylated enzymes with water to produce acetic acid. It has been estimated that one

enzyme can hydrolyze 300,000 molecules of acetylcholine every minute (Ragnarsdottir, 2000). Organophosphorus compounds inhibit the normal activity of the acetylcholine esterase by establishing covalent bonding to the enzyme, thereby changing its structure and function. They bind to the serine residue present in active site of acetylcholine esterase enzyme. Binding of op compounds to acetylcholinesterase brings phosphorylation of the enzyme and this phosphorylated enzyme is unable to function as an effective catalyst for the hydrolysis of acetylcholine (Raushel, 2002). The regeneration of acetylcholine esterase enzyme is very slow and may take hours or days, resulting in the accumulation of acetylcholine at the synapses. Nerves are then over stimulated (Manahan, 1992), leading to convulsion, paralysis and finally death for insects and mammals (Ragnarsdottir, 2000) (Fig.1.4).

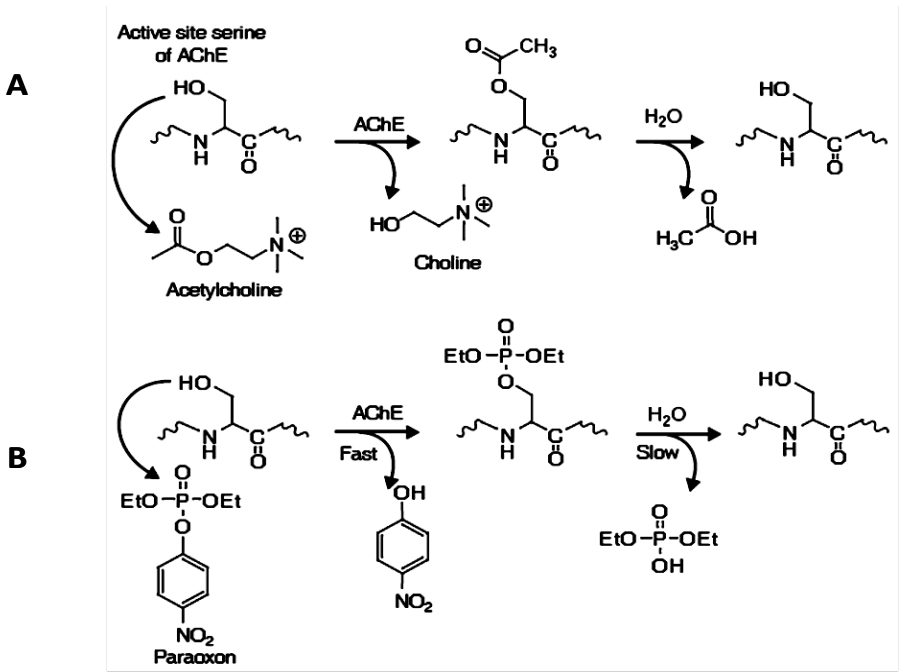


Fig.1.4. Panel A. Acetylcholinesterase (AchE) mediated hydrolysis of acetylcholine. Panel B. Mechanism of inhibition of acetylcholinesterase (AchE) by organophosphates.

b. Organophosphorus pesticides as environmental pollutants and its health hazards

Though organophosphate pesticides are less persistent, their continuous and extensive use has led to contamination of several ecosystems in different parts of the world. Even traces of pesticides residues are seen in rain, fog and snow due to successive cycles of volatilization and condensation (Rice, 1996; Dubus et al., 2000). Several organophosphorus compounds are used on animals for the control of body pests. As several of them are fat soluble and can thus enter the body readily through the skin and potentially find their way into meat and milk as well (MAFF/HSE, 1995). Contamination of grains, vegetables and fruits with organophosphorus compounds is also well documented (Pesticide Trust 1996; National Consumer Council 1998). Another potential and more dangerous source of organophosphorus contamination arises from their use as chemical warfare agents. It is estimated that about 200,000 tons of extremely toxic organophosphorus chemical warfare agents such as Sarin, Soman, and VX were manufactured and are stored. As required by the Chemical Weapon Convention (CWC) 1993, these stocks must be destroyed by the member states (Singh, 2008).

The prolonged use of these pesticides has resulted to its chronic poisoning. The classic example of the pesticide poisoning is mercury poisoning reported in Japan and parathion poisoning in Missisipi of the United States of America (Clarion-Ledger, a Mississippi News Paper, November, 1996). It is estimated that OP pesticides cause around 3 million poisonings and 200,000 human deaths annually, mostly in developing countries (Karalliedde and Senanayake, 1999). This problem is mainly

due to the toxicity of the pesticides which were used by farmers, who do not wear appropriate protective clothing due to inadequate knowledge on pesticide poisoning. The U.S. Environmental Protection Agency (EPA) reports annually about 3000 to 5000 cases of acute pesticide poisoning in USA annually. Hence the toxicity of pesticides is observed not only in developing countries but also in developed countries (Steenland, 1995).

It has been estimated that of the total quantity of pesticides used, less than 1% is utilized by plants while the remaining 99% goes directly into soil, indirectly affecting biota and food chain, leading to pollution of the environment (Thomson and Abbot 1966; Pimentel and Leviton, 1986). Apart from that, pollution of the environment also arises due to accidents in transportation or in manufacturing (Munnecke, 1979a; Kearney et al., 1987; Copella et al., 1990; Smith et al., 1992).

In India, the first report of poisoning due to pesticides was from Kerala in 1958, where over 100 people died after consuming wheat flour contaminated with parathion (Karunakaran, 1958). According to report of Central Bureau of Investigation, Government of India, as many as 4536 persons died while handling poisonous substances. A sizable number of men, women and children especially from agricultural families of Handigodu village, Malanadu district, Karnataka, as well as from cluster of villages surrounding Sagar in Shimoga district have been attacked by horrible bone disease. This disease which typically crippled the affected people is named after the village as Handigodu syndrome. Scientific investigations conducted in this regard have revealed that consumption of fish and crabs caught in paddy fields that were intensely sprayed with pesticides of high toxicity (notably parathion and

endrin) was responsible for this disease (Bhat and Krishnamachari, 1977). In 2007, Center for Science and Environment (CSE) reported the presence of pesticides in soft drinks. Of the reported pesticides, two of them belong to OP class of compounds (Malathion and Chlorpyriphos). Related investigations revealed that the water was used for manufacturing these soft drinks are contaminated by pesticides (cseindia.org). Long term, low-level exposure to a pesticide, rotenone (widely used in horticulture) is suspected to be a cause of Parkinson's disease in humans (Giasson and Lee, 2000). Recently it is shown that the reason for illness of Gulf War Veterans is mainly due to their exposure to acetylcholinesterase inhibitors like toxic nerve agents and organophosphorus compounds (Golomb, 2008).

Increased use of pesticides has led to the entry of these chemicals into the environment and has contaminated soils, waters, dairy products and other food commodities (Caldwell and Raushel, 1991). This has caused an environmental concern for the mankind since many of the pesticides used have been found to be highly toxic; thus indiscriminate dumping of chemical wastes needs immediate attention.

The Government of India has taken steps to ensure the safe use of pesticides. The Insecticide Act , promulgated in 1968 and enforced on 1st August ,1971 envisages to regulate the import, manufacture, sale , transport, distribution and use of insecticides with a view to prevent risks to human beings or animals and for matters connected therewith (The Insecticide Act,1968).

1.2. Microorganisms and Biodegradation

The toxicity of insecticides has come into light for the first time through the book “Silent spring” written by Rachel Carson that has drawn attention of general public and various governmental agencies about the toxic effects of pesticides on humans. Since then, numerous programmes have concentrated on the proper disposal of pesticides without any toxic effects to environment.

The fate of pesticides applied is dependent on various abiotic and biotic factors. The abiotic factors include physical and chemical factors acting independently or in combination. In general practice the detoxification of pesticides was done by physical and chemical methods which include landfills and incineration. Landfills function adequately in the short term but it leads to leaching of pesticides into surrounding soil and ground water. Incineration, a method for the detoxification of pesticides, though approved by the US Environmental Protection Agency (EPA), has met with serious public opposition because of the toxic emissions and high costs involved; incineration requires large amounts of energy to reach high temperatures that are needed to destroy pesticides.

The biotic factors that are known to reduce the toxicity of pesticides include plants, fungi, bacteria etc. It is well documented that bacteria exhibit versatility in degrading pesticides and are very well known for detoxification of xenobiotics. Fungi and yeasts are the minor components of this biodegradation process. Compared to the disadvantage of conventional methods for decontamination of pesticides, bioremediation using microorganisms has been considered as a potentially convenient, effective, low-cost, and environmentally friendly method. Microbial

degradation of toxic compounds offers a promising strategy by which many toxic wastes may be economically and safely detoxified.

Biodegradation is the term used to describe the process of using microorganisms to degrade or remove toxic components from the environment (Glazer and Nikaido, 1995). Biodegradation is a form of biotransformation that mainly converts the toxic parent compound to products of less toxicity. Biodegradation has distinct advantages over physicochemical methods as it can be cost-effective and achieve the complete degradation of toxic compounds without destruction of the site material or associated indigenous flora and fauna (Timmis and Pieper, 1999). Most of the pesticides applied, regardless of the site or purpose of application, enter the environment either in its original or modified form (Hill and Wright, 1978; Pimentel and Leviton, 1986).

Of the various genera of bacteria, *Pseudomonads* are shown to be versatile for the degradation of xenobiotics. Due to the presence of an array of enzymes, pathways and control mechanisms they have occupied a unique status in the degradation of xenobiotics. They catalyze different chemical reactions such as oxidation, hydroxylation, hydrolysis, dehalogenation, dealkylation, nitro group reduction and aromatic ring cleavage. The ability of microbes in degradation has been widely reviewed in the literature (Dagley, 1975; Alexander, 1985; Lal, 1983; Tiedje et al., 1987; Reinecke and Knackman, 1988; Chaudhry and Chapalamadugu, 1991; Cork and Krueger, 1991; Mohn and Tiedje, 1992; Deo et al., 1994; Moorman, 1994; Singh et al., 1999).

Several bacterial strains that are involved in degradation of different classes of pesticides have been isolated from different geographical regions. Most of the microorganisms responsible for detoxification of the toxic compounds belong to the genera *Flavobacterium*, *Pseudomonas*, *Alcaligenes*, *Arthrobacter* and *Agrobacterium*. In particular, some strains of *Pseudomonas* have been reported to degrade a large number of organic chemicals. For example, strains of *Pseudomonas* sp. and *Klebsiella pneumoniae* have been shown to possess hydrolase enzymes that are capable of breaking down s-triazine herbicides, such as atrazine. Strains of *Alcaligenes* possess several enzymes like oxygenases, hydroxylases, and isomerases that are involved in the degradation of herbicide 2, 4 - D (2, 4 – dichlorophenoxyaceticacid) (Tiedje et al., 1964; Don et al., 1985). A few strains of *Pseudomonas* have shown the ability to degrade 2, 4, 5–T (2, 4, 5 – Tichlorophenoxyaceticacid) which is poorly biodegradable as compared to 2, 4 – D (Karns et al., 1983; Karns et al., 1984). Several microorganisms are known to degrade organochlorine pesticides like DDT [1, 1–bis (4 – chlorophenyl)-2, 2, 2-trichloro ethane], both aerobically and anaerobically. Major chemical reactions in microbial degradation of organochlorine pesticides include dechlorination, dehydrochlorination, oxidation and isomerization (Lal and Saxena, 1982). Under anaerobic conditions, strains like *Aerobacter aerogenes*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae* dechlorinated DDT to DDE, an important step in degradation of DDT (Wedemeyer, 1966). Aerobic degradation of DDT via 4-chlorobenzoic acid (4-CBA) was reported in *Alcaligenes eutrophus* A5 (Nandeau et al., 1994).

Apart from bacterial strains, few fungal strains also exhibit predominant ability to eliminate toxic environmental pollutants. Mainly fungal strains belonging to

Phanerochaete, *Aspergillus*, *Trichoderma*, *Rhizopus* and *Penicillium* genus have the demonstrated ability to eliminate different groups of pesticides like organochlorines, carbamates and organophosphates. The white - rot fungi *Phanerochaete chrysosporium* possess enzymes that not only enable them to degrade lignin and cellulose, but also to degrade many recalcitrant chemicals including halogenated-phenol ring-containing compounds, such as pentachlorophenol (wood preservative), which is a persistent pollutant in industrial wastes emanating from paper and leather tanning industry (Evans and Bucke, 1998).

Both bacteria and fungi can degrade OP pesticides through hydrolytic cleavage, and pyrethroids (e.g. permethrin) through cleavage of the ester bonds. With the exception of dithio-carbamates, microbial degradation of all types of carbamate pesticides has also been demonstrated; for example, a rapid hydrolysis of carbaryl has been reported due to presence of the esterase enzyme in *Pseudomonas* sp. (Mulbry and Kearney, 1991).

a. Biodegradation of Organophosphorus pesticides

Organophosphate pesticides are comparatively less persistent in the environment, but possess high mammalian toxicity (Singh and Singh, 1997; Singh and Gautam, 1997). Due to acute toxicity, they adversely affect soil biotic factors and other soil properties, and also harm non-target organisms (Kaplainis et al., 1959; Knapp, 1962; Nacemann, 1970; Zakrevsky and Mallet, 1975). The action of microbial communities, either individually or in a consortium, is solely responsible for the less persistence of OP pesticides in soil. A report on biodegradation of OP pesticides became available in 1946 describing the degradation of diisopropyl fluorophosphate

(DFP), involving cleavage of the phosphorus-fluorine bond by an enzyme in animal tissues (Mazur, 1946). Since then, a number of workers have studied microbial degradation of organophosphates in plants, soils, water and animals (Beynon et al., 1973), in flooded soil and in anaerobic cultures (Sethunathan, 1973) and by soil microorganisms (Laveglia and Dahm, 1977; Munnecke, 1981; Read, 1983). Bacteria from flooded soil were found to hydrolyze selected organophosphorus insecticides (Adhya et al., 1981). Daughton and Hsieh (1977) are the first ones to report the biodegradation of OP pesticides by a consortium of defined microbial cultures. OP pesticides are either cleaved or degraded in the environment. Microbial cleavage is responsible for degradation of organophosphorus insecticides (Rosenberg and Alexander, 1979). This is the most possible reason for the lesser persistence of OP pesticides in the environment (Felsot, 1989). The susceptibility of OP compounds to microbial degradation has been reported for several insecticides such as parathion (Sethunathan and Yoshida, 1973), methyl parathion (Chaudry et al., 1988; Somara and Siddavattam, 1995; Zhongli et al., 2001; Pakala et al., 2007), glyphosate (Wacket et al., 1987; Kertesz et al., 1994a), diazinon (Sethunathan, 1971; Sethunathan and Pathak 1972), coumaphos (Mulbry, 2000; Horne et al., 2002b), isofenphos (Racke and Coats, 1987), monocrotophos (Bhadbhade et al., 2002a; Singh and Singh, 2003), chlorfevinphos (Suett et al., 1996), fenitrothion (Adhya et al., 1981; Hayatsu et al., 2000), phorate (Suett and Jukes, 1997) and the nematicides cadusafos (Karpouzas et al., 2004b), ethoprophos (Smelt et al., 1987; Karpouzas et al., 1999b), phorate (Mich and Dahm, 1970) and fenamiphos (Anderson and Lafuenza, 1992; Stirling et al., 1992; Davis et al., 1993;Singh et al., 2003; Karpouzas et al., 2004a).

Being esters, they have many vulnerable sites and are potentially hydrolyzable. Degradation of organophosphate pesticides is generally faster than that of organochlorines. Soil is an excellent medium for both biological and nonbiological degradation of organophosphorus pesticides. The principal reactions in soils are hydrolysis, oxidation, alkylation, and dealkylation (Singh et al., 1999). Microbial degradation through hydrolysis of *P*-*O*-alkyl and *P*-*O*-aryl bonds is considered the most significant step in the detoxification of organophosphorus compounds. Hydrolysis of organophosphorus compounds leads to a reduction in their mammalian toxicity by several orders of magnitude.

b. Parathion (*O, O*–diethyl–*O*–*p*–nitrophenyl phosphorothioate)

The degradation of parathion by microorganisms is well documented (Licheinstein and Schultz, 1964; Sethunathan and Yoshida, 1973; Siddaramappa et al., 1973; Munnecke et al., 1974; Daughton and Hsieh, 1977; Rosenberg and Alexander, 1979; Nelson, 1982; Serdar et al., 1982; Mulbry et al., 1986; Misra et al., 1992). The major degradation products of parathion are *p*-nitrophenol and diethyl thiophosphoric acid. (Munnecke and Hseih, 1976).

Sethunathan & Yoshida (1973) isolated the first organophosphorus degrading bacterium, *Flavobacterium* sp. that could degrade parathion and diazinon. Siddaramappa et al. (1973) isolated a *Pseudomonas* sp. that was able to hydrolyze parathion and utilize the hydrolysis product *p*-nitrophenol as a carbon or nitrogen source. Later, *P. stutzeri* was isolated, and demonstrated to hydrolyze parathion, although *p*-nitrophenol was metabolized by a separate bacterium (Daughton & Hsieh, 1977). Rosenberg & Alexander (1979) isolated two *Pseudomonas* sp. that were able

to hydrolyze a number of organophosphorus compounds including parathion, and using ionic cleavage products as a sole source of phosphorus. An *Arthrobacter* sp. isolated from parathion-treated soil was found to hydrolyze parathion and uses the *p*-nitrophenol product as sole carbon source (Nelson, 1982). Horne et al. (2002) isolated bacterial strain *Agrobacterium radiobacter* P230 which is capable of degrading a wide variety of OP compounds. A *Pseudomonas* sp. and *Xanthomonas* sp. were isolated from a pesticide disposal site in northern Israel, both degraded parathion in two stages involving initial release of *p*-nitrophenol by parathion hydrolysis followed by subsequent degradation of *p*-nitrophenol (Tchelet et al., 1993).

According to available reports, the major degradation products of parathion are *p*-nitrophenol and diethyl thiophosphoric acid (Munnecke and Hsieh, 1976) (Fig.1.5).

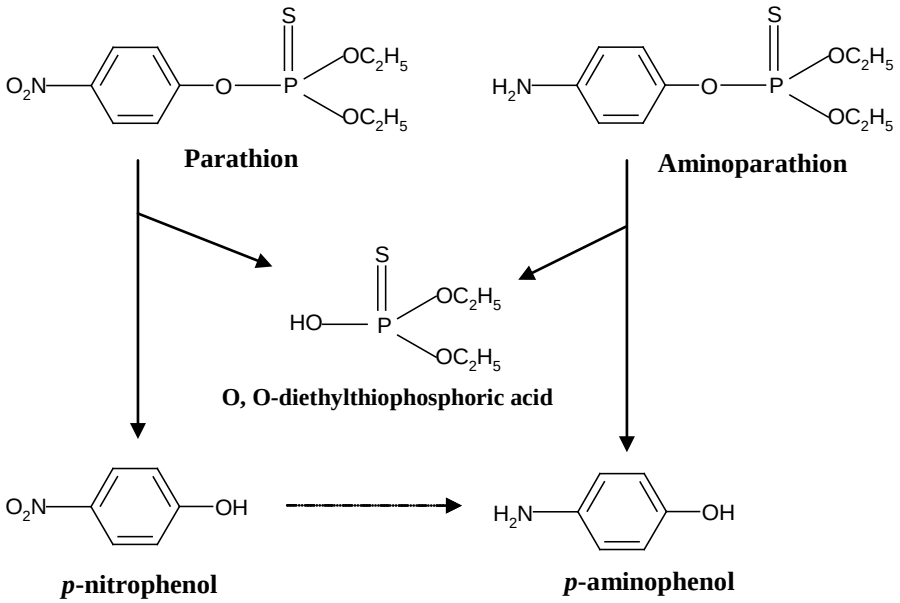


Fig.1.5. Proposed pathway of parathion degradation in flooded soils by *Flavobacterium* sp. (Adhya et al, 1981).

The complete mineralization of the hydrolytic products of parathion is well documented by Munnecke and Hsieh, (1974). The hydrolytic product *p*-nitrophenol follows two different pathways for degradation to maleylacetate. Spain and Gibson (1991) proposed the formation of maleylacetate from *p*-nitrophenol via *p*-benzoquinone, hydroquinone, and γ-hydroxymuconic semialdehyde (Fig.1.6).

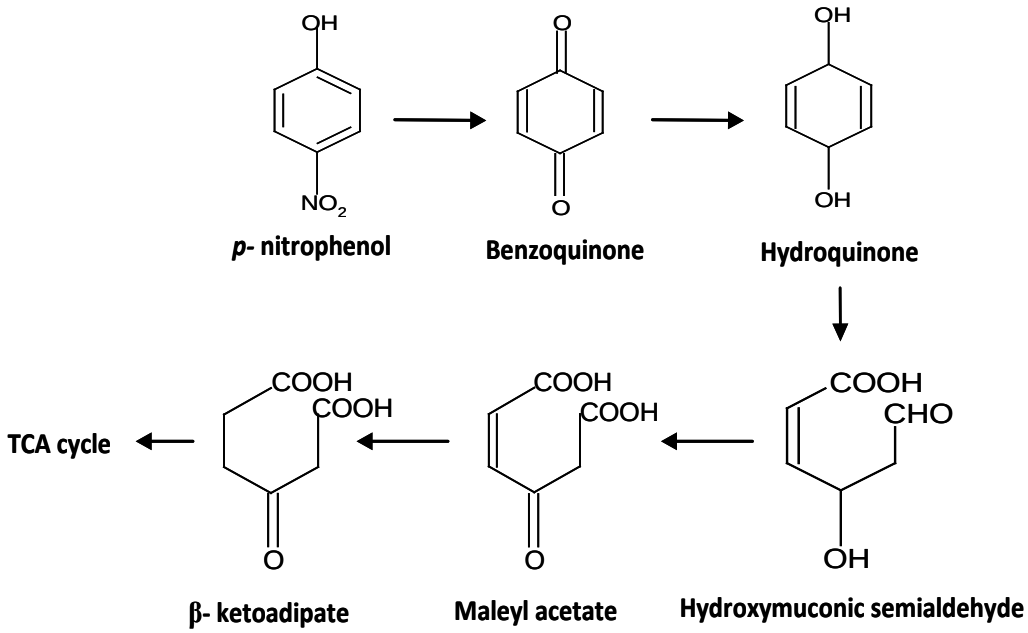


Fig.1.6. Proposed pathway of *p*-nitrophenol degradation by gram negative bacteria (Spain and Gibson, 1991; Prakash et al, 1996).

In the second pathway, maleylacetate was produced from *p*-nitrophenol via 4-nitrocatechol, 1, 2, 4 – benzenetriol shown in an *Arthobacter* sp. and a *Bacillus* sp. (Jain et al., 1994; Kadiyala and Spain, 1998) (Fig.1.7).

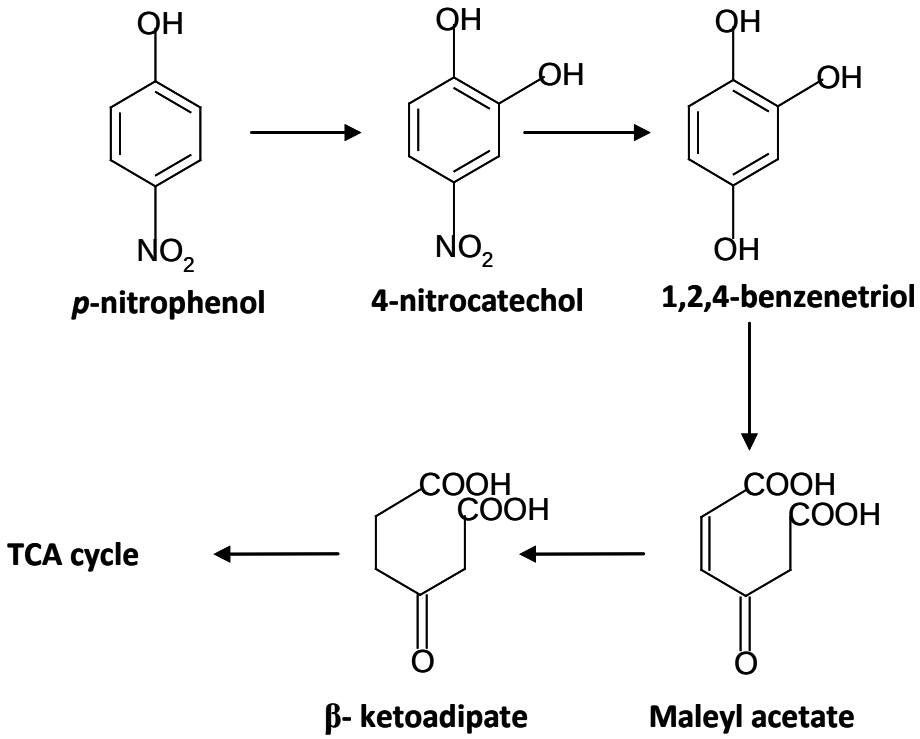


Fig.1.7. Proposed pathway of *p*-nitrophenol degradation by gram positive bacteria (Hanne et al, 1993; Jain et al, 1994; Kadiyala and Spain, 1998).

Alternative metabolic pathways have also been reported for parathion. Munnecke and Hsieh (1976) reported a secondary metabolic pathway of parathion, which involved the oxidation of parathion to paraoxon that was further hydrolyzed to *p*-nitrophenol and diethylphosphoric acid. In some other microorganisms, parathion is reduced to aminoparathion (Yasuno et al., 1965; Mick and Dahm, 1970).

Some microbes that are reported to degrade parathion are *Chlorella pyrenoidosa, Trichoderma viride, Rhizobium japonicum, Rhizobium meliloti, Penicillium waksmanii, Bacillus subtilis, Pseudomonas* sp., *Corynebacterium simplex,* mixed cultures with at least nine different bacterial isolates (*Pseudomonas* sp.,

Brevibacterium sp., *Azotomonas* sp., *Xanthomonas* sp.), *Flavobacterium* sp., (Zuckerman et al., 1970;Yasuno et al., 1965; Hirakoso, 1969; Sethunathan, 1973; Rao and Sethunathan, 1974; Siddaramappa et al., 1973; Munnecke and Hseih, 1976; Brown, 1980; Adhya et al., 1981; Serdar et al., 1985; Mulbry et al., 1989), *Agrobacterium radiobacter* P230 (Horne et al., 2002), *Flavobacterium balustinum* (Somara and Siddavatam, 1995), *Plesiomonas* sp. (Zhongli et al., 2001) *Pseudomonas diminuta* (Serdar et al., 1982), *Pseudomonas putida* (Rani and Lalitha kumari,1994).

However, detailed investigations were carried out on the activities of only two bacterial isolates viz., *Flavobacterium* sp. strain ATCC 27551 and *Pseudomonas diminuta* MG. *Flavobacterium* sp. was isolated from the rice fields of International Rice Research Institute (IRRI), Philippines whereas *Pseudomonas diminuta* was isolated from the agricultural fields of Texas, USA through enrichment cultures using parathion as sole source of carbon (Sethunathan et al., 1973; Serdar et al., 1982).

c. Methyl parathion (*O, O*–dimethyl–*O*-*p*-nitrophenyl phosphorothioate)

As compared to parathion degradation, reports on methyl parathion degradation by various microbial communities are limited. Methyl and ethyl parathion have identical chemical structures except for the ethyl groups of the P chain of parathion, which are replaced by methyl groups. Miyamoto et al. (1966) observed the degradation of methyl parathion and fenitrothion by a *Bacillus subtilis*. Reports on methyl parathion degradation include isolation of *Flavobacterium balustinum* that utilizes methyl parathion as sole source of carbon (Somara & Siddavattam, 1995). In this bacterium, the organophosphorus degrading (*opd)* gene was found to be linked with a novel gene involved in degradation of *p*-nitrophenol (Siddavattam et al., 2003).

A *Pseudomonas* sp. was isolated that can co-metabolically degrade methyl parathion (Chaudry et al., 1988). A *Bacillus* sp. was reported to degrade methyl parathion by hydrolysis as well as reduction of the nitro group (Sharmila et al., 1989). Ou and Sharma (1989) isolated another *Bacillus* strain that utilized methyl parathion as a carbon and energy source. Misra et al. (1992) reported the isolation of two bacterial isolates which rapidly hydrolyzed methyl parathion, parathion and fenitrothion. Degradation of methyl parathion and utilization of its degradation product *p*-nitrophenol as a source of energy was reported in *Pseudomonas putida* (Rani and Lalithakumari, 1994). A *Pseudomonas* sp. that degrades methyl parathion in soil and on sodium alginate beads was reported (Ramanathan & Lalithakumari, 1996). Hayatsu et al. (2000) isolated *Burkholderia* sp. NF100 which utilized both fenitrothion and methyl parathion as carbon sources. In a study, a *Burkholderia cepacia* was isolated from a methyl parathion treated site which utilized methyl parathion and *p*-nitrophenol as sole sources of carbon (Keprasertsup et al., 2001). Zhongli et al (2001) reported a *Plesiomonas* sp. strain M6 that degrades methyl parathion by means of a novel methyl parathion degrading gene. This isolate also utilizes *p*-nitrophenol and other aromatic compounds as a sole source of carbon (Zhongli et al., 2002). Another strain of *Pseudomonas* sp. WBC capable of complete degradation of methyl parathion that utilizes it as sole source of carbon and nitrogen was reported (Yali et al., 2002). The hydrolysis product of methyl parathion is also *p*-nitrophenol. The proposed pathway of methyl degradation is presented in Fig.1.8.

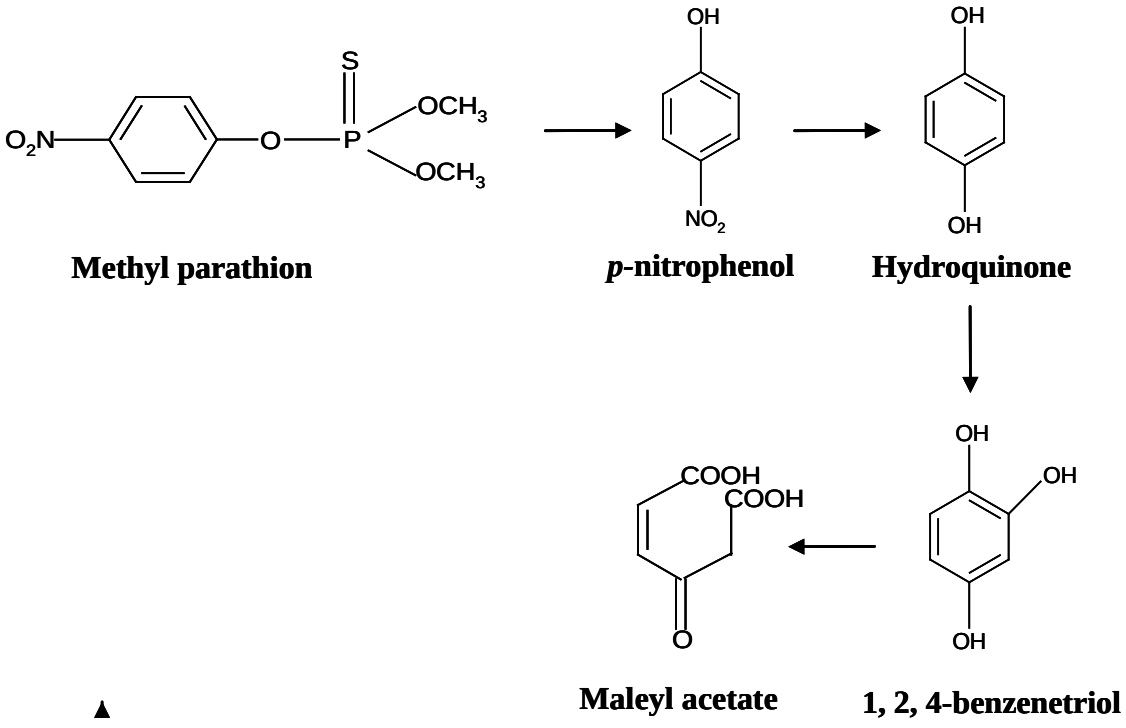


Fig.1.8. Proposed pathway of methyl parathion degradation by *Pseudomonas putida* (Rani and Lalithakumari, 1994).

d. The role of bacterial enzymes in detoxification of organophosphates

Over billions of years bacteria have perfected the skills of using various compounds for their survival. To survive and succeed in hostile environments, bacteria have evolved various catalytic mechanisms. These mechanisms serve as a store for the evolution of more efficient enzymes (Butler and Mason 1997; Ellis 2000). Of the various catalytic enzymes that are available to bacteria, the class of hydrolases plays an important role in the degradation of OP pesticides. The OP hydrolyzing enzymes are known variously as organophosphorous acid anhydrases (OPA), phosphotriesterases (PTE), organophosphorous triesterases, parathion hydrolases (PH), paraoxonases, somanases or organophosphate hydrolases (OPH)

depending on the substrate used while assaying the enzyme activity (Brown, 1980; Hoskin et al., 1982, Cheng et al., 1993; DeFrank and Cheng, 1991; Abd-Alla, 1994).

e. Organophosphorus hydrolase (OPH), a remarkable enzyme for detoxification of organophosphorus compounds

Organophosphorus hydrolase (OPH) has been isolated from several bacteria (Serdar et al., 1982; Mulbry & Karns, 1989a; Singh et al., 1999). Among them the organophosphorus hydrolase from *Pseudomonas diminuta* and *Flavobacterium* are well studied. The ability of OPH to hydrolyze various OP pesticides has been described in detail (Efremenko and Sergeeva, 2001; Singh, 2008). This enzyme hydrolyzes the compounds that are structurally related to parathion exhibiting broad temperature range, pH optima and high stability (Brown, 1980; Donarski et al., 1988; Dumas et al., 1989). Several structurally discrete enzymes that hydrolyze OP pesticides have been purified and characterized (De Frank and Cheng, 1991; Mulbry and Karns, 1989). However, only the enzymes from *Flavobacterium* sp. strain ATCC 27551 and *Pseudomonas diminuta* MG are worth mentioning since they are studied in more detail because of their broad substrate range and high K_{cat} values (Dumas et al., 1989; Hong and Raushel, 1996).

The gene that encodes the active OPH from *Pseudomonas* and *Flavobacterium* is *opd*, (*opd*, for organophosphate-degrading) which is localized on large indigenous plasmids in both cases (Serdar et al., 1982; Mulbry et al., 1986). The *opd* gene was first isolated and sequenced from *P. diminuta* and reported to be present on a 66 kb plasmid, pCMS1 (Serdar et al., 1982). The *opd* gene isolated from *Flavobacterium* sp. ATCC25 was also located on a plasmid, pPDL2 (Mulbry et al., 1987). The *opd* gene

sequences obtained from these two sources were found to be identical (Harper et al., 1988).

OPH hydrolyzes wide range of OP compounds, which include parathion, methyl parathion and fensulfothion, among many others (Dumas et al., 1989) and OP chemical warfare agents (CWA) (Dumas et al., 1990). OPH is a homodimer metalloprotein with a molecular weight of ~72 kDa (Bening et al., 1994). It is a member of the amidohydrolase superfamily (Holm and Sander, 1997) and consists of two identical subunits containing 336 amino acids. High-resolution X-ray structure analysis showed that OPH protein folds into an $(\alpha\beta)_8$- barrel motif with the active site located at the carboxy-terminal end of the central β-sheet core (Bening et al, 2001) (Fig.1.9).

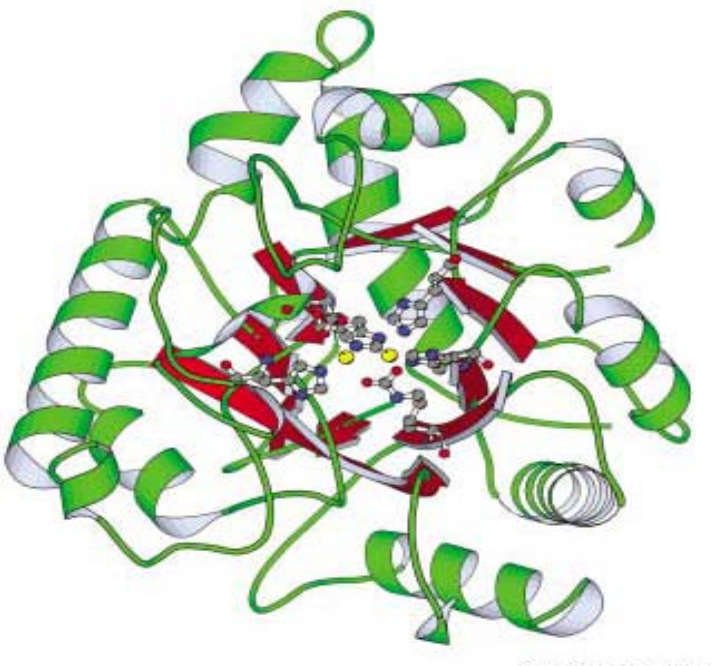


Fig.1.9. Ribbon diagram for the structure of the bacterial phosphotriesterase (Bening et al., 2001).

The active site of OPH contains two zinc ions per subunit. OPH has a wide range of substrate specificities and was demonstrated to catalyze hydrolysis of P–O,

P–F, P–CN and P–S bonds to different extents. It has the highest activity against the P–O linkage (with Kcat/Km value of 5.5 x 10^{-7} $mol^{-1}L$ s^{-1} for paraoxon) and least specificity for the P–S bond (with Kcat/Km value of 6.8 x 10^{-2} $mol^{-1}L$ s^{-1} for VX) (Efremenko and Sergeeva, 2001). OPH requires zinc for its activity and it has been demonstrated that replacement of zinc ions with other divalent metals such as cobalt, cadmium, copper, iron, manganese and nickel had effects on its catalytic activity. Enzymatic activity of Co^{2+} - reconstituted OPH has the greatest activity against paraoxon (Omburo et al., 1992). The hydrolysis of OP compounds proceeds via S_N2 reaction (Lewis et al., 1988). The *opd* gene from *Pseudomonas* has been sub-cloned into a variety of expression vectors, to achieve OPH expression in hosts including *Escherichia coli* (Serdar et al., 1989), *Streptomyces* (Steiert et al., 1989) and insect cells (Dumas et al., 1990), among others.

The OP hydrolyzing *Agrobacterium radiobacter* P230, isolated in Australia (Horne et al., 2002a) has been subject of intense research in recent times. Studies pertaining to the genetics and biochemistry of OP degradation have led to the identification of novel organophosphate degrading gene designated as *opdA*. Though similarities between *opd* and *opdA* are noticeable, there exist critical differences in the primer sequence of the gene which justify its nomenclature as *opdA*. The product of *opdA*, despite of showing similar mechanism of catalysis like that of OPH, due to minor differences in sequence, the OPDA effectively degraded fenthion and phosmet for which OPH, the product of *opd* gene has no activity (Horne et al., 2002a) Based on obvious sequence similarities and overlapping catalytic principles between *opd* and *opdA*, and the *opdA* has been shown to be evolved more recently than *opd* (Horne et al., 2003).

1.3. Engineering of microorganisms for detoxification of toxic environmental pollutants

Metabolic engineering can be defined as purposeful modification of cellular metabolism using recombinant DNA technology and other molecular biological techniques (Bailey, 1991). Advances in genetic and protein engineering techniques have opened up new avenues to move towards the goal of genetically engineered microorganisms (GEMs) to function as biocatalysts in which certain desirable biodegradation pathways or enzymes from different organisms are brought together in a single host with the aim of performing specific reactions. The development of novel organisms for use in biodegradation has been a key challenge throughout this period. In particular, the growing understanding of the genetic and biochemical basis of the metabolism of xenobiotic compounds holds considerable potential for the development of recombinant microorganisms useful for the bioremediation of environmental pollution caused by urban and industrial activities (van der Meer et al., 1992; Timmis et al., 1994; Knackmuss, 1996; Reineke, 1998).

Many scientific groups have developed genetically engineered microorganisms by introducing novel genes responsible for degradation of many organophosphates. For example Zhi Liu et al., (2006) constructed a genetically engineered microorganism that degrades both organophosphates and carbamates. Methyl parathion degrading (*mpd*) gene from *Pseudomonas putida* DLL-1was cloned into a broad host vector pBBR1MCS-2 to produce a recombinant plasmid pBBR-*mpd* that was later transformed into carbofuran degrading *Sphingomonas* sp. CDS-1. This genetically engineered microorganism (GEM) has shown degradation of both methyl

parathion and carbofuran and thus appears to be a promising GEM candidate for environmental bioremediation. In a similar manner, a number of attempts have been made to manipulate natural isolates to impart genetic ability needed to degrade simultaneously on wide range of pesticides and herbicides (Shao et al., 1995b, Lan et al., 2006) and to completely mineralize parathion (Walker and Keasling, 2002, Mattozzi et al., 2006).

1.4. Catalytic properties of OPH

As stated before organophosphorus hydrolase (OPH) hydrolyzes triester linkage found in wide range of op compounds. For example OPH can successfully hydrolyze P-O, P-F, P-C and P-S bonds that are found in prominent op compounds like paraoxon, parathion, methyl parathion, coumaphos, diazinon, acephate demeton-S, phosalone and fensulfothion (Dumas et al., 1989; Efremenko and Sergeeva, 2001). In addition to the aforementioned insecticides, OPH also possess the ability to hydrolyze the nerve agents DFP, sarin, soman, and VX (Dumas et al., 1990b; Kolakowski et al., 1997; Efremenko and Sergeeva, 2001). Though natural substrate for OPH remains unknown, the purified enzyme hydrolyzes the insecticide paraoxon, at rate that approaches the diffusion-controlled limit (Efremenko and Sergeeva, 2001). However the catalytic efficiency of OPH goes down when other class of organophosphates are used as assay substrates. The kinetic properties of OPH with various op compounds are presented in table 1.0. It the table is examined acephate, the predominant insecticide serves as poor substrate. High Km value and poor turnover number (shown in bold case in table 1.0) indicates acephate as one of the most resistant organophosphate for OPH hydrolysis.

Table 1.1 Kinetic constants for the hydrolysis of organophosphorus insecticides.

Substrate	Bond Type	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
Paraoxon	P-O	3170	0.058	5.5×10^7
Parathion	P-O	630	0.24	2.6×10^6
Methyl parathion	P-O	189	0.08	2.4×10^6
Coumaphos	P-O	610	0.39	1.6×10^6
Diazinon	P-O	176	0.45	3.9×10^5
Fensulfothion	P-O	67	0.46	1.5×10^5
DFP	P-F	465	0.048	9.7×10^6
Sarin	P-F	56	0.7	80×10^3
Soman	P-F	5	0.5	10×10^3
Acephate	P-S	2.8	160	18
Demeton – S	P-S	1.3	0.78	1.6×10^3
Phosalone	P-S	0.63	0.26	2.4×10^3

(Dumas et al., 1989; Lai et al., 1995; Efremenko and Sergeeva, 2001).

1.5. Hypothesis

Perusal of available literature on biodegradation of op compounds highlights the necessity of isolating acephate mineralizing bacterial strain. If such isolate doesn't possess innate ability to degrade op compounds other than acephate, its manipulation by expressing OPH is expected to generate a "super bug", which would be capable of degrading wide range of op compounds, besides thriving on acephate.

a. Definition of the problem

Isolation and characterization of acephate degrading bacterium from activated sludge and its genetic manipulation to improve degradation range and efficiency.

1.6. Brief description of work done in the study

The present study describes isolation, taxonomic characterization of acephate mineralizing bacterium isolated from activated sludge collected from pesticide manufacturing unit. This strain identified as *Pseudomonas jinjuensis* biotype *acephalyticus* hydrolyzed the amide linkage found in acephate generating acetic acid and methamidophos. Methamidophos is further metabolized to serve as source of carbon and nitrogen. A tentative pathway followed in mineralization of acephate is elucidated based on the identification of metabolites using mass spectrometry. Further, OPH is expressed in *P. jinjuensis* biotype *acephalyticus* to target inner membrane and cytoplasm. This manipulated strain when tested successfully degraded wide range of op compounds.

Materials & Methods

2.1. Materials

2.1.1. Antibiotics

Name of the antibiotic	Name of the Supplier
Ampicillin sodium salt	Amersham Pharmacia Biotech, UK
Kanamycin Sulfate	Amersham Pharmacia Biotech, UK
Tetracycline hydrochloride	Amersham Pharmacia Biotech, UK
Chloramphenicol	Amersham Pharmacia Biotech, UK
Streptomycin	Amersham Pharmacia Biotech, UK
Gentamycin	Amersham Pharmacia Biotech, UK
Spectinomycin	Amersham Pharmacia Biotech, UK

2.1.2 Biochemicals

Name of the Chemical	Name of the Supplier
Acrylamide	Amersham Pharmacia Biotech,UK
Acephate	Rallis India Limited, Hyderabad
Ammonium persulphate	Amersham Pharmacia Biotech,UK
Ammonium nitrate	Qualigens
Bovine serum albumin	Amersham Pharmacia Biotech, UK
Bromophenol blue	Amersham Pharmacia Biotech,UK
Butanol	Qualigens
Calcium chloride	Qualigens

Calcium nitrate	Qualigens
Chloroform	Qualigens
Coomassie Brilliant blue R	Amersham Pharmacia Biotech,UK
Cobalt chloride	SRL
α-cyano-4-hydroxycinnamic acid	Sigma Aldrich
Deoxynucleotide triphosphates	Amersham Pharmacia Biotech,UK
Dipotassium hydrogen orthophosphate	Merck
N,N□-Dimethylformamide	SRL
Ethidium bromide	SRL
Ethylenediaminotetraacetic acid disodium salt (EDTA)	SRL
Ethyl acetate	SRL
Ferrous Sulphate	Qualigens
Glucose	Qualigens
Glycerol	Qualigens
Glycine	SRL
Hydrochloric acid	Qualigens
Isopropanol	SRL
Isopropyl thiogalactopyranoside (IPTG)	Amersham Pharmacia Biotech, UK
Lysozyme	Amersham Pharmacia Biotech, UK
Magnesium chloride	Amersham Pharmacia Biotech, UK
Magnesium sulphate	SRL

Methanol	SRL
N,N'-Methylene bis acrylamide	SRL
β-mercaptoethanol	Sigma Aldrich
α-napthylamine	SRL
Phenol Saturated	Bangalore GeneI
Phosphoric acid	Merck
Potassium chloride	Qualigens
Potassium dihydrogen ortho phosphate	Merck
Sodium citrate	SRL
Sodium chloride	SRL
Sodium dodecyl sulfate	SRL
Sodium hydroxide	SRL
Sodium Sulphate	Merck
Sulphanilamide	Merck
Sucrose	SRL
RNase A	Amersham Pharmacia Biotech, UK
Tetra ethyl methylene diamine (TEMED)	Sigma
Tris	SRL
Tryptone	Himedia
X-gal	Amersham Pharmacia
Xylene cyanol	Amersham Pharmacia Biotech, UK

Yeast extract	Himedia
Zinc chloride	SRL
Trifluoroacetic acid	Sigma Aldrich
Monocrotophos	Rallis India Limited, Hyderabad
Phosalone	Rallis India Limited, Hyderabad
Ethion	Rallis India Limited, Hyderabad
Methyl parathion	Rallis India Limited, Hyderabad
Methamidophos	Sigma Aldrich

2.1.3. Restriction and other enzymes

Name of the enzyme	**Name of the Supplier**
*Bam*HI	MBI, Fermentas
*Dra*I	MBI, Fermentas
*Eco*RI	MBI, Fermentas
*Sma*I	MBI, Fermentas
T_4 DNA Ligase	MBI, Fermentas
Taq DNA polymerase	MBI, Fermentas

2.2. Bacterial strains

Name of the strain	Genotype / phenotype	Source
E. coli DH5α	*supE44 ΔlacU169 (Δ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1*	Hanahan, 1983.
E. coli S17-1	*thi pro hsdR hsdM recA RP4 2-Tc::Mu-Km^r::Tn7 (Tp^r, Sp^r, Sm^r)*	Simon et al., 1983.
Pseudomonas jinjuensis biotype *acephalyticus*	*Cm^r, Amp^r, Km^s*	This work

2.3. Plasmids

Plasmid	Relevant Genotype	Reference
pSS15	Amp^r , 1.5 kb DNA fragment containing complete *opd* gene of *Flavobacterium balustinum* cloned in pUC18 vector	Siddavattam et al., 2006.
pHYS400	Amp^r, complete *opd* gene encoding preOPH cloned in pET23b as *Nde*I - *Xho*I fragment	Siddavattam et al., 2006.
pHNS400	Amp^r, *opd* sequence encoding mature OPH cloned in pET23b as *Nde*I - *Xho*I fragment	Pandey et al., 2009.
pMMB206	Cm^r, broad-host-range low-copy-number mobilizable expression vector	Morales et al., 1991.
pMMBKM	Km^r, Kanamycin cassette cloned into pMMB206 vector by replacing the chloramphenicol resistance gene.	This work

pMMBKMOPD	Km^r, *opd* gene cloned with its native promoter as *Bam*HI fragment into pMMBKM expression vector	This work
pHLCKMOPD	Km^r, *opd* gene cloned with signal sequence as *Eco*RI and *Bam*HI fragment into pMMBKM expression vector	This work
pHNSKMOPD	Km^r, *opd* gene cloned without signal sequence as *Eco*RI and *Bam*HI fragment into pMMBKM expression vector	This work

2.4. Preparation of stock, working solutions and buffers

Acephate (*O, S* – dimethyl acetyl phosphoramidothioicacid)

Technical grade acephate (97% pure), procured from pesticide manufacturing company, Rallis India Limited, Patancheru, Hyderabad, and kept in a specially designed container before storing in a refrigerator. When necessary the container having acephate was taken to a fume cupboard to make 1M acephate stock solution. Initially acephate taken in a clean screw cap tube was weighed and dissolved in adequate amounts of milli Q water to get 1M solution. The contents were filter sterilized before storing them in aliquots of 500 µl. When necessary 500 µl of 1M acephate is used to supplement 50 ml of mineral salts medium to get 10 mM of acephate as carbon/nitrogen/sulfur supplement.

Methamidophos (*O, S* – dimethyl phosphoramidothioicacid)

Methamidophos was procured from Sigma – Aldrich. 1M stock solution of methamidophos was prepared by dissolving 141.12 mg in 1 ml milli Q water. The filter sterilized stock solution was distributed in to 500 µl aliquots and stored at 4^0C. When necessary an aliquot of 500 µl is used as carbon supplement in 50ml of minimal salts medium.

Methylparathion (MP) (*O, O*-dimethyl-*O*-paranitrophenyl phosphorothioate)

Methyl parathion was procured from Sigma Aldrich in a powder form and stored in a refrigerator. A 100 mM stock solution of methyl parathion was prepared by dissolving 26.32 mg in 1 ml of methanol and stored at 4^oC until further use. Whenever necessary 1 µl of stock of methyl parathion was added to 1ml of assay buffer while assaying OPH activity.

Monocrotophos

Monocrotophos (97% pure, technical grade) was procured from pesticide manufacturing company, Rallis India Limited, Patancheru, Hyderabad, and kept in a specially designed container before storing in a refrigerator. When necessary the container having monocrotophos was taken to a fume cupboard to make 1M monocrotophos stock solution. Initially monocrotophos taken in a clean screw cap tube was weighed and dissolved in adequate amounts of milli Q water to get 1M solution. The contents were filter sterilized before storing them in aliquots of 1 ml. When necessary 500 µl of 1M monocrotophos is used to supplement 50 ml of mineral salts medium to get 10 mM of monocrotophos as carbon supplement.

Ethion

Ethion (90% pure, technical grade) was procured from pesticide manufacturing company, Rallis India Limited, Patancheru, Hyderabad, and kept in a specially designed container before storing in a refrigerator. When necessary the container having ethion was taken to a fume cupboard to make 1M ethion stock solution. Initially ethion taken in a clean screw cap tube was weighed and dissolved in adequate amounts of methanol to get 1M solution. The contents were filter sterilized before storing them in aliquots of 1 ml. When necessary 500 µl of 1M ethion is used to supplement 50 ml of mineral salts medium to get 10 mM of ethion as carbon supplement.

Phosalone

Technical grade phosalone (94% pure), procured from pesticide manufacturing company, Rallis India Limited, Patancheru, Hyderabad, and kept in a specially designed container before storing in a refrigerator. When necessary the container having phosalone was taken to a fume cupboard to make 1M phosalone stock solution. Initially phosalone taken in a clean screw cap tube was weighed and dissolved in adequate amounts of methanol to get 1M solution. The contents were filter sterilized before storing them in aliquots of 1 ml. When necessary 500 µl of 1M phosalone is used to supplement 50 ml of mineral salts medium to get 10 mM of phosalone as carbon supplement.

Ampicillin

Ampicillin stock solution was prepared by dissolving 500 mg of ampicillin in 5 ml of milli Q water and sterilized by filtration. The sterilized solution was stored in 1 ml aliquots at -20°C. When required 1 µl of ampicillin stock solution is added to 1 ml of medium after cooling it to 45°C to get the working concentration of 100 µg/ml.

Chloramphenicol

Chloramphenicol stock solution was prepared by dissolving 250 mg of chloramphenicol in 10 ml of 100% ethanol and sterilized by filtration. The stock solution was stored in 1 ml aliquots at -20°C. When required 1 µl of chloramphenicol stock solution was added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 30 µg/ml.

Tetracycline

Tetracycline stock solution was prepared by dissolving 250 mg of tetracycline hydrochloride in 10 ml of 70% ethanol/water and sterilized by filtration. The stock solution was stored in 1ml aliquots at -20°C. When required 1 µl of tetracycline stock solution was added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 25 µg/ml.

Kanamycin

Stock solution of kanamycin was prepared by dissolving 250 mg of kanamycin sulfate in 10 ml of milli Q water and sterilized by filtration. The stock solution was stored in 1 ml aliquots at -20°C. When required 1 µl of kanamycin stock

solution is added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 25 µg/ml.

Gentamycin

Stock solution of gentamycin was prepared by dissolving 200 mg of gentamycin in 10 ml of milli Q water and sterilized by filtration. The stock solution was stored in 1 ml aliquots at -20°C after filter sterilization. When required 1 µl of gentamycin stock solution is added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 20 µg/ml.

Streptomycin

Stock solution of streptomycin was prepared by dissolving 200 mg of streptomycin in 10 ml of milli Q water. The stock solution was stored in 1 ml aliquots at -20°C after filter sterilization. When required 1 µl of streptomycin stock solution was added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 20 µg/ml.

Spectinomycin

Stock solution of spectinomycin was prepared by dissolving 200 mg of spectinomycin in 10 ml of milli Q water. The stock solution was stored in 1 ml aliquots at -20°C after filter sterilization. When required 1 µl of spectinomycin stock solution is added to 1 ml of medium after cooling it to 45^{o}C to get the working concentration of 20 µg/ml.

IPTG

1M IPTG stock solution was prepared by dissolving 236.8 mg IPTG in 1 ml of autoclaved milli Q water and filter sterilized using 0.2 mμ Sartorius filter. The stock solution was stored in 1 ml aliquots at -20°C. When required the stock solution was thawed on ice bath and adequate amount is added to the cooled medium (45^{o}C) to get 1-2 mM working concentrations of IPTG.

X-Gal

2% of X-gal stock solution was prepared by dissolving 20 mg of X-gal in 1 ml of N,N'-dimethyl formamide. When required 2□l of stock solution is added to each 1 ml of medium after cooling it to 45^{o}C.

2.5. Preparation of buffers and solutions for SDS-PAGE

Acrylamide solution (30%)

100 ml acrylamide solution was prepared by dissolving 30 g acrylamide, 0.8 g N, N-methylene-bis-acrylamide in 70 ml of distilled water. The contents were then filtered and the solution was finally adjusted to 100 ml. The stock solution prepared in this manner is stored at 4^{o}C until further use.

Staking gel buffer for SDS-PAGE

3.93 g of Tris was dissolved in 50 ml of double distilled water and the pH of the solution was adjusted to 6.8 using 1N HCl and finally the volume of the buffer was made up to 100 ml with distilled water.

Running gel buffer for SDS-PAGE

59.93gm of Tris was dissolved in 400ml of distilled water and pH of the solution was adjusted to 8.8 using 1N HCl. Finally the volume of the buffer solution was made up to 500 ml using distilled water.

Tank buffer (pH 8.5) for SDS-PAGE

3.03 g of Tris, 14.4 g of glycine and 1 g of SDS was dissolved in 500 ml of distilled water and pH of the solution was adjusted to 8.5 with 2N HCl. Finally the volume of the buffer was made up to 1000 ml. The buffer at 1X concentration contains 0.025M Tris, 0.192M glycine and 0.1% SDS.

Protein Markers

Low molecular weight markers supplied by MBI Fermentas were used. Size of the protein markers include, 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa.

Sample loading buffer for SDS-PAGE

1.2 ml of 0.5 M Tris (pH 8.0), 2 ml of 10% SDS, 1 ml of 10% glycerol, 0.5 ml of β-mercaptoethanol, 0.001 g of bromophenol blue were taken in a 10 ml reagent bottle. The contents were mixed well before the volume was made up to 10 ml and solution was stored at 4°C until further use. When necessary adequate amounts of loading buffer was used to prepare protein samples.

Staining solution

0.2 gm of coomassie brilliant blue was dissolved in 30 ml of methanol. To this 10 ml of acetic acid was added and finally the volume was made up to 100 ml

using distilled water. The contents were stored at room temperature in amber color bottle until further use.

Destaining solution

30 ml of methanol is mixed with 10 ml of glacial acetic acid before making up the volume to 100 ml using distilled water. The contents were freshly made and used once to destain the SDS-PAGE gels.

2.6. SDS-polyacrylamide gel electrophoresis

The protein samples were separated for SDS-Polyacrylamide gel electrophoresis following the procedures of Laemmli (1970). 12.5% running acrylamide gel solution was prepared by mixing 3.6 ml 30 % acrylamide solution, 4.5 ml running buffer (0.98 M Tris-Cl, pH, 8.8), 0.9 ml distilled water, 90 µl of 10% SDS, 4.5 µl of TEMED and 45µl of freshly prepared 10% ammonium persulphate. The solution was mixed well and degassed before adding ammonium persulphate. The contents were then poured in between two sealed glass plates containing 1.5 mm spacers to form a slab. These contents were over-layered with 0.1 ml water saturated n-butanol and allowed to polymerize for 40 minutes at room temperature. After polymerization of running gel, butanol was removed by repeated washing and the traces of water were removed by wiping with filter paper strips. The 7.5 % stacking acrylamide gel solution was prepared by taking 1.25ml of 30 % acrylamide solution, 2.5 ml of stacking gel buffer (0.325 M Tris-Cl, pH, 6.8), 1.25ml of water, 50 µl of 10% SDS, 5 µl of TEMED and 50 µl ammonium persulphate. The stacking gel solution was carefully over layered on the running gel. Immediately a comb of required size was placed to form the wells. The stacking gel was allowed to

polymerize for 30 minutes. After 30 minutes the comb was removed and the wells were washed with tank buffer (50 mM Tris-Cl, 0.384 M glycine, 0.1% SDS, pH 8.5). Protein samples were mixed with equal volumes of sample loading buffer (50 mM Tris HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM 2-mercaptoethanol) and kept in a boiling water bath for 5 minutes. The contents were then briefly centrifuged before loading the sample into the wells. The electrophoresis was carried at 100 volts till the tracking dye reached the anode end of the gel. The gel was removed from the glass plates and the protein bands were stained with staining solution containing 0.2% W/V coomassie brilliant blue in methanol:water:acetic acid (30:60:10) for 6-8 hrs. The gel was then destained in the destaining solution containing methanol:water:aceticacid (30:60:10) for another 6-8 hrs.

2.7. Preparation of buffers for Western blotting

Protein transfer buffer

3.03 gm of Trizma-base and 14.4 gm of glycine was dissolved in 650 ml of distilled water. To this 200 ml of methanol was added and final volume was made up to 1000 ml with distilled water. The buffer was stored at 4^{o}C until further use.

TBS-T Buffer

2.42 gm of Tris and 8 gm of sodium chloride were dissolved in 500ml of distilled water. The pH of the solution was adjusted to 7.6 using 1N HCl before adding 1ml of 0.1% Tween-20. Finally the volume of the buffer solution was made up to 1000 ml with milli Q water.

Blocking Buffer

Blocking buffer was prepared by adding 10 gm of skimmed milk powder to 10 ml of 1X Tris buffered saline with 0.1% Tween-20 and the contents were stirred well. The solution was prepared fresh before blocking the membrane.

2.8. Western blotting

Western blotting was performed using ECL+Plus western blotting detection system supplied by Amersham Pharmacia Biotech, UK. After performing SDS-PAGE, the unwanted and unused areas of the gel was removed to keep the size of the gel as minimum as possible. Left-hand corner on the top of the gel was cut to mark the orientation of the gel. Four pieces of Whatman filter paper soaked in towbin buffer (0.3M Tris-Cl pH 10.4; 20% methanol) were placed on anode graphite plate. On top of these filter papers, 2 pieces of Whatman filter paper soaked in towbin buffer were placed. The polyvinylidene fluoride (PVDF) membrane (Highbond-P) was kept in methanol for 5 seconds to prewet the membrane and immediately rinsed with towbin buffer and placed on to the anode plate. Then the SDS-PAGE gel submerged in towbin buffer was placed on top of the membrane. On top of the gel, 2 pieces of filter papers soaked in towbin buffer were placed. Transfer of proteins onto PVDF membrane was carried out at (15 volts) constant voltage at room temperature. The process of protein transfer was continued for 45 min.

Once the transfer process was finished, membrane was stained with ponceau reagent to check the rate of proteins transfer on to PVDF membrane followed by removing the ponceau reagent by rinsing with 1X TBS-T buffer. Then the membrane was blocked with blocking reagent such as 10% skimmed milk powder in 1X TBS-T

buffer (20 mM Tris-base; 137 mM NaCl; pH 7.6). Blocking was continued for 1 hr at room temperature with constant shaking. Then the membrane was washed three times for 15 min in large volumes of 1X TBS-T buffer. Primary antibody (mouse IgG) raised against OPH or commercially procured anti-His antibodies were diluted in a ratio of 1:5000 in 1X TBS-T buffer containing 10% blocking agent and the membrane was incubated at room temperature with constant shaking for 3 hrs. After incubation the membrane was washed with 1X TBS-T three times for 15 min each wash to remove excess / unbound primary antibody. After primary antibody incubation, the membrane was incubated for 45 min at room temperature with a secondary antibody (anti-mouse IgG supplied by ECL+Plus kit, Amersham Pharmacia Biotech, UK) diluted in a ratio of 1:5000 in 1X TBS-T buffer containing 10% blocking reagent. Then the membrane was washed three times with 1X TBS-T as mentioned above and protein signals were detected by following the manufacturer's instructions.

2.9. Detection

Detection was performed using ECL+Plus kit procured from Amersham Pharmacia Biotech, UK by taking Solution A and B in a ratio of 1:40 respectively and mixed them gently in dark room. Membrane was then incubated in the reagent mix for exactly one min and dried with tissue papers to remove excess detection reagent before wrapping in a clean cling film. Then the membrane was exposed to Biomax X-ray film for 30 seconds. The film was developed by immersing it in a Kodak developer solution and fixed by transferring it to Kodak - fixer solution for 5 min. The film was then washed in water and dried before analyzing the results.

2.10. Preparation of solutions for Agarose gel electrophoresis

Tris Borate EDTA (TBE) buffer

A stock solution of 10 X TBE buffer was prepared by adding 108 g of Tris, 55 g of boric acid and 40 ml of 0.5 M EDTA (pH 8.0) to 900 ml of distilled water and dissolved properly before adjusting the volume of the contents to1000 ml with distilled water. The buffer was then stored in a container fixed with a tap. When necessary adequate volume of the stock buffer was diluted to get 1X TBE with distilled water and used for preparing agarose gels.

TE buffer

TE buffer was prepared by dissolving 121 mg of Tris and 37.2 mg of EDTA (pH 8.0) in 80 ml of distilled water. The pH of the buffer was adjusted to 8.0. Finally the volume was made up to 100 ml with distilled water to get the concentration of 10 mM Tris and 1 mM EDTA.

Sample Loading buffer (6X) for Agarose gel electrophoresis

5 mg of bromophenol blue, 25 mg of xylene cyanol and 4 g of sucrose was dissolved in few ml of distilled water and finally volume was made up to 10 ml and stored at 4°C.

2.11. Agarose gel electrophoresis

Agarose gel electrophoresis was performed by the following standard procedures described by Sambrook *et al*., 1989. Required amount of agarose was dissolved in TBE by heating in micro oven. The solution was then cooled to 50 -

55°C and poured on a clean sterile gel tray fitted with combs. The gel was allowed to solidify at room temperature. After solidification the gel along with the gel tray was immersed in the TBE buffer poured in the electrophoretic tank. Buffer level was adjusted to cover the gel to a depth of about 1 mm. Appropriate amount of DNA samples were mixed with 4 µl of 10 X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose) and loaded into the wells of submerged gel. Electrophoresis was carried out at 100 volts till the bromophenol blue reaches the end of the gel. The gel was then stained in staining solution (0.5 µg/ml ethidium bromide in water) for 15 minutes and was put in destaining solution (distilled water) for 15 minutes. Then the gel was visualized under UV transilluminator and the electrophoretic mobility of DNA was recorded by taking the photograph of the gel using gel documentation system.

2.12. DNA ligation

Before ligation reaction was set, the concentration of vector and insert was measured and were then taken in a sterile eppendorf tube in 1:3 (vector : insert) reaction. One unit of T_4 DNA ligase and 2µl of 10X ligation buffer was added to the reaction tube. Finally the reaction volume was adjusted to 20µl and ligation reaction was performed at 16^0C for 18 hours. A portion of ligation mixture was taken to transform *E. coli* competent cells. The colonies obtained on selective plates were used to isolate recombinant plasmid. Presence of insert was established by restriction digestion and subsequence analysis of the generated fragments on 0.8% agarose gel.

2.13. Transformation

Preparation of competent cells

Competent cells were prepared following the procedures of Mandel and Higa, (1970) with slight modifications. *E. coli* cells required to make competent were grown in LB broth of 100 ml at 37°C with an initial inoculum of 1%. The cultures were allowed to grow till the cell density reached to 0.3-0.4 OD at 600 nm. The culture was chilled on ice for 30 minutes and centrifuged at 6000 rpm for 10 minutes to harvest the cells. The cell pellet was then suspended in 50 ml of ice cold 0.1M $CaCl_2$ and incubated on ice for 30 minutes. After incubation the cells were sedimented and gently resuspended in 10 ml of ice cold 100mM $CaCl_2$ containing 15% glycerol and stored in aliquots of 200 µl at -70°C.

Transformation

The frozen competent cells were thawed by placing them on ice bath. The ligation mixture/plasmid of interest was added and incubated on ice for 30 minutes. After 30 minutes, the cells were subjected to heat shock at 42°C for exactly 90 seconds and immediately chilled on ice for 2 min. Further, 800 µl of LB broth was added and incubated at 37°C for 45 minutes. The cells were collected by centrifugation and resuspended in 200 µl of LB broth before plating. When needed 60µl of 2% X-gal or 60 µl of 2% IPTG was added and plated on LB agar plates containing appropriate antibiotic. The plates were then incubated at 37°C for 12 hr for colonies to appear.

Conjugation

All the three expression plasmids were mobilized into *Pseudomonas jinjuensis* biotype *acephalyticus* by non-quantitative biparental plate mating method (Simon et al., 1983). All the *E coli* S17-1 strains carrying expression plasmids were taken as donors and *Pseudomonas jinjuensis biotype acephalyticus* as recipient strains. Donor and recipient strains grown to mid log phase were taken in the ratio of 1:3 and plated on LB plate. The cells were allowed to grow at 30^0C for 72hrs. After incubation the mating mixture was scraped into an eppendorff tube containing 1 ml of 0.9% steriled NaCl solution and suspended gently. The cell suspension was then serially diluted and the exconjugants were selected on LB agar plates containing kanamycin (25 µg/ml) and chloramphenicol (30 µg/ml) depending on the resistance marker available on plasmid. Selection on chloramphenicol and kanamycin eliminates *E. coli* donor strain and allows to grow only exconjugants of *Pseudomonas jinjuensis biotype acephalyticus.*. Presence of expression plasmid was again confirmed by performing PCR using gene specific primers.

2.14. Media and Growth Conditions

The following media were used to propagate the bacteria. All media were sterilized by autoclaving for 20 min at 15 lb/Sq. When required appropriate amounts of antibiotics of Ampicillin (100 µg/ml), Tetracycline (25 µg/ ml), Chloramphenicol (30 µg/ ml), Streptomycin (20 µg/ml), Spectinomycin (20 µg/ml) Gentamycin (20 µg/ml) and Kanamycin (25 µg/ml) were added after cooling the media to 45^oC.

Luria Bertani (LB) broth

The LB medium was prepared by dissolving 10 g of peptone, 5 g of yeast extract and 10 g of NaCl in 500 ml of milli Q water. The contents were stirred and finally made up to 1 liter with milli Q water. The pH of the medium was adjusted to 7.0 with 1N NaOH and then sterilized it by autoclaving. The LB agar plates were prepared by adding 2% agar to LB broth.

Minimal Medium

The minimal medium was prepared by dissolving 4.8 g of K_2HPO_4, 1.2 g of KH_2PO_4 and 1 g of NH_4NO_3 in a few ml of milli Q water and finally the volume was made up to 997.85 ml. Then the salt solution was sterilized by autoclaving for 15 minutes at 15 lb pressure. $MgSO_4.7H_2O$ (1 g/10 ml), $Fe_2(SO4)_3$ (1 gm/100 ml) and $Ca(NO_3)_2.4H_2O$ (4 gm/20 ml) were prepared separately and sterilized by autoclaving as described above. About 2 ml of $MgSO_4.7H_2O$, 100 µl of Ca $(NO_3)_2.4H_2O$ and 50 µl of Fe_2 $(SO4)_3$ were added to sterile 1 litre of minimal medium under aseptic conditions.

Preparation of acephate plates

The acephate plates were prepared in minimal medium supplemented with 10 mM acephate. Initially the minimal medium was prepared by dissolving 2.4 g of K_2HPO_4, 0.6 g of KH_2PO_4 and 0.5 g of NH_4NO_3 in few ml of milli Q water and agar was added at 2% concentration. The volume was finally made up to 498.925 ml. Then this solution was sterilized by autoclaving for 15 minutes at 15 lb pressure. $MgSO_4.7H_2O$ (1 g/10 ml), Fe_2 $(SO4)_3$ (1 gm/100 ml) and Ca $(NO_3)_2.4H_2O$ (4 gm/20

ml) were prepared separately and sterilized by autoclaving as described above. After cooling to 45°C 1 ml of $MgSO_4.7H_2O$, 50 µl of Ca $(NO_3)_2.4H_2O$ and 25 µl of $Fe_2(SO4)_3$ were added to the sterile minimal medium under aseptic conditions. The contents were mixed properly after adding 5 ml of 1M acephate solution. These plates after solidification were stored at 4°C by keeping them in a special container.

2.15. Enrichment culture technique to isolate acephate (*O, S* – dimethyl acetyl phosphoramidothioicacid) degrading bacterium

Enrichment culture technique used by Chaudhry et al, (1988) for isolating methylparathion degrading soil microbes was used with slight modifications. Activated sludge brought from pesticide manufacturing unit located at Patancheru, Hyderabad, India was used as source of inoculum to isolate acephate degrading bacterium by enrichment culture technique. About, 10 gm of effluent treated sludge was added to a 250 ml flask containing 50 ml of minimal medium supplemented with 10 mM acephate as sole source of carbon. The contents were then incubated at 30°C in incubator shaker for a period of 7 days. After incubation the flask was left on a bench top for few minutes to allow the sludge particles to settle. The clear suspension (1ml), containing microorganisms was used to inoculate a fresh 50 ml minimal medium supplemented with 10 mM acephate. In similar way four such transfers were made and every time the enriched population was spreaded both on LB and minimal medium plates containing 10 mM acephate. After the fourth transfer bacterial colonies that were observed on acephate plates were independently inoculated in the minimal salts medium supplemented with acephate as carbon source.

2.16. Growth of *Pseudomonas jinjuensis* biotype *acephalyticus* using acephate as source of carbon, nitrogen and sulfur source

As the pure culture can grow on acephate by utilizing it as carbon and energy source an attempt was made to check whether acephate also serves as source of nitrogen, phosphorus and sulphur. Strain DS004 was inoculated into four different flasks containing the 50 ml of minimal medium (K_2HPO_4, 4.8 g, KH_2PO_4, 1.2 g and NH_4NO_3, 1 g, $MgSO_4.7H_2O$, 0.2 g, Ca $(NO_3)_2.4H_2O$, 0.04 g and Fe_2 $(SO4)_3$, 0.001 g in 1000 ml of water). When acephate was used as sole source of carbon minimal medium containing all salts was used as growth medium. However when acephate was used as source of nitrogen usage of Ca $(NO_3)_2.4H_2O$ and NH_4NO_3 was avoided in the medium. The sulfur containing salts were avoided when acephate is used as source of sulfur. In places of these salts sterile acephate solution (10 mM) was supplemented to the minimal medium. After preparing the nitrogen and sulfur source media a single colony that was propagated on acephate plate was used to inoculate these media. The flasks were then incubated at 30^{o}C in an incubator shaker with 200 rpm. The growth of the cultures was monitored by measuring the culture O.D. at 600 nm. A typical growth curve was generated by plotting a graph taking OD values on Y axis and time on X axis.

2.17. Identification of the bacteria

After obtaining the pure bacterial culture capable of growing on acephate, the bacteria was first characterized by using conventional microbiological techniques. Primarily the bacterial morphology was studied by using phase contrast, scanning electron and transmission electron microscopy.

Sample preparation for Phase contrast microscopy

In the preparation of samples for microscopic examination, the cells of strain DS004 were grown in minimal medium containing 10 mM acephate. After growing the culture to mid log phase, the cells were harvested and extensively washed with citrate saline buffer. Further, appropriate dilutions of cells were layered on microscopic slides before observing them under Phase contrast microscope.

Sample preparation for Scanning electron microscopy

To observe under Scanning electron microscope the cells were placed on a small piece of cover slip (0.5 cm), which is washed with acetone. About 5 µl of bacterial suspension was placed on the cover slip and air dried for 5 min. Then the cover slip was placed in Gold coating chamber and the gold coating was done under vacuum for 5 min. Then the cover slip was removed and observed under the scanning electron microscope**.**

Staining methods

Gram staining

A loopful of logarithmically growing culture was taken on a clean slide, to prepare a bacterial smear. The slide was then flooded with crystal violet (1 g of crystal violet is dissolved in 10 ml of absolute ethanol, filter sterilized and the volume made to 100 ml with double distilled water), and stained for a minute. Then the cells were washed extensively by keeping the slides under running tap water. In the next step Gram's Iodine solution (Iodine 1 g, potassium iodide 2 g, dissolved in 300 ml double distilled water.) a mordant was added to the slide for a minute. After washing with

water, the decolorization of dye was done with acetone. In the final step safranin is applied as a counter stain, washed with running water, and the air dried slide was observed under microscope (Olympus B201) at 100 X magnification.

Transmission Electron Microscopy (TEM) for negative staining of flagella

About 5 ml of well grown culture was centrifuged at 8000 rpm for 5 minutes and the pellet was suspended in 0.1 M phosphate buffer. The cells were again collected by centrifugation before dissolving in 1 ml of fresh phosphate buffer. A small drop of this sample was placed on a piece of Para-film and a carbon coated EM grid was placed on that drop. After 20 minutes the grid was removed and the excess sample was drained with filter paper. The grid was washed with distilled water and stained with 2% uranyl acetate. The grid was washed and allowed for air drying. The grid with sample was observed under transmission electron microscope (Model: Hitachi, H-7500).

Physiological and Biochemical Characterization

Utilization of organic/inorganic compounds as electron donor and/or carbon source

Over night culture was used as one percent inoculum to inoculate minimal salts medium containing organic or inorganic compounds shown in table (Table.1) to test if they can serve as electron donor and/or carbon source. Growth was monitored turbidometrically at O.D 600 nm. The tested organic compounds were used at a concentration of 0.3%, w/v.

Organic/Inorganic compounds tested for carbon source/electron donor
Glucose
L-glutamic acid
Sodium citrate
Potassium sodium tartrate
Potassium gluconate
DL-Malate
Succinic acid
Salicylic acid
Anthranilate
Benzoic acid
***p*-Aminobenzoic acid**
Acephate
Acetamide
Methanol

Gelatin liquefaction

Ability of the strain to liquefy gelatin was tested by inoculating 10% of culture in to fully filled screw cap test tubes containing modified Biebl and Pfennig's (1981) medium supplemented with pyruvate (0.3%, w/v) as carbon source, ammonium chloride (0.068%, w/v) as nitrogen source and gelatin (12%, w/v). Uninoculated test tube served as control. The test tubes were incubated at 30°C. After growth of the

culture tubes along with controls were refrigerated at 4°C for 10 minutes and the liquefaction of the gelatin is tested by comparing with the control.

Nitrate Reduction Test

To determine the strains ability to reduce nitrate 10 ml of a sterile nitrate reduction broth, (heart infusion broth (25 gm), potassium nitrate (2 gm) in distilled water 1000 ml) was inoculated with fresh over night culture of strain DS004. After incubation, alpha-napthylamine and sulfanilic acid are added. These two compounds react with nitrite if available in the medium and turn red in color.

2.18. Surface peptide analysis by MALDI-TOF/TOF

Three layer sample deposition method was used in the analysis of cell surface peptides by following the procedures described elsewhere (Ruelle et al., 2004). About 1 µl of bacterial preparation from mid log phase culture was applied to the MALDI-TOF sample plate. The air dried sample was considered as first layer. This first layer was then overlaid with 1 µl of ethanol and the contents were carefully dried by placing the plate under cool and dry place. After the contents are dried, 1 µl of matrix solution (1% α -cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile and 1% trifluoroacetic acid) was placed on top of the second layer. The contents were dried at room temperature before analyzing the sample in MALDI-TOF/TOF Autoflex (Bruker Daltonics) in reflectron mode.

2.19. 16S rRNA gene sequence analysis

Isolation of genomic DNA from acephate degrading bacterium

Genomic DNA from the bacterial cells was isolated by using QIAGEN DNeasy tissue kit by following manufacturer's protocols. Cells were collected from 5 ml of the overnight culture by centrifuging for 10 minutes at 7500 rpm. Cell pellet was then resuspended in 180 µl of ATL buffer (tissue lysis buffer). Further the cells were mixed with 20 µl of Proteinase K (100 mg/ml) before incubating the contents at 55^{o}C for 1 hour. The incubation was continued till a clear cell suspension is seen in the tubes ensuring the complete lysis. After complete lysis, 4 µl of DNase free RNase (100 µg/ml) was added and incubated at room temperature for 2 minutes. To this, 200 µl of buffer AL (lysis buffer) was added and incubated at 70^{o}C for a further period of 10 minutes. After incubation the contents were brought to the room temperature and 200 µl of 95% ethanol was added before mixing the contents thoroughly. The contents were loaded on to a DNeasy mini column and centrifuged at 8000 rpm for 1 minute to facilitate passing of contents through matrix. The flow through was discarded and washed with 500 µl of buffer AW1 (wash buffer 1) followed by 500 µl of buffer AW2 (wash buffer 2). After washing the DNeasy mini column was kept in a clean 1.5 ml eppendorf tube and the genomic DNA was eluted with 200 µl of buffer AE (elution buffer).

PCR amplification of 16S rRNA gene of acephate degrading bacterium

A number of studies are available on amplification of 16S rRNA gene (Hayatsu et al., 2000; Horne et al., 2002; Foster et al., 2004). The universal forward (5'-CGggatccAGAGTTTGATCCTGGCTCAGAACGAACGCT-3') and reverse (5´-

CGggatccTACGGCTACCTTGTTACGACTTCACCCC-3') primers designed by Chen et al., (1997) were adapted in the study to amplify 16S rRNA gene from acephate mineralizing bacterium. The 50 µl typical reaction contained 1 unit of Taq polymerase, 2. 5 mM dNTPs, Taq polymerase buffer besides 10 ρmoles of chromosomal DNA and 5 ρmoles of universal forward and reverse primers. A typical three stage PCR programme containing the following steps was used for amplifying 16S rRNA gene from strain DS004.

Stage 1

97^{o} C - 1. 30 minutes

Stage 2

94^{o} C - 1 minute

60^{o}C - 1 minute

72^{o}C - 1 minute

Repeated 29 cycles.

Stage 3

72^{o} C - 10 minutes

Hold step - 4^{o}C

The same PCR conditions were used for amplification of *opd* gene, except that the primers described elsewhere were used in PCR reaction mixture.

Purification of PCR product

After performing the polymerase chain reaction (PCR), the total PCR reaction mixture (100 µl) was taken into an eppendorff tube and the PCR product was purified following the manufactures protocol using QIAgen PCR purification kit. The 100 µl PCR mixture was mixed with 500 µl of buffer PB (Binding buffer) and passed through Qia quick spin column fitted in a collecting tube. The flow through was discarded and the Qia quick spin column was washed with 0.75 ml of wash buffer PE (Wash buffer). The column was then placed in a new eppendorff tube and subjected for brief centrifugation to remove the traces of ethanol. Then the column was placed in a 1.5 ml eppendorff tube and the DNA was eluted by adding 50 µl of buffer EB (10 µl Tris-Cl, pH 8.5). DNA sample was collected by centrifuging the tube for 1min and stored at -20^{0}C until further use.

Cloning of PCR product in to pGEM-T easy vector

In order to clone the PCR product into pGEM-T easy vector, 200 ng of PCR product was taken in clean eppendorff tube and 50 ng of pGEM-T easy vector was added (Purchased from Promega, USA) before keeping it for ligation by adding appropriate amounts of 10X T4 ligase buffer and 0.1 U T4 DNA ligase. The ligation was performed for 16 hours at 16^{o}C. After completion of ligation, the ligation mixture was transformed into *E. coli* DH5α competent cells as described elsewhere and the transformants were selected by plating the cells on LB plate containing 100 µg/ ml of Ampicillin, IPTG (1mM) and X-gal. The colonies containing recombinant plasmids were selected based on blue white screening.

Purification of plasmids using QIAgen Mini preparation kit method

Plasmid vectors were purified using QIAgen mini preparation kit especially when used for cloning. A single bacterial colony carrying plasmid was inoculated into a 5 ml of LB medium containing appropriate antibiotic and was incubated overnight at 37°C with vigorous shaking (~150 rpm). The overnight culture was centrifuged at 8000 rpm for 10 minutes and supernatant was discarded.. The bacterial cell pellet was resuspended in 250 µl of buffer TE containing RNase (2 µg/ml). To lyse the cells, 250 µl of buffer P2 (0.2N NaOH and 1% SDS) was added and immediately the tube was gently inverted 4-6 times to mix up the contents. After lysis of the cells 350 µl of buffer N3 (3M Sodium acetate, pH 4.8) was added and the tube was inverted immediately for 4-6 times. Then tube was centrifuged at 12000 rpm for 10 minutes to pellet down the cell debris. After centrifugation the supernatant was directly transferred to a QIA preparation column placed in a collecting tube and centrifuged for 1 minute at 10000 rpm. The column was then washed with 0.75 ml of buffer PE (70% alcohol). To remove the residual wash buffer PE, column fitted with the collection tube was centrifuged at 12000 rpm for 1 minute. Finally plasmid DNA was eluted from the column by adding 50 µl buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O to the center of QIA preparation column followed by brief centrifugation at 12000 rpm. The plasmid DNA was stored at –20°C until further use.

Isolation of plasmid DNA by Alkaline Lysis method

Mini preparations of plasmid DNA was carried out by the following procedures of Birnboim and Doly (1979), and Ish-Horowicz and Buker (1981) with slight modifications. A single bacterial colony carrying plasmid to be isolated was

inoculated into 3 ml of LB medium containing appropriate antibiotic and was incubated overnight at 37°C with vigorous shaking (150 rpm). 1 ml of overnight culture was centrifuged at 6000 rpm for 10 minutes and supernatant was discarded. Bacterial pellet was resuspended in 100 µl of ice-cold solution I (50mM glucose, 25mM Tris -Cl, pH 8.0; 10mM EDTA, pH 8.0) by vigorous vortexing. To the above bacterial suspension a 200 µl of freshly prepared solution II (0.2N NaOH, 1% SDS) was added and the contents were mixed by inverting the tube 5-6 times. Then 150 µl of ice-cold solution III (3M potassium acetate, pH 4.8) was added to the above bacterial lysate and mixed by inverting the tube 4-5 times. Then tube was kept on ice for 3-5 minutes. A white precipitate formed in the above mixture was removed by spinning the contents at 12000 rpm for 10 minutes. Then the supernatant was transferred into a fresh tube and extracted with equal volumes of phenol: chloroform mixture. The contents briefly mixed by vortexing were centrifuged at 12000 rpm for 10 minutes, to separate aqueous and organic layers. The volume of the aqueous phase was measured before transferring to a fresh tube. A 1/10th volume of 3 M sodium acetate and 2 volumes of absolute alcohol were added to the aqueous phase and the tubes were kept at –20°C for 30 minutes to facilitate precipitation of plasmid DNA. Then the tubes were centrifuged at 12000 rpm for 10 minutes at 4°C to pellet down the plasmid DNA. The DNA pellet was further washed with 70% ethanol to remove traces of salts associated with plasmid. Subsequently the plasmid DNA was dried before redissolving it in 50 □l of TE (pH 8.0) and stored at -20°C until further use.

Purification of DNA fragments from agarose gel

After performing agarose gel electrophoresis, appropriate portion of the gel containing desired DNA fragment was sliced, weighed and carefully taken into a micro centrifuge tube. Then 3 volumes (W/V) of buffer QG was added and incubated at 50°C for 10 min to dissolve the gel. After the gel slice is completely dissolved 1 volume (w/v) of isopropanol was added. Meanwhile the QIA quick column was placed in a collection tube and the contents were carefully transferred into the column. Immediately the column fitted in collection tube was placed in a microfuge and the contents were centrifuged at 12000 rpm for 1 minute. The flow-through was discarded and 0.75 ml of buffer PE (70% alcohol) was added to QIA quick column. The column was washed by briefly centrifuging the contents at 12000 rpm. The column was subjected to one more spinning to remove residual ethanol from buffer PE. The QIA quick column was then placed in a 1.5 ml eppendorff tube and the DNA was eluted in 50 µl of buffer EB (10µM Tris-Cl, pH 8.5) or H_2O. DNA sample was collected by centrifuging the tube for one minute and stored at –20°C until further use.

Sequencing of 16S rRNA gene

16S rRNA gene was sequenced by following Sanger's method (Sanger et al., 1977). When necessary internal primers were designed and used for getting complete 16S rRNA gene sequence.

Sequence analysis and construction of phylogenetic tree

The 16S rRNA gene sequence of length 1514 bp was submitted to the NCBI-BLAST search in order to know the nearest phylogenetic relatives to strain DS004.

EzTaxon server (Chun et al., 2007) is more useful for comparing unknown of 16S rRNA gene sequences with type strain 16S rRNA gene sequences. Based on the blast search results, type strain sequences of the closely related members and an out group sequence were obtained in fasta format from National Center for Biotechnology Information (NCBI) – Nucleotide search or from Ribosomal Database Project-II (RDP-II) Release 9.56 (Cole et al., 2009; www.rdp.cme.msu.edu/). The type strain numbers were either obtained from Bergey's Manual of Systematic Bacteriology (2005) or from List of prokaryotic names with standing in nomenclature (LPSN – http://www.bacterio.cict.fr/index.html). All the closely related type strain sequences along with an out-group sequence and the 16SrRNA gene sequence of strain DS004 were aligned using the CLUSTAL X program (Thompson *et al*., 1997). The alignment file was opened with Bio Edit software and the alignment was corrected manually and the file was saved with ".phy" as extension which served as an input file for the programs used for phylogenetic analysis.

Methods for phylogenetic tree construction

Distance and character based methods were used for phylogenetic analysis.

Distance based methods

Two distance based methods were used for phylogenetic tree construction; one is with neighbor joining and the other with Fitch.

Neighbor joining method

The evolutionary distance matrix was calculated using the distance model of Jukes and Cantor (1969). The evolutionary tree was constructed using the neighbor-

joining method (Saitou and Nei, 1987) and the resultant tree topologies were evaluated by bootstrap analysis based on 100-1000 resamplings, using the SEQBOOT and CONSENSE programs in the PHYLIP package.

Fitch method

The evolutionary distance matrix was calculated using the distance model of Jukes and Cantor (1969). The evolutionary tree was constructed using the Fitch-Margoliash Program (Fitch and Margoliash, 1967) and the resultant tree topologies were evaluated by bootstrap analysis based on 100 resamplings, using the SEQBOOT and CONSENSE programs in the PHYLIP package.

Character based methods

Maximum likelihood method in both phylip package and PhyMl were used for phylogenetic dendrogram construction.

Maximum likelihood method in Phylip package

The CLUSTAL X alignment file with ".phy" extension was used as the input file to the program SEQBOOT in the PHYLIP package (Felsenstein, 1993) and the output file of SEQBOOT was used as the input file for maximum likelihood (Felsenstein, 1981) analysis with 100 datasets and 5 times jumbling. One single tree was produced using 100 trees generated during maximum likelihood analysis using the program CONSENSE. A final dendrogram with evolutionary distances was constructed by taking the alignment file with ".phy" extension as the infile and the consensus tree as the intree in the maximum likelihood program of the PHYLIP package.

Maximum likelihood method in PhyML program

The dendrogram was constructed using PhyML (Guindon and Gascuel, 2003) program using 100 replicates of non parametric bootstrap analysis, GTR model of nucleotide substitution and 4 substitution rate categories.

2.20. DNA-DNA hybridization

DNA-DNA hybridization was performed by the membrane filter method (Tourova and Antonov, 1987) which essentially involves three steps

i. Immobilization of the DNA

ii. Radioactive labeling of DNA to be used as a probe

iii. Hybridization between the immobilized DNA and the radioactive DNA

Immobilization of DNA

DNA samples were prepared from the type strains and from DS004 by following the procedures described in the earlier sections of this chapter. After quantifying the DNA samples about 10 µg of DNA was taken in a microfuge tube and adequate amount of 20X SSC (3M Sodium chloride and 0.3 M Sodium citrate) was added to get a final concentration of 6X SSC. The contents were then boiled for 10 min and immediately chilled by placing the tube on ice. The sample was then immobilized on to a Hybond N^+ membrane using a dot blot apparatus. The wells were then washed with 100 µl of 0.5N NaOH after which the filter was removed, dried and the DNA sample absorbed on the membrane was cross linked with the help of UV cross linker.

Radioactive labeling of the DNA to be used as a probe

Labeling of the DNA was carried out using nick translation kit procured from M/s Fermentas, USA, following manufacturer's protocols. In a microfuge tube in a 100 µl reaction mix consisting 1 unit of klenow fragment, dNTP mix, 200 ng of DNA sample and 5 µl (50 µCi) α-^{32}P-dATP was taken in a microfuge tube and incubated at 37^{o}C for 30 minutes. After incubation the reaction was stopped with the addition of 8 µl of 0.25 M EDTA. Subsequently the labeled DNA was precipitated by adding 56 µl of 5M ammonium acetate, 50 µl of carrier DNA and 500µl of ice cold alcohol. The contents were then incubated at –70^{o}C for 1 h or over night at –20^{o}C and spun at 15000 rpm for 20 min at 4^{o}C. The DNA pellet was then washed with 70% alcohol and briefly dried under vacuum before dissolving in 6X SSC. The labeled DNA was then denatured for 10 min by placing in boiling water bath and immediately chilled on ice and used for hybridization.

Hybridization

The filters containing DNA was soaked in the prehybridization buffer (0.5 M Phosphate buffer pH 7.2 and 0.1% SDS) for 1 h at a 62^{o}C. Subsequently, the prehybridization buffer was discarded and the probe dissolved in the prehybridization buffer was added to the filter and hybridization was done for 16 h at the same temperature. The filter was then washed with 0.5 X SSC containing 0.1% SDS for 10 min at room temperature and then with 0.1X SSC containing 0.5% SDS for 20 min at 50^{o}C. Subsequently, the filters were dried and radioactivity found on each filter was determined using Tri-carb liquid scintillation counter (Model No. B1500, Zurich, Switzerland). The percent hybridization was calculated as follows:

$$\% \text{ Hybridization} = \frac{\text{Counts obtained from heterologous hybridization}}{\text{Counts obtained from homologous hybridization}} \times 100.$$

2.21. Extraction and characterization of catabolic intermediates of acephate

Preparation of samples for analysis in GC-MS

Pseudomonas jinjuensis biotype *acephalyticus* was grown to log phase in the minimal medium supplemented with acephate as sole source of carbon. Then the cells were removed from the medium by performing centrifugation at 8000 rpm for 10 minutes. The spent medium was taken in a clean conical flask before acidifying the medium by adding 1.5 ml of 6N HCl per 100 ml. After acidification the spent medium was extracted thrice with ethyl acetate. All the solvent fractions were pooled and dehydrated by the addition of solid sodium sulphate. After dehydration the solvent fractions were taken to a fume cup board and evaporated to dryness at room temperature. Finally the residue was dissolved in methanol and stored in -20^{o}C until further use. The minimal medium having acephate without cells served as the control.

GC-MS analysis

The isolated sample was then analysed in GCMS- QP 5050A (Shimadzu) chromatograph. The column (25 m x 0.2mm ID x 0. 33µ) used was packed with 100% SPB1, Supelco (Sigma). The column, injector port and detector temperatures were maintained at 210, 230 and 250^{o}C respectively. Helium gas was used as the carrier gas and the flow rate was maintained at 1ml/min. Initially the compounds extracted from the controls were analyzed. The data obtained on GCMS was taken as reference

for the identification of any new peaks found in the extracts prepared from the spent medium of acephate grown cultures.

Preparation of samples for analysis in LC-MS

Pseudomonas jinjuensis biotype *acephalyticus* was grown to log phase in the minimal medium supplemented with acephate (10 mM) as sole source of carbon. Then the cells were removed from the medium by performing centrifugation at 12, 000 rpm for 10 minutes. The cell pellet collected was thoroughly washed with citrate saline buffer and then resuspended in 1ml of milli Q water. The cell suspension was then used to make dilutions to get cell suspensions with different OD's of 0.1, 0.2, 0.5, 1.0 and 2.0. Filter sterilized acephate (10 mM) was added to each of these cell suspension before incubating at 30^{o}C for 15 minutes. After incubation about 100 µl of the sample was withdrawn from the tube and cells were removed by a brief centrifugation at 13000 rpm. The clear supernatant was directly loaded on LC-MS.

LC-MS analysis

The above prepared samples were injected by autosampler into Eclipse XDB-C18 column (5 X 4.6 X 150 mm) using a binary pump HPLC (Agilent 1200 series). An isocratic buffer system of 95% (v/v) water having 5% (v/v) acetonitrile was used at a flow rate of 1.0 ml min^{-1} for 20 min. The spectrum was recorded for 10 min at 220 nm in the UV range and part of the elute was diverted to ESI source of Q-TOF (Bruker Daltonics). Mass spectra were acquired in positive mode with collision energy 5 eV, capillary temperature 200^{o}C and a source voltage of 3.8 kV. Mass was acquired in the *m/z* range of 50 –1000. The data was averaged for each peak and smoothed using Gaussian algorithm. Calibration of the instrument was done using ES

Tunning mix (Agilent technologies). MS/MS of the base peaks obtained at at 6.0, 4.2 and 2.7 min were done in MRM mode with 10–15 eV collision energy

2.22. Construction of expression plasmids

Expression vector pMMB206 (Morales et al., 1991) is used as source plasmid to construct expression plasmid pMMBKM used for expressing *opd* gene in *P.jinjuensis* biotype *acephalyticus*. The chloramphenicol resistance gene is replaced with Km gene to facilitate its selection in Chloramphenicol resistant *P.jinjuensis* biotype *acephalyticus*. The *opd* gene and its variants coding preOPH and mOPH were taken from the expression plasmids described elsewhere (Siddavattam et al., 2006; Pandey et al., 2009) through PCR amplification and cloned in pMMBKM as *Eco*RI and *Bam*HI fragments.

2.23. Expression of OPH in *P.jinjuensis* biotype *acephalyticus*

Single colonies of *P.jinjuensis* biotype *acephalyticus* containing expression plasmids (pHLCKMOPD and pHNSKMOPD) were inoculated in 3 ml of LB medium and grown for overnight with appropriate antibiotics. The overnight culture (1%) was used as inoculum to get 10ml (LB medium) of mid log phase culture. The culture was then allowed to grow until the cell density reached to 0.5 OD units at 600 nm. The log phase culture was then induced with 1mM IPTG and the culture was allowed to grow for a further period of 3 hrs to achieve maximum expression of OPH. The control cultures having expression vector pMMB206 was maintained under identical conditions and used them as negative controls. After induction, the cells were pelleted and the pellet was suspended in sample buffer (62 mM Tris-Cl (pH 6.8); 2% SDS; 10% glycerol; 5% β-mercaptoethanol; 0.01% bromophenol blue). Protein extracts

were boiled in water bath for 5 minutes and samples were briefly centrifuged. Then the proteins obtained in clear supernatant were analyzed on 12.5% SDS-PAGE.

2.24. Sub-cellular localization of OPH in *P. jinjuensis* biotype *acephalyticus* DS004

Samples from 10 ml cultures were withdrawn at different time points and the OPH activity was assayed with Methyl Parathion as substrate, following standard procedures (Chaudhry et al, 1988). The cultures that demonstrated good OPH activity were used for preparing sub-cellular fractions. In the preparation of sub-cellular fractions initially the cells were harvested at 6500 rpm for 10 min. These cells were resuspended in adequate amount of phosphate buffer pH 7.2. The cell suspensions thus prepared were sonicated (35 amplitude, pulse on for 15 sec and off for 45 sec (one cycle), total no. of cycles 10, temperature 4^{o}C). After sonication the sample centrifuged at 10,000 rpm for 30 min. The unbroken cells and cell debris was removed as pellet fraction where as the supernatant was collected for preparing sub-cellular fractions. Then supernatant fraction was centrifuged in a ultracentrifuge at 45,000 rpm for 1 h. After centrifugation the supernatant was collected in a clean tube and named it as cytosolic fraction. The pellet collected at the bottom of the tube was treated as membrane fraction. These sub-cellular fractions were used for assaying OPH activity and to perform western blots analysis for detection of OPH (Gorla et al, 2009).

2.25. Protein estimation

Protein content of the bacterial lysate as well as various sub-cellular fractions was estimated by the following procedure described by Lowry *et al*.,

(1951). In a clean test tube 100 µl of sample was taken and mixed with 900µl of water and 5 ml of alkaline solution (2% Na_2CO_3 and 0.5 g copper sulphate in 50:1 ratio). Then the contents were thoroughly mixed before allowing to stand at room temperature for 10 min. Immediately after incubation 0.5 ml of Folin-Ciocalteau reagent (diluted with distilled water in 1:1 ratio before use) was added and thoroughly mixed before allowing them to stand for 30 min at room temperature. After incubation blue color developed was measured at 750 nm. A solution containing 5 ml of alkaline solution and 0.5 ml of Folin-Ciocalteau reagent served as blank. Known concentrations of bovine serum albumin was used for preparation of protein standards.

2.26. Parathion Hydrolase Assay

A spectrophotometric assay described elsewhere was adapted to determine organophosphate hydrolase (OPH) activity (Chaudhry et al., 1988). The reaction was carried out in the test tubes containing 100 µmol of methyl parathion in 1 ml of 50 mM Tris glycine buffer (pH 9.0). The reaction was started by adding one of the components such as crude extract, cytoplasmic and membrane fractions as source of OPH. The tubes were incubated at 37°C for 60 minutes. An increase in the absorbance at A_{410}nm due to formation of *p*-nitrophenol was determined (Chaudhry et al, 1988). The concentration of *p*-nitrophenol formed in the reaction was determined using the extinction coefficient of PNP (16500 M^{-1} Cm^{-1}). The specific activity of the enzyme was expressed as micromoles of PNP produced per milligram of protein per minute. The specific activity of the enzyme was calculated using the formula given below.

$$\text{Enzyme activity} = \frac{\text{Absorbance at 410nm}}{\text{16,500 (Extinction coefficient of PNP at 410nm)}}$$

Specific activity = Enzyme activity / mg of protein / min

Results and Discussion

Chapter I

In the introductory chapter the reasons for persistence of acephate in the environment and need to study biodegradation of acephate has been highlighted. The present chapter is entirely devoted on isolation and characterization of acephate mineralizing bacteria from the activated sludge collected from a pesticide manufacturing unit, Rallis India Ltd., Hyderabad. Acephate (*O, S*-dimethyl acetyl phosphoramidothioic acid), like other organophosphates is extensively used to control various insect pests (Marquis, 1986; Aspelin, 1994).

After reporting isolation of methyl parathion degrading *Flavobacterium* sp. from rice fields of IRRI a number of attempts have been made to isolate bacterial strains capable of degrading organophosphorus compounds from diversified environments (Graetz et al., 1970; Sethunathan and Yoshida, 1973; Rao and Sethunathan, 1974; Daughton and Hsieh, 1977; Barles et al., 1979; Somara and Siddavattam, 1995; Zhongli et al., 2001; Horne et al., 2002; Pakala et al., 2007; Aleem et al., 2010 (manuscript submitted)). While isolating the bacterial strains capable of degrading structurally diverse group of op compounds the investigators have essentially used enrichment culture techniques using environmental samples as source of microorganisms. In this study activated sludge is used as microbial source to isolate a bacterium capable of mineralizing acephate.

3.1 Isolation of acephate mineralizing bacterium from activated sludge

The activated charcoal is mainly used to trap residues of insecticides while treating effluents generated from the pesticide manufacturing units. Once activated charcoal is saturated with the residues of insecticides and other chemicals used while manufacturing insecticides, it is collected and stored for prolonged periods before

disposal. This material, otherwise known as activated sludge, due to existence of organic waste is a rich source for microbes contributing for biodegradation of toxic and recalcitrant compounds. The present study has exploited activated sludge for isolation of bacterial strains capable of mineralizing acephate residues. Initially the minimal salts medium (M9 medium) supplemented with acephate as carbon source was added with one gram of activated sludge and enrichment of acephate mineralizing bacteria was performed as described in materials and methods. While performing enrichment culture technique every alternative day about one ml of clear culture media was collected and an appropriate dilution of the medium was plated both on LB and M9 plates containing acephate as carbon source. A portion of the clear culture medium was also used to reinoculate fresh M9 medium having acephate as carbon source. The process was repeated for at least 30 days.

In the first transfer a very few colonies were seen on acephate plates and all of them have shown uniform colony morphology. However, the colonies appeared on LB plates have shown variations in colonies size and morphology. In subsequent transfers the colony count on acephate plate has gone up. Even the morphological diversity of colonies noticed on LB plates during first transfer has disappeared in subsequent transfers. In the final transfer most of the non-specific colonies have disappeared and pure colonies with identical morphology and color were found both on acephate and LB plates. The bacteria that failed to use acephate as source of carbon slowly disappeared during the process of enrichment and pure white bulky colonies got enriched on acephate and LB plates. These pure colonies were further propagated on acephate plates to ensure the purity of acephate utilizing bacterium. This bacterium thus obtained was designated as strain DS004.

3.2 Substrate tolerance

After obtaining pure culture through enrichment culture technique, the strains ability to tolerate high concentrations of acephate was tested. While assessing its tolerance to various concentrations of acephate, minimal salts medium was supplemented with different concentrations of acephate i.e. 0.5 mM, 1 mM, 10 mM, 20 mM, 50 mM, 60 mM, 80 mM and 100 mM and an appropriate amounts of overnight cultures of the strain was inoculated. The growth of strain DS004 was frequently monitored by recording the culture turbidity at OD600. As shown in Fig. 3. 1, the culture has grown without any hindrance in all flasks that contain either 50 mM or less than 50 mM acephate.

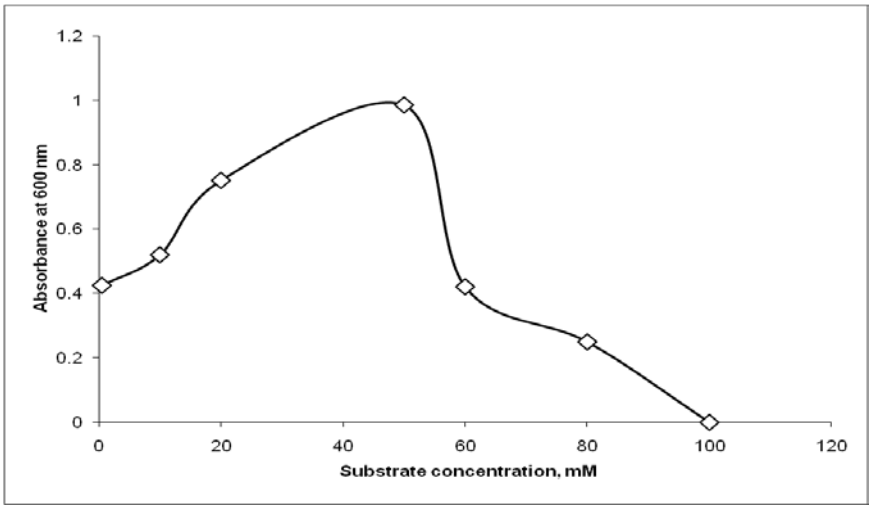


Fig. 3.1 Growth of strain DS004 at different concentrations of acephate.

However, when acephate concentration is increased beyond 50 mM growth got steadily decreased and finally at 100 mM concentration no growth was recorded even after incubating the culture for 48 hours (Fig.3.1).

As optimal growth of strain DS004 was observed in 10 mM acephate all further studies pertaining to establishment of growth behavior of the strain were carried out in minimal medium containing 10 mM acephate. The strain DS004 reached to late log phase after 12 hours of incubation and no further growth was observed even after incubating the culture for 24 hours and thus indicating the existence of cells in stationery phase. Due to existence of characteristic amide bond, acephate shows maximum absorption at 220nm. Therefore, while recording the growth of the culture, the acephate concentration was also recorded in the culture medium by monitoring the absorbance at 220. As shown in Fig. 3. 2, with the increase in culture OD, a concomitant decrease of acephate concentration is seen in culture medium suggesting that strain DS004 is using acephate for its growth (Fig.3.2).

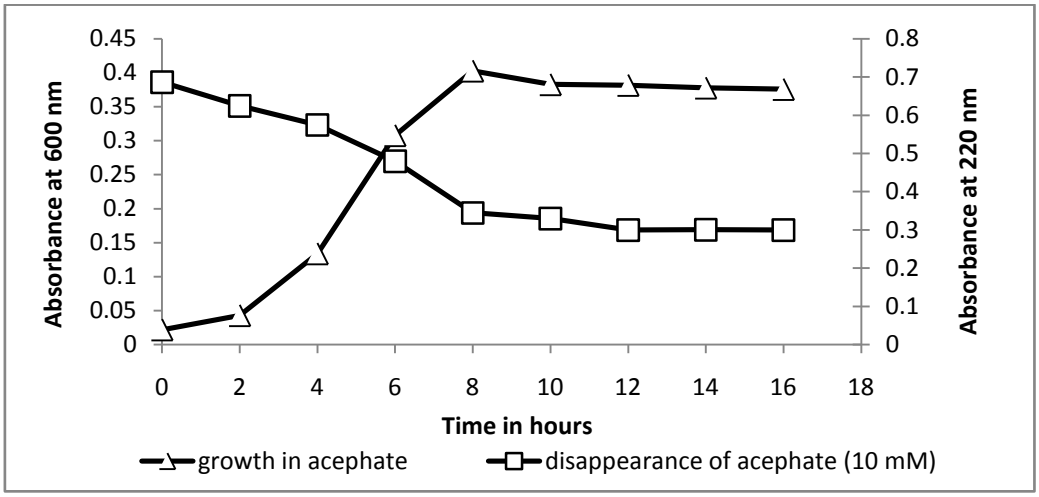


Fig. 3.2 Graph showing inverse relationship between growth of the strain DS004 and disappearance of acephate.

3.3 Usage of acephate as source of nitrogen and sulfur

Acephate having molecular formula ($C_4H_{10}NO_3PS$) can also serve as source of nitrogen and sulfur. As its ability to use acephate as source of carbon is established

further studies were conducted to test if acephate can serve as source of nitrogen and sulfur. As described in materials and methods sections nitrogen and sulfur free minimal salts medium was prepared and strain DS004 was inoculated in nitrogen and sulfur free media. Interestingly in both nitrogen and sulfur free minimal medium the strain DS004 has grown and culture has reached to late log phase after 12 hours of incubation. Further incubation has recorded no growth indicating the existence of cells in stationery phase. This clearly shows the ability of the strain DS004 in using acephate as nitrogen and sulfur source (Fig.3.3)

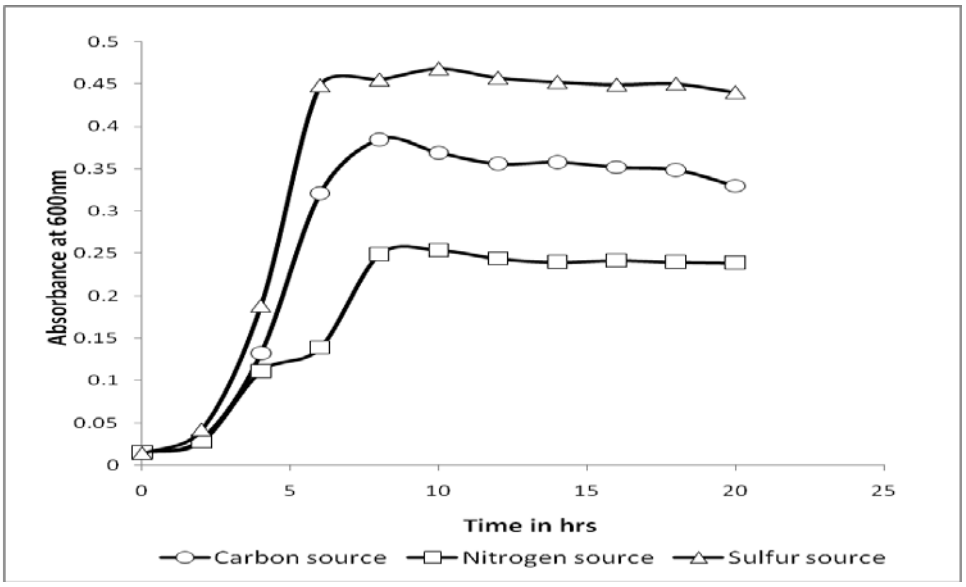


Fig. 3.3 Growth curves of strain DS004 by utilizing acephate as carbon, nitrogen and sulfur source.

3.4. Taxonomic characterization of strain DS004

A number of biochemical and microbiological tools are available for establishing the taxonomy of new microbial isolates. In the present study the taxonomic identity of strain DS004 is established by using both conventional, biochemical and modern molecular biological tools. Here results obtained from all the

sources are presented in a sequential manner to establish a clear view on the taxonomic status of DS004.

3.41 Morphological Characterization

The microscopic examination of acephate degrading bacterium was done using Phase contrast, Scanning and Transmission electron microscopy. Under phase contrast microscope, the acephate degrading bacterium appeared as rod shaped, motile bacterium (Fig.3.41 A). However, under scanning electron microscopy, strain DS004 appeared as a rod shaped bacterium with 1.3 µm long and 0.47 µm width (Fig. 3.41 B). A number of cells were found to be doublets suggesting that cells are dividing via binary fission. After gaining preliminary insights on the morphology of DS004, a single cell was taken for a detailed examination under transmission electron microscopy. As shown in Fig. 3.41 C strain DS004 is seen as a rod shaped bacterium with a single polar flagellum (Fig.3.41 C). Such observation is supportive of observing motile bacteria under scanning electron microscope.

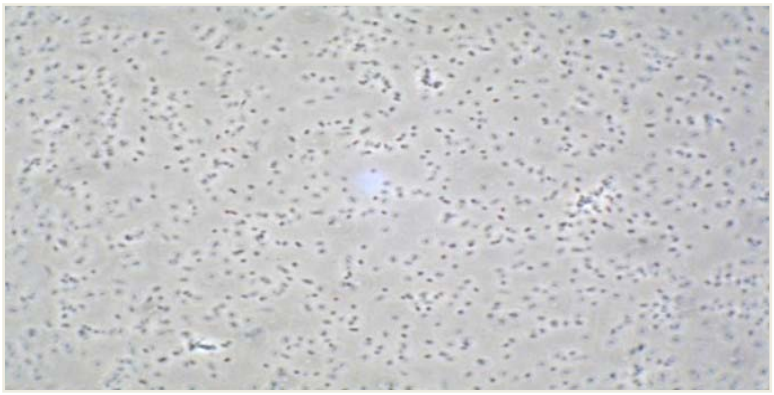


Fig. 3.41 A Phase contrast micrograph of strain DS004.

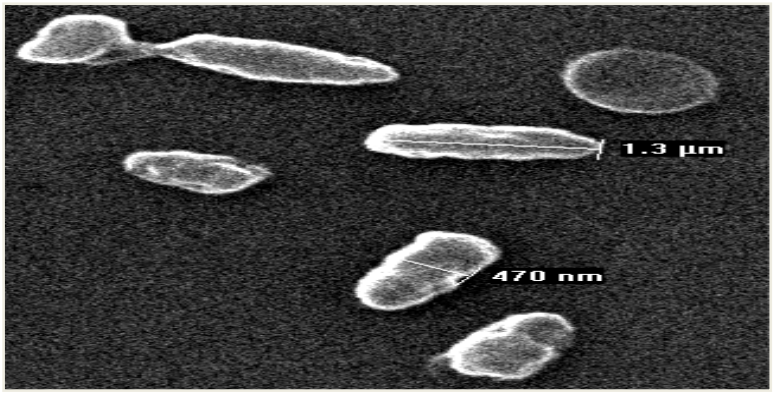


Fig. 3.41 B Scanning electron micrograph of strain DS004.

Fig. 3.41 C Transmission electron micrograph of strain DS004.

The characteristic features of DS004 obtained by using various microscopic techniques are shown in table 3. 1.

Table 3.1: Morphological characteristics of strain DS004.

Morphology	Result
Size	1.5-2.0 µm long and 0.7-0.9 µm wide
Shape	Round
Arrangement	Single
Motility	Polar flagellum
Gram staining	Gram negative

3.42 Biochemical properties of strain DS004

After studying the morphological properties, the strain DS004 was used to conduct number of biochemical studies as described in materials and methods section. The results obtained in these studies are listed in table 3. 2. These properties of strain DS004 were used to compare with similar biochemical properties of type strains that showed more than 97 % 16S rRNA gene identity to have a better taxonomic understanding.

Table 3.2: Biochemical Properties of strain DS004.

Carbon source	**Result**
Glucose	+
L-glutamic acid	+
Sodium citrate	+
Potassium sodium tartrate	-
Potassium gluconate	+
DL-Malate	+
Succinic acid	+
Salicylic acid	-
Anthranilate	-
Benzoic acid	+
p-Aminobenzoic acid	+
Acephate	+
Acetamide	+
Methanol	+
Acid production from sugars	

Dextrose	-
Maltose	-
Lactose	-
Galactose	-
Xylose	-
Mannitol	-
Dulcitol	-
Cellobiose	-
Inositol	-
Trehalose	-
Gas production from sugars	
Dextrose	-
Maltose	-
Lactose	+
Galactose	-
Xylose	-
Mannitol	-
Dulcitol	+
Cellobiose	+
Inositol	-
Trehalose	-
Starch hydrolysis	-
Gelatin liquefaction	+
Nitrate Reduction	+
Susceptability to Antibiotics	
Ampicillin	+
Chloramphenicol	+

Kanamycin	-
Tetracycline	+
Gentamycin	+
Streptomycin	+
Spectinomycin	+

3.43 Description of acephate degrading bacterium strain DS004

Based on the morphological and biochemical examination, the cells of strain DS004 can be described as aerobic, gram-negative rods having 1.5-2.0 μm long and 0.7-0.9 μm wide. They are motile due to existence of a single polar flagellum. They are propagated through binary fission and occur in pairs. The cells are capable of growing on acephate as sole source of carbon, nitrogen and sulfur at the temperatures ranging 25^{o}C – 37^{o}C and pH between pH 7.0 - 8.0, while showing optimum growth at 30^{o}C and pH 7. 0. It doesn't require any vitamin supplements for growth. The strain DS004 successfully hydrolyzed gelatin and failed to hydrolyze starch. Glucose, citrate, malate, gluconate, L-glutamic acid, succinic acid, benzoate, *p*-aminobenzoic acid served as good carbon/energy sources for strain DS004. However, growth is not supported by salicylate, anthranilate and tartrate. Acid is not produced from sugars like glucose, galactose, xylose, maltose, lactose, mannitol, dulcitol, cellobiose, inositol, and trehalose. Gas production was observed from lactose, dulcitol and cellobiose but not from dextrose, galactose, xylose, maltose, mannitol, inositol and trehalose. Nitrate reduction was found.

If the strain DS004 morphological characteristics, such as shape, motility with single polar flagellum and physiological characteristics like recording growth under aerobic conditions at 30^{o}C temperature and pH 7.0 – 8.0, are seen together with biochemical characteristics like absence of gas formation from glucose and using citrate as carbon source, which are considered to be the key characteristics of genus *Pseudomonas*, a place for strain DS004 in genus *Pseudomonads* is justifiable. However, to reinforce the claim a mass spectrometric analysis was done to identify the *Pseudomonas* genus specific surface peptides in strain DS004.

3.5 Analysis of cell surface peptides

Biochemical and morphological tests are to some extent useful for establishing strains genus identity. However, most of the times they provide misleading information creating confusion while establishing taxonomic status of the microbial isolates. Therefore to strengthen our claim on the taxonomic status of the strain DS004, a mass finger print profile was generated for its surface peptides. Mass finger print profiles of cell surface peptides are extensively used in taxonomic characterization of new bacterial isolates (Karas et al., 1987). MALDI-TOFMS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) is a soft ionization method allowing desorption of intact peptides (Dai et al., 1999) and proteins from whole bacterial cells (Holland et al., 1996; Krishnamurthy et al., 1996; Easterling et al., 1998; Wang et al., 1998; Arnold et al., 1999; Holland et al., 1999; Lynn et al., 1999; Evason et al., 2001; Ryzhov and Fenselau, 2001) or cell fractions without extensive separation (Chong et al., 1997; Nilsson 1999). The advantages of MALDI-TOF MS include high sensitivity, speed and reproducibility (Saenz et al.,

1999). Different investigations have shown application of MALDI-TOF/TOF MS for characterizing intact bacterial cells (Saenz et al., 1999), bacterial spores (Ryzhov et al., 2000; Birmingham et al., 1999; Demirev et al., 2001; Elhanany et al., 2001) and other microorganisms such as viruses (Thomas et al., 1998; Birmingham et al., 1999) and fungi (Welham et al., 2000). The identification is based on the existence of a characteristic peaks considered as genus, species, or strain specific biomarkers. For instance, in *Escherichia coli*, two species specific biomarkers (at m/z 9226±3 and 9537±2) and two strain-specific biomarkers for ATCC11775 (at m/z 9065±2 and 9736±3) were identified (Ruelle et al., 2004). To confirm the genus identity of strain DS004, its cell surface peptides were extracted and analyzed on MALDI-TOFMS as described in materials and methods section. After establishing the mass profile, it was compared with similar profiles generated for *Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* PAO11610 that were used as reference strains. In accordance with the reported data, in mass fingerprints of reference strain *E.coli* ATCC 11775 a characteristic mass peaks at m/z value 9067 was obtained (Fig.3.5A), suggesting that the technique used to characterize the strain DS004 is appropriate and is in consistent with earlier reports (Ruelle et al, 2004, Nicolas, 2008). Similarly, the surface peptide finger print profile generated for another reference strain *P. aeruginosa* has also shown the mass ions corresponding to m/z value 4544, 5212, 6363, 6912, 7546, 8509 and 9090 (Fig.3.5B). The mass spectrum is in total agreement with the reported mass profile generated for the surface peptides of reference strain *Pseudomonas aeruginosa* PAO11610 (Nicolas, 2008).

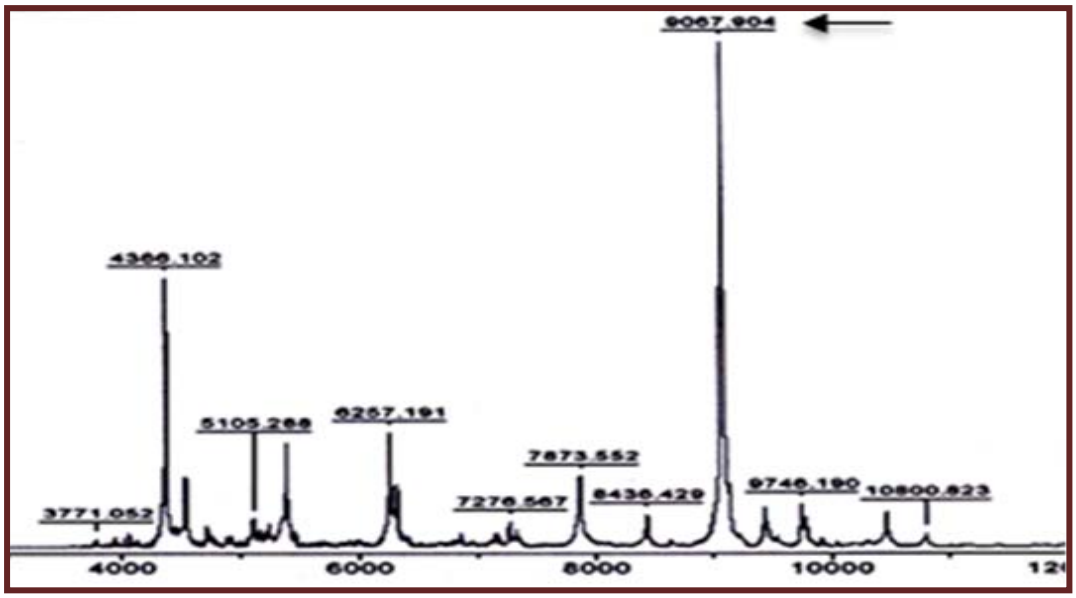


Fig. 3.5 A Mass spectrum showing the *E.coli* genus specific peak in reference strain *E.coli* ATCC1775.

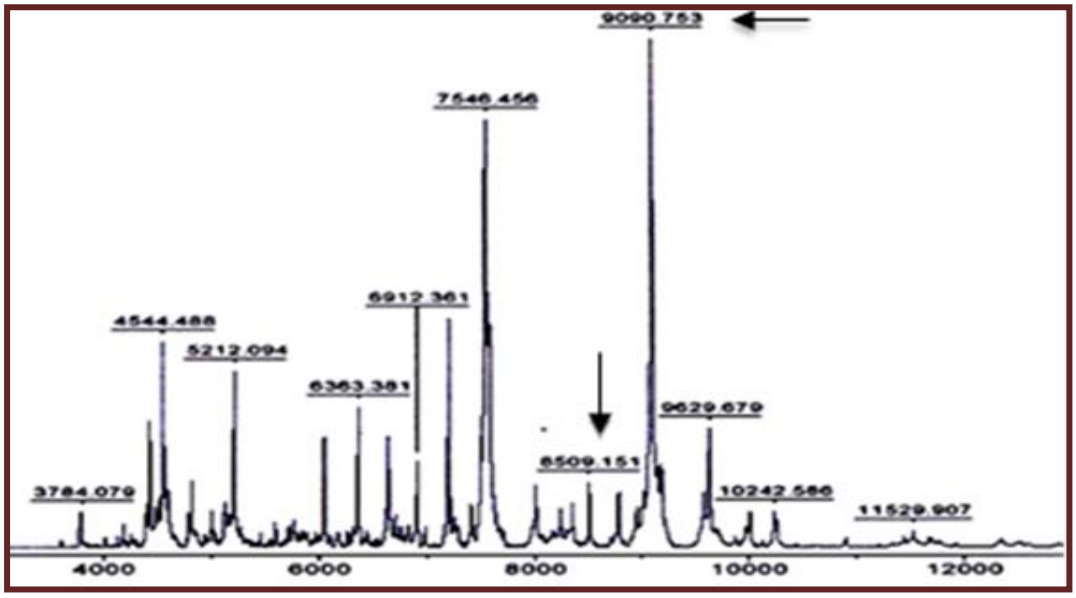


Fig. 3.5 B Mass spectrum of *Pseudomonas aeruginosa* PAO11610 surface peptides. *Pseudomonas* genus specific peaks are shown with arrows.

After establishing the validity of the methodology, the surface peptides of strain DS004 were extracted as described in materials and methods section and mass profile was established. As presented in Fig. 3.5 C, the mass profile has indicated existence of mass ions corresponding to m/z values of 4544, 5210, 6633, 7543, 8508,

and 9088 (Fig.3.5C). All of them when compared perfectly match with the mass profile generated to the surface peptides of reference strain *Pseudomonas aeruginosa* PAO11610. This result clearly establishes belongingness of strain DS004 to genus *Pseudomonas*.

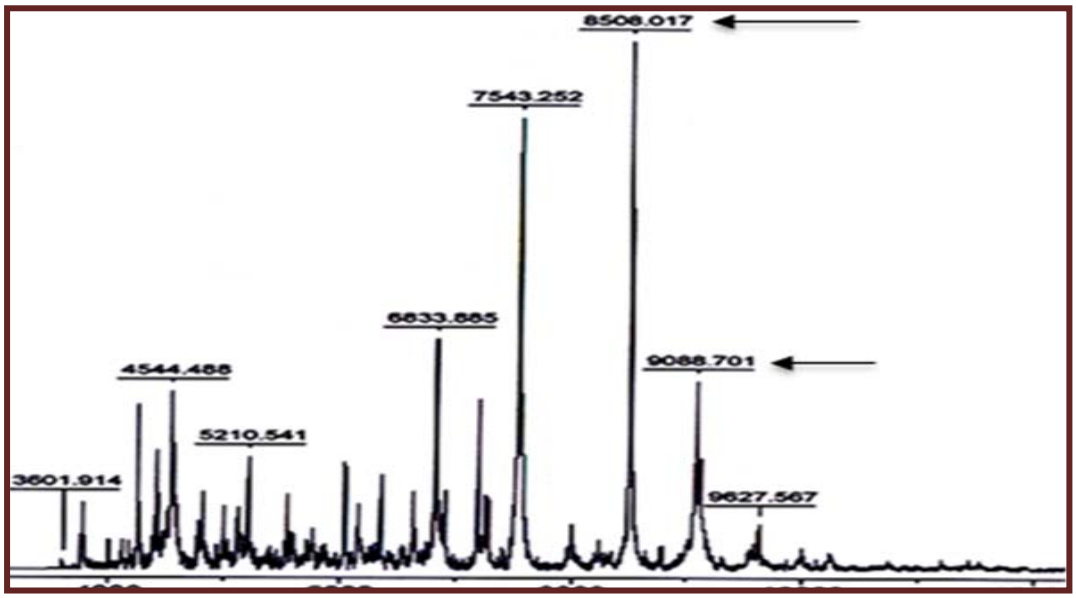


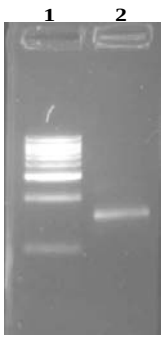
Fig. 3.5 C Mass spectrum of strain DS004 surface peptides. The mass peaks that match with reference strain *Pseudomonas aeruginosa* PAO116100 are shown with arrow marks.

Genus *Pseudomonas* contains number of species that use rare and recalcitrant carbon compounds as source of carbon and energy (Benzet and Matsumara, 1973; Kilbane et al., 1982; Karns et al., 1982; Lillis et al., 1983; Bachmann et al., 1988; Sahu et al., 1990; Chapalamadugu and Chaudhry, 1993). In fact a number of strains belonging to genus *Pseudomonas* have been shown to degrade op pesticides successfully (Doughton and Hsieh, 1967; Boush and Matsumura, 1967; Gibson and Brown, 1974; Mohapatra and Awasthi, 1977; Serdar et al., 1982; Chapalamadugu and Chaudhry, 1993). Although the aforementioned techniques have helped to establish the genus identity, they failed to provide accurate taxonomic position to strain DS004. In order to overcome this problem, and to establish an authentic taxonomic position to

DS004, an attempt is made to generate phylogenetic tree based on 16S rRNA gene sequence.

3.6 Amplification of 16S rRNA gene from strain DS004

In order to amplify 16S rRNA gene, the genomic DNA from strain DS004 was isolated using the procedures described in materials and methods section. After purification of the genomic DNA, the concentration of the DNA was measured and approximately 100 ρmols of DNA was used as template for amplification of 16S rRNA. Taking published results in to consideration an universal forward primer 5'-CGggatccAGAGTTTGATCCTGGCTCAGAACGAACGCT-3' and a reverse primer 5′-CGggatccTACGGCTACCTTGTTACGACTTCACCCC-3', corresponding to positions 8 to 37 and 1479 to 1506 of *E. coli* 16S RNA gene, were designed and used for amplification of 16S RNA gene of strain DS004 (Chen *et al.*, 1997). These primers are designed with restriction site *BamHI* to facilitate cloning (Chen *et al.*, 1997). The 16S rRNA gene amplified from strain DS004 was found to be1.5 Kb and is well within the limits of standard 16SrRNA gene size and hence was used for further studies (Fig.3.6).



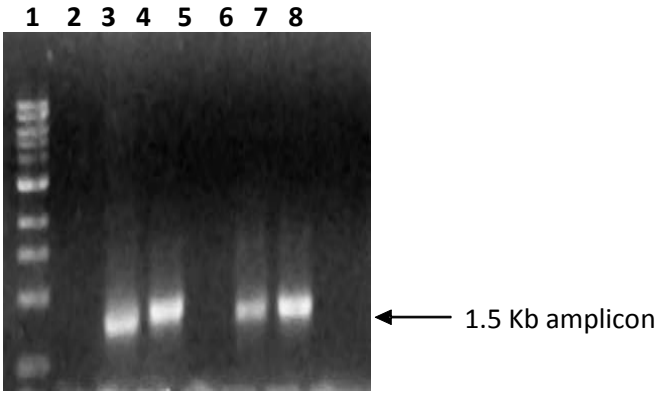
Lane1 represents 1 Kb DNA ladder; Lane2 represents 1.5 Kb amplified 16S rRNA gene

Fig. 3.6. Amplification of 16S rRNA gene from strain DS004.

As size of the PCR product is equal to the size of the 16S rRNA gene reported from number of bacterial strains it was assumed that the PCR amplified product is 16S rRNA gene of strain DS004 and attempted to clone and generate sequence of the PCR product (Rotthauwe et al., 1995; James and Russel, 1996; Khadhair and Evans, 2000; Muraji and Nakahara, 2001).

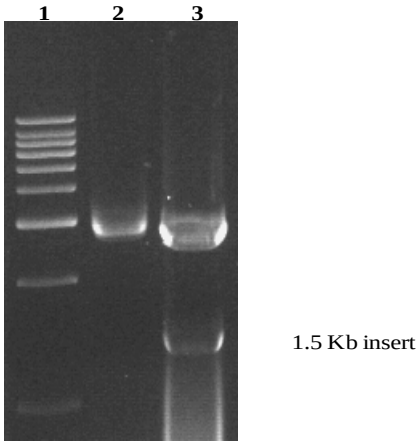
3.7 Cloning of 16S rRNA gene in pGEM-T Easy vector

The amplicon assumed to be the 16S rRNA of DS004 was cloned in pGEM-T Easy vector (Robles and Doers, 1994). As this vector is having 3´-T overhangs at the 3' end, it facilitates ligation of a PCR product. The ligation was carried out by following the procedures described in materials and methods section. The colonies having recombinant DNA were selected on LB agar plates having Amp + X-gal + IPTG following general blue white selection procedure. In order to identify the clone having 16S rRNA gene, few white colonies were selected to perform colony PCR using universal M13 forward primer 5´-GGAAACAGCTATGACCAT-´3 and reverse primer 5´- GTAAAACGACGGCCAGT-´3. The colonies that gave 1.5 Kb amplicon were selected to isolate plasmid and to reconfirm existence of 16S rRNA gene. (Fig.3.7). The colonies that gave positive results with colony PCR were sub cultured and the recombinant plasmid DNA was isolated by alkaline lysis method. The isolated plasmid was further digested with *Bam*HI and analyzed on 0. 8% agarose gels. The plasmid that gave 1.5 Kb insert (Fig.3.8), which was similar in size with PCR product, was selected and designated as pAS1and used for further sequencing.



Lane 1 represents 1 Kb DNA ladder; lanes 2 to 8 represent colonies screened by colony PCR using M13 forward and reverse primers

Fig. 3.7 Colony PCR for identification of clones with recombinant plasmid.



Lane1 represents1Kb DNA ladder, lane 2 represents Uncut pAS1 plasmid, lane 3 represents pAS1 plasmid cut with *Bam*HI restriction enzyme

Fig. 3.8 Agarose gel showing the presence of 1. 5 Kb 16SrRNA gene in plasmid pAS1.

3.8 16S rRNA gene sequencing

In order to generate complete sequence of the 1.5 Kb 16S rRNA gene from strain DS004, the plasmid pAS1 was sequenced using M13 universal forward and reverse primers. A 523 base pair long sequence was obtained with forward primer and a 516 base pair long sequence was obtained with reverse primer. As the insert to be sequenced is 1.5 Kb there was obviously no overlap in the sequence. Therefore, a 20

mer internal primer was designed based on the sequence generated with forward primer. Using this internal primer (5' -TCCAGCCATGCCGCGTGTGT- 3'), designated as AS003, an additional 600 base pair long sequence was generated, which overlapped with the sequence generated using the M13 reverse primer. A clear strategy used to sequence entire 1. 5 Kb sequence is shown in (Fig.3.9) and the complete 16S rRNA sequence of strain DS004 shown in Fig.3.10 is deposited in the GenBank/EMBL/DDBJ with an accession number AM 407893.

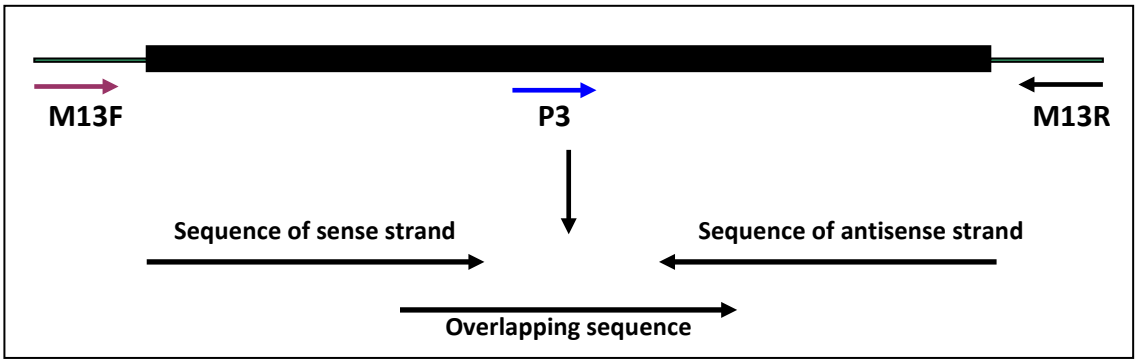


Fig. 3.9 Strategy used for obtaining complete sequence of 16S rRNA gene of strain DS004. Filled bar indicates 16S rRNA gene. Vector sequences corresponding to the M13 forward and reverse primer binding sites are shown with thick lines. Sequence obtained using various primers are shown with thick arrows.

GAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAGTGGAGCTTGCTCCATGATTC
AGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACC
AAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTT
GGGAGGAAGGACAGTAAGTTAATACCTTgGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGGTTTGGTAAGATGGATGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATCCATAACTGCCTGACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG
TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATA
AGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCCGGAATCTTGCAGAGATGCGAGAGTGCCTTCGGGAATCGGAACA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCA
GCACGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG
GCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGT
CCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGCCACGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAG
TGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGATCCCGAATC

Fig. 3.10 Complete sequence of 16S rRNA gene of strain DS004.

3.9 Construction of Phylogenetic tree

Phylogenetic analysis based on 16S rRNA gene sequence has been recognized as the most important method for inferring relationships of the genus *Pseudomonas* (Moore et al., 1996; Anzai et al., 1997, 2000). Therefore, the 1514 bp long 16S rRNA gene sequence of strain DS004 was compared with corresponding sequences of closely related species and other reference microorganisms retrieved from the EMBL database. Bacterial strains which have showed highest similarity to the 16S rRNA gene sequence of strain DS004 were taken into consideration for further studies. Among these sequences only the sequences of type strains were selected instead of taking the sequences of uncultured bacterial strains and strains approved by the local culture collection centres (Table.3.3).

Table 3.3: List of type strains that have showed highest similarity with the 16S rRNA gene sequence of strain DS004

Isolate	**Organism**	**Identity**	**Accession No.**
Strain DS004	*Pseudomonas azelaica* DSM 9128^T	98.7	AM088475
	Pseudomonas multiresinivorans ATCC 700690^T	98.6	X96787
	Pseudomonas nitroreducens DSM 14399^T	98.5	AM088474
	Pseudomonas jinjuensis LMG 21316^T	98.2	AF468448
	Pseudomonas knackmussii DSM 6978^T	97.9	AF039489
	Pseudomonas citranellolis DSM 50332^T	97.7	Z76659
	Pseudomonas alcaligenes ATCC 12815^T	96.4	AF09472
	Pseudomonas aeruginosa DSM 50071^T	96.1	Z76672
	Pseudomonas pseudoalcaligenes DSM 50018^T	95.6	Z76675
	Pseudomonas alcaliphila DSM 17744^T	95.5	AJ550466
	Pseudomonas oleovorans DSM 1045^T	94.8	Z76665

Reinforcing the data obtained from biochemical and morphological studies, strain DS004 exhibited highest level of 16S rRNA gene sequence similarity (95 – 99%) to strains of the most closely related valid or invalid *Pseudomonas* species. However, as stated before the type strains, available in authentic culture collection centre's (DSMZ, Germany;) were taken and aligned using CLUSTALW (www.ebi.ac.uk/tools/clustalw2) to obtain percentage Score values. These scores were used as input for MEGA 4 software (www.megasoftware.net) while constructing phylogenetic tree (Fig.3.11). As shown in phylogram the strain DS004 has shown highest relationship to the type strain *Pseudomonas jinjuensis* LMG 21316^T (Fig.3.11).

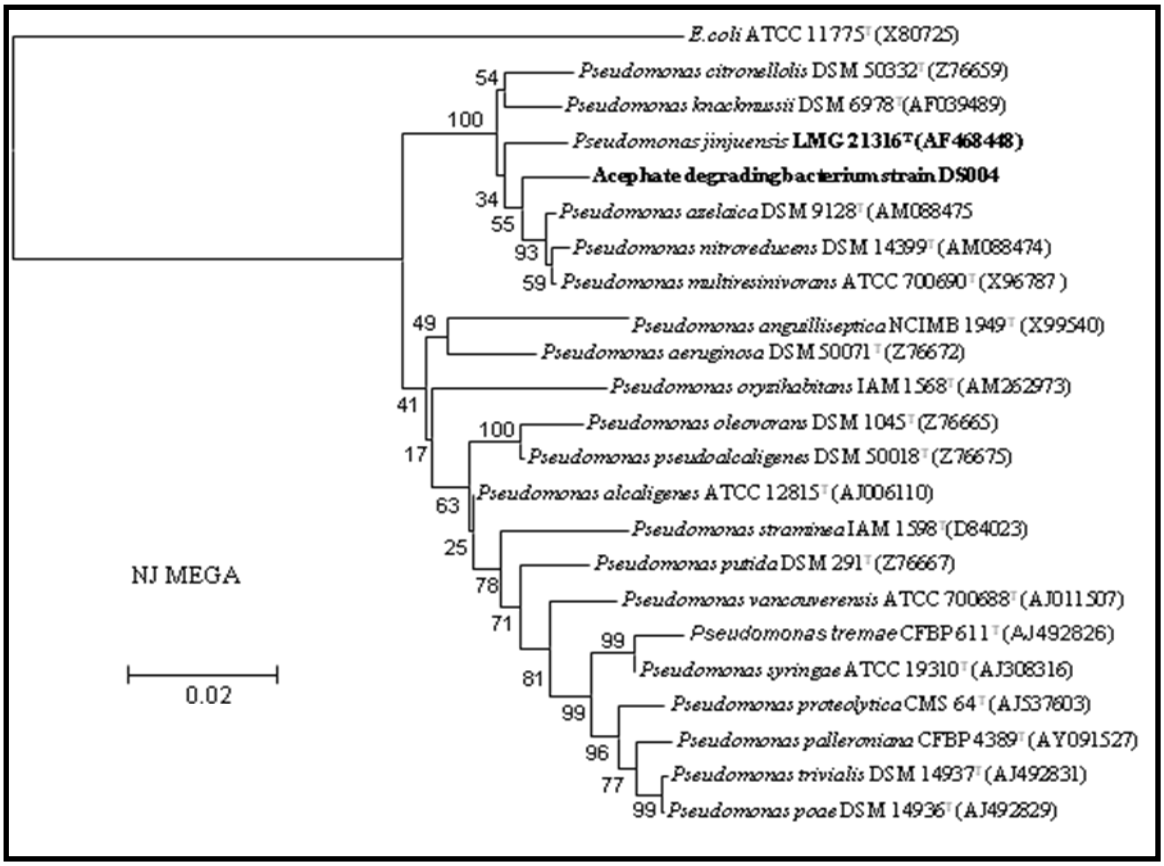


Fig. 3.11 Phylogenetic tree of strain DS004 constructed using 16S rRNA gene sequence.

The phylogenetic tree is in total agreement with the findings of Anzai and his associates who kept type strains *P. jinjuensis* LMG 21316^T, *P. azelaica* DSM 9128^T, *P. knackmussii* DSM 6978^T, *P. multiresinivorans* ATCC 700690^T, *P. citronellolis* DSM 50332^T, and *P. nitroreducens* IAM 1439^T, as one cluster designating them as '*P. aeruginosa group*' (Anzai et al., 2000) which belong to class *Gammaproteobacteria*.

Though not a critical tool, the level of 97% 16S rRNA gene sequence similarity is shown to be a threshold for the species delineation of bacteria (Stack Brandt & Goebel, 1994). However, as an exception *Pseudomonas koorensis* which has 99% 16S rRNA gene similarity with *Pseudomonas pavonaceae* is shown as a novel species (Kwon et al., 2003). Therefore, species delineation within the genus *Pseudomonas* cannot be assessed solely on the basis of 16S rRNA gene sequence similarity (Achouak et al., 2000; Andersen et al., 2000; Sikorski et al., 2001). However, it can certainly be an invaluable tool used to reliably establish phylogenetic relationships within the genus *Pseudomonas*.

3.10 DNA-DNA Hybridization

DNA–DNA hybridization values have been used as a decisive means of demarcating taxonomic positions at the species level (Kwon et al., 2003). The current species concept suggests that only those strains with at least approximately 70% DNA–DNA relatedness and aΔ *Tm* value of 5 °C or less constitute a single species (Wayne et al., 1987). In the present study the total DNA of strain DS004 was hybridized with the closely related type strains (Table 3.3) to ascertain the genome wide homology between the type strains and strain DS004 (Tourova and Antonov,

1987). The results of the study is shown in table 1.4, which indicates that strain DS004 has highest DNA relatedness (95%) with *Pseudomonas jinjuensis* LMG 21316$^{T.}$ If the entire data generated in the present chapter is seen together with the DNA-DNA hybridization studies it justifies assigning the name of *Pseudomonas jinjuensis* DS004 to the acephate mineralizing strain DS004.

Table 3.4: DNA – DNA Hybridization of strain DS004 with type strains of *Pseudomonas* genus showing more than 95% sequence similarity at 16S rRNA gene level.

	Pseudomonas nitroreducens **DSM 14399^{T}**	***Pseudomonas azelaica*** **DSM 9128^{T}**	**Strain DS004**	***Pseudomonas citranellolis*** **DSM 50332^{T}**	***Pseudomonas jinjuensis*** **LMG 21316^{T}**	***Pseudomonas knackmussii*** **DSM 6978^{T}**
Pseudomonas nitroreducens **DSM 14399^{T}**						
Pseudomonas azelaica **DSM 9128^{T}**	**55%**					
Strain DS004	**56%**	**55%**				
Pseudomonas citranellolis **DSM 50332^{T}**	**60%**	**85%**	**56%**			
Pseudomonas jinjuensis **LMG 21316^{T}**	**54%**	**71%**	**95%**	**55%**		
Pseudomonas knackmussii **DSM 6978^{T}**	**61%**	**48%**	**30%**	**48%**	**60%**	
Pseudomonas multiresinivorans **ATCC 700690^{T}**	**69%**	**62%**	**86%**	**52%**	**75%**	**58%**

As shown in table 1.4, *Pseudomonas jinjuensis* DS004 shows high genome wide similarity (95%), with type strain *Pseudomonas jinjuensis* LMG 21316$^{T.}$ Such similarity has prompted to test the type strains ability to grow in acephate containing minimal medium. However, the type strain failed to use acephate as carbon source. On further examination, the type strain *Pseudomonas jinjuensis* LMG 21316^{T} has differed with *Pseudomonas jinjuensis* DS004 with respect to a number of biochemical characteristics. All of them are indicated in table 3.5. Considering the genome wide similarities and noted differences on certain biochemical properties, *Pseudomonas jinjuensis* DS004 is justified in giving the status of biotype. As acephate utilization is the key and prominent difference it is proposed to give a name of *acephalyticus* for the biotype. Therefore, the acephate mineralizing *Pseudomonas jinjuensis* DS004 is designated as *Pseudomonas jinjuensis* biotype *acephalyticus*. The culture is deposited in German culture collection centre in the name and accession number of *Pseudomonas jinjuensis* biotype *acephalyticus* = DSM 19477^{T}.

Table 3.5: List of similar and differentiating characteristics between strain DS004 and *Pseudomonas jinjuensis* LMG 21316^{T}.

Characteristics	**Strain DS004**	***Pseudomonas jinjuensis* LMG 21316^{T}**
Glucose	+	+
Sodium citrate	+	+
Potassium gluconate	+	+
DL-Malate	+	+
Succinic acid	+	+
Acephate	+	_
Methanol	+	_
p-Aminobenzoic acid	+	_
Gelatin hydrolysis	+	_

3.11. Discussion

Microorganisms are identified and classified for more than one reason. Identification and classification of microorganisms involves the study of several characteristics starting from morphological level of characterization to genetic level of characterization. Classification is more complicated and routinely uses more advanced methods than identification.

The first and important characteristic used to classify and identify microbes is analysis of morphological characteristics like basic shape, size, characteristic arrangement, the presence of flagella, presence of a capsule, presence of endospores, etc. Once after identifying the morphological characteristics of microbe, Gram staining was done to differentiate into gram negative or gram positive. As so many bacteria look alike it is necessary to do biochemical tests for identification. These tests mainly look the ability of an organism to produce certain enzymes which can make use of certain nutrients and also look the type of products that are produced in the process. The biochemical tests that are routinely used in the identification of microbes include utilization of different types of carbon / energy sources, acid and gas production from sugars, gelatin liquefaction, starch hydrolysis, catalase, oxidase tests and nitrate reduction. Since characteristics vary with different strains, the more tests that are done the better are the identification. In order to establish a evolutionary relationship, phylogenetic analysis was done using 16S rRNA gene sequence. 16S rRNA gene sequence based phylogenetic tree, while providing a very clear picture on genus identity, fails to give a clear picture on the status of the species of the isolate. As a convention, if new isolates 16S rRNA gene is 98% similar with the similar sequence of type strain, the isolate is given type strains species name. In number of

occasions, this convention on naming new isolates is challenged (Kwon et al., 2003). DNA-DNA hybridization is more acceptable while establishing the species identity. This is due to the fact that it provides a clear picture on genome wide similarity of the isolate with the type strains (Kwon et al., 2003).

In addition to the aforementioned techniques, some of the clinical isolates were also require some other biochemical tests for their identification and classification. Those are mainly serology tests and Phage Typing. Serological testing can even distinguish between strains of same species. Strains with different antigens are called serovars or biovars.

The present study has used biochemical and modern techniques to classify strain DS004. Initial morphological and biochemical studies have given a weak identity to strain DS004. Based on these studies the strain is tentatively identified as member of *Pseudomonas*. The claim is further strengthened from the data obtained through mass profile generated for surface peptides. Atleast three genus specific mass peaks were identified to reinforce the claim of DS004 as a member of genus *Pseudomonas* (Fig.3.5C). The phylogenetic tree generated based on 16S rRNA gene sequence has provided convincing data to claim strain DS004 as a member of genus *Pseudomonas* (Fig.3.11).

Though these are independent experiments done taking altogether different approaches, all of them have unequivocally shown strain DS004 as a member of genus *Pseudomonas*. The data generated through independent studies are supportive of each other and gave no ambiguity with respect to the identification of genus of the strain DS004.

The 16S rRNA gene sequence based phylogenetic tree is constructed mainly to have a species identity. The tree has shown more than 98% similarity to the type strain *Pseudomonas azelaica* DSM 9128^T, *Pseudomonas multiresinivorans* ATCC 700690^T, *Pseudomonas nitroreducens* DSM 14399^T and *Pseudomonas jinjuensis* LMG 21316^T (Table 3.3). As per the established norms if the new isolate's 16S rRNA gene sequence shows more than 98% identity with the type strains 16S rRNA gene sequence, the isolate can be given species name of the type strain. If 16S rRNA gene homology is taken into consideration three type strains showed more than 98% homology. However, when genome wide similarity was tested by performing DNA-DNA hybridization studies only the genome of *Pseudomonas jinjuensis* LMG 21316^T is shown to have 95% genome wide similarity (Table 3.4). Such high degree genome wide similarity rather justify giving the name of species *jinjuensis* to strain DS004. Despite of having such high degree genome wide similarity, the type strain failed to grow on acephate. Further with a number of key biochemical factors, the strain DS004 differed from the type strain (Table 3.5). Such differences have indeed stood as primary supporting factors to claim strain DS004 as a biotype of *Pseudomonas jinjuensis*. Therefore the acephate mineralizing strain DS004 is given the name of *Pseudomonas jinjuensis* biotype *acephalyticus*.

Before the emergence of modern techniques like 16S rRNA gene analysis and DNA-DNA hybridization experiments only morphology, differential staining, and biochemical tests were the identification tools available to microbiologists. The novel methods now available are accurate in classifying the organisms and even they have changed some previous classifications (Anzai et al., 2000). Isolation of organophosphate degrading bacterial strains is not uncommon in literature. A number

of soil bacteria that degrade op compounds found their way into literature through extensive research activities. An elegant reviews has recently appeared highlighting the physiology and biochemistry of the organism (Singh and Walker, 2006; Singh, 2008). If detailed degradation pathways and organisms ability to use them as source of carbon are taken into consideration they can be grouped two categories. The first category soil microbes are those that hydrolyze op compounds due to existence of a novel esterase. They hydrolyze op compounds and thus make them incapable of inhibiting acetyl cholinesterase. These microbes may not use degradation products as source of carbon (Sethunathan and Yoshida, 1973; Munnecke and Hsieh, 1974; Serdar et al., 1982; Tchelet et al., 1993; Zhongli et al., 2001). This is rather passive degradation process and is mainly due to existence on endogenous substrate that has considerable structural similarities with op compounds.

The second category of biodegradation is rather a dynamic process. They thrive on these toxic op residues and use them as source of carbon, nitrogen, sulfur and phosphorus (Siddaramappa et al., 1973; Daughton and Hsieh, 1977; Rosenberg and Alexander, 1979; Nelson, 1982; Pakala et al., 2007). They have acquired genetic machinery to code for enzymes that convert these toxic and recalcitrant compounds into TCA cycle intermediates. These are real nature's gift to mankind and they are the preferential candidates which can be used for developing bioremediation strategies.

If biochemistry of op compound degradation is clearly examined, existence of a metal dependent triesterase which hydrolyzes the characteristic triester bond found in organophosphates is evident in most of the organisms (Mulbry et al., 1986; Chaudhry et al., 1988; Serdar et al., 1989; Somara and Siddavattam, 1995). Most of the times as stated before, the hydrolytic products are not used as source of carbon.

They either accumulate causing disruption to the soil ecosystem or form carbon source for other soil microbes living in the habitat. Finally elimination of op compounds from the environments is rather an effort of a microbial consortium.

In very few cases scientists have successfully isolated microbes that mineralize op compounds including the byproduct generated due to hydrolytic cleavage of triester linkage of op compounds. Though such reports are rare, microbiologists have succeeded in isolating such novel strains ((Munnecke and Hsieh, 1976; Rosenberg and Alexander, 1979; Rani and Lalithakumari, 1994; Pakala et al., 2007). In any case if degradation pathways are examined, most of them essentially start with the hydrolysis of triester linkage, due to existence of existence of triesterase popularly known as OPH. Infact for OPH, organophosphates with P-O linkage serve as preferred substrate. The phosphonates, phosphoramidothioates which contain P-C or P-S bonds with central phosphorus atom serve as poor substrates to OPH (Efremenko and Sergeeva, 2001). Precisely due to this reasons phosphoramidothioates are more persistent in nature. Their residues are found in environments for long time. Acephate, a phosphoramidothioate is one of the most prominent insecticides used all over the world. Its recalcitrance became rather evident through the work of Antonius and his coworkers (Antonius and Snyder., 1994; Antonius, 1995). Despite of having such published evidences there exists no report in literature on isolation of acephate mineralizing/degrading strains. This is rather first isolate that thrive on acephate using it as carbon, nitrogen and sulfur source.

3.12. Conclusions

1. A bacterium capable of using acephate as source of carbon, nitrogen and sulfur is isolated from the activated sludge collected from a pesticide manufacturing unit.
2. Based on the morphological, biochemical and molecular biological tools, the bacterium is identified as *Pseudomonas jinjuensis* biotype *acephalyticus.*

Results and Discussion

Chapter II

In the previous chapter we have described isolation and characterization of acephate degrading bacterium isolated from activated sludge collected from a pesticide manufacturing unit. Employing both modern molecular biology tools and microbiological studies, the isolate which uses acephate as source of carbon, nitrogen and sulfur is designated as *Pseudomonas jinjuensis* biotype *acephalyticus*. It's in fact the first report on isolation of acephate mineralizing bacteria. The potential of such novel isolate can only be exploited for bioremediation of acephate, only when its catabolic pathway and mechanisms that contribute for its regulation are known. This chapter is mainly devoted for understanding the acephate degradation pathway in *Pseudomonas jinjuensis* biotype *acephalyticus.* While understanding the catabolic pathway of acephate, a systematic investigation is conducted to identify the catabolic intermediates generated and to know the time of their formation and duration of their presence either in culture medium or in resting cell assay. Based on identification and time of formation of catabolites of acephate, a putative pathway followed for degradation of acephate is presented in this chapter.

4.1. Identification of catabolic intermediates of acephate by GC-MS

While attempting to identify catabolites of acephate two independent methods have been followed. One of them is extracting and identifying the degradation products from the culture medium where *Pseudomonas jinjuensis* biotype *acephalyticus* is grown using acephate as sole source of carbon. The second approach is through resting cell assay, which typically contained acephate grown *Pseudomonas jinjuensis* biotype *acephalyticus* cells in various densities in milli Q water. Addition of fresh acephate to the resting cells and time dependent extraction and analysis of

cell free extracts to detect acephate degradation products in a time dependent manner has indeed provided substantial clues on identification of catabolic intermediates of acephate formed at different time intervals.

While following the first approach, acephate degradation products were extracted from a mid log phase culture of *Pseudomonas jinjuensis* biotype *acephalyticus* grown in minimal medium supplemented using acephate as source of carbon. The extracted metabolites were then analyzed both in GC-MS and LC-MS. Before analyzing acephate degradation products the parent compound and its mass spectrum was generated using GC-MS. As shown in Fig 4.1A the parent compound was detected at the retention time 13. 8 minutes with a characteristic mass pattern shown in Fig. 4.1B. After analyzing the parent compounds the extracts prepared from the medium were analyzed using GC-MS by keeping the conditions described in materials and methods section. In a typical chromatogram two characteristic peaks were obtained in addition to the peak that corresponds to parent compound acephate.

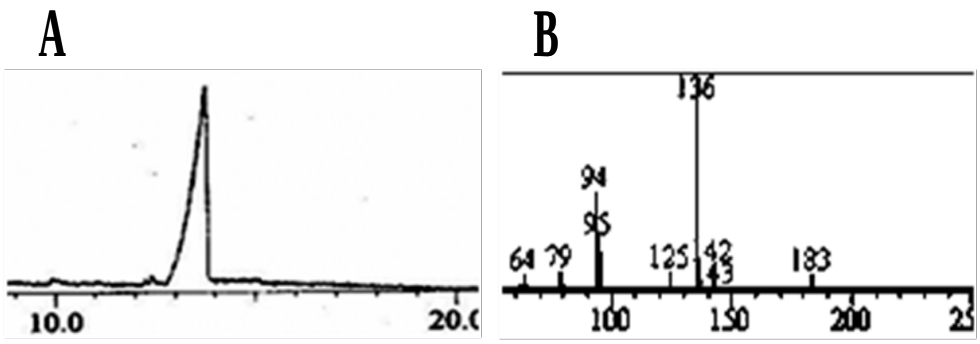


Fig. 4.1. Panel A shows gas chromatogram showing single peak of acephate with retention time 13.8 min. Panel B shows mass spectrum of acephate showing the presence of acephate with molecular mass 183.

One of them was found at the retention time 12.0 minutes (Fig 4.2 A). As it is new and prominent, a mass spectrum was generated for this peak. Its mass spectrum perfectly matched with the mass spectrum generated for the authentic methamidophos obtained from Sigma-Aldrich and used as standard. As shown in Fig 4.2 B, the major molecular ion [M^+] at m/z 141, perfectly coincided with the mass of methamidophos. Even, the other major fragmentation ions were found to be identical when compared to the mass spectrum of authentic methamidophos.

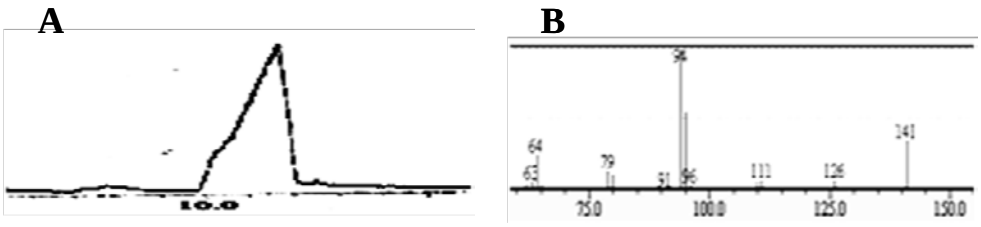


Fig. 4.2. Panel A shows gas chromatogram showing single peak of methamidophos with retention time 12.0 min. Panel B shows mass spectrum of methamidophos showing the presence of methamidophos with molecular mass 141.

Similarly the second characteristic peak was seen at retention time of 4.3 min (Fig 4.3 A). In its mass spectrum a molecular ion [M^+] at m/z 60 perfectly coincided with the mass of acetic acid (Fig 4.3 B).

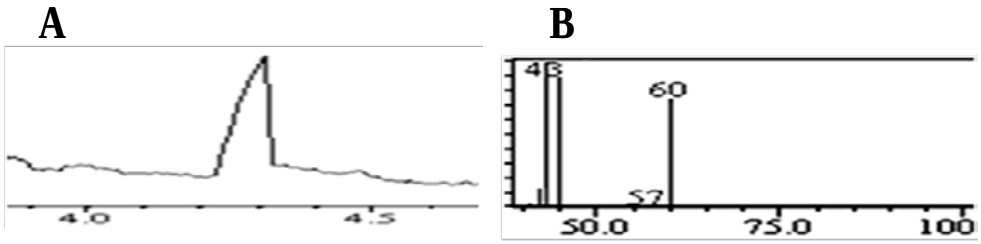


Fig. 4.3. Panel A shows gas chromatogram showing a single peak of acetic acid with retention time 4.3 min. Panel B shows mass spectrum of acetic acid showing the presence of acetic acid with molecular mass 60.

Identification of these two prominent compounds in the spent medium clearly indicates that acephate is hydrolyzed to form methamidophos and acetate. If the structure of acephate is examined methamidophos and acetate are linked together through an amide linkage. If formation of acetate and methamidophos are taken as any indication, it would clearly suggests that the amide linkage which keeps these two moieties together in acephate, is hydrolyzed through existence of a novel amidase found it *Pseudomonas jinjuensis* biotype *acephalyticus* (Fig 4.4). However, the other metabolites of methamidophos were not detected in GC-MS. This may be due to high solubility of methamidophos degradation products in water. In order to overcome such problem LC-MS was employed.

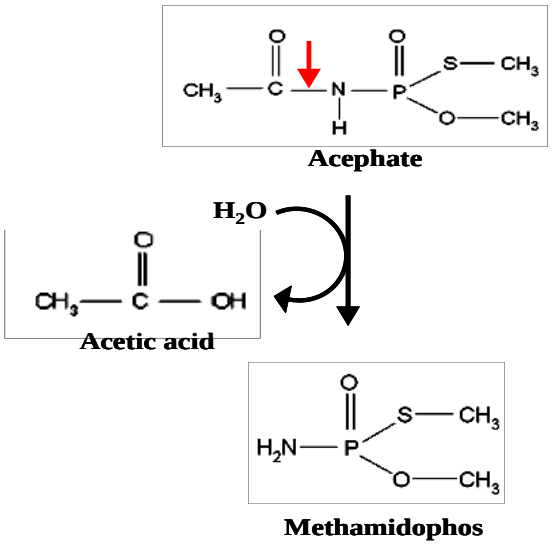


Fig. 4.4 Hydrolysis of acephate to methamidophos and acetic acid by *P.jinjuensis* biotype *acephalyticus*.

4.2. Identification of Catabolic intermediates of acephate by LC-MS analysis

While employing LC-MS for identification of metabolites of acephate and methamidophos resting cells were prepared to obtain acephate grown *Pseudomonas jinjuensis* biotype *acephalyticus* in various cell densities. While varying the cell

densities in each assay mixture, the acephate concentration and incubation time were kept constant. Each resting cell preparation was incubated with 10 mM acephate for 15 min with shaking at 200 rpm. After incubation, cells were pelleted and the supernatant was directly taken for LC-MS analysis. Before analyzing the acephate metabolites formed during incubation the parent compounds acephate and methamidophos were analyzed and their retention times were recorded. These two parent compounds have appeared in the chromatogram at retention time's 6.2 and 4.3 min (Fig 4.5 & 4.6). After standardization, the metabolites formed in resting cells were analyzed. As expected two characteristic peaks that match both in retention time and mass spectrum of acephate and methamidophos were detected. This observation reinforces the data generated by GC-MS study and rather confirms that the initial step of acephate degradation is due to cleavage of amide linkage, probably due to existence of novel amidase in *Pseudomonas jinjuensis* biotype *acephalyticus*.

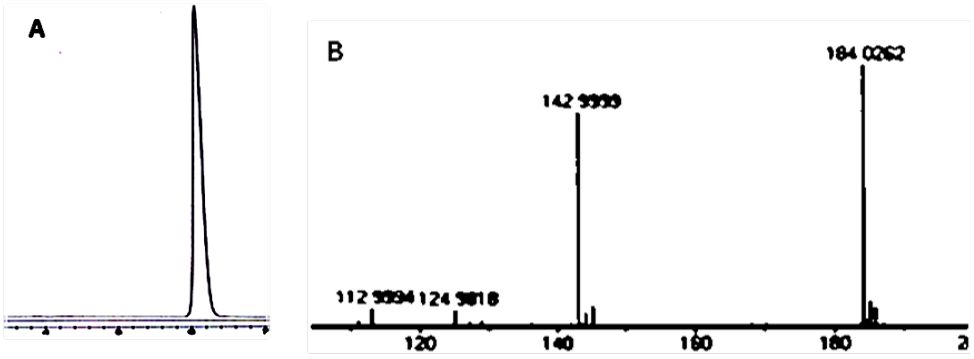


Fig. 4.5. Panel A shows liquid chromatogram showing single peak of acephate at retention time 6.2 min Panel B shows mass spectrum of acephate showing molecular mass as 184.

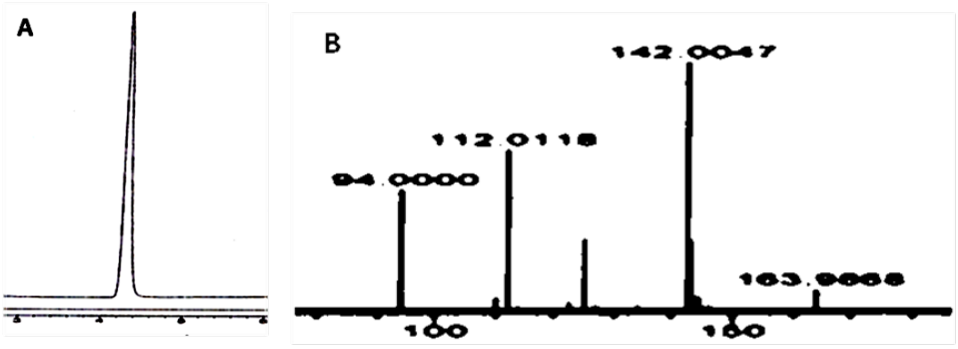


Fig. 4.6. Panel A shows the liquid chromatogram showing single peak of methamidophos with retention time 4.2 min. Panel B shows mass spectrum of methamidophos showing molecular mass as 142.

Further, formation and disappearance of these two peaks followed very characteristic pattern. Either with increase in cell density or with the increase of time the acephate concentration has gone down leading the increase in the peak height of methamidophos. With the further increase of time the methamidophos peak disappeared, suggesting that the methamidophos is being either used as source of carbon or further metabolized (Fig 4.7).

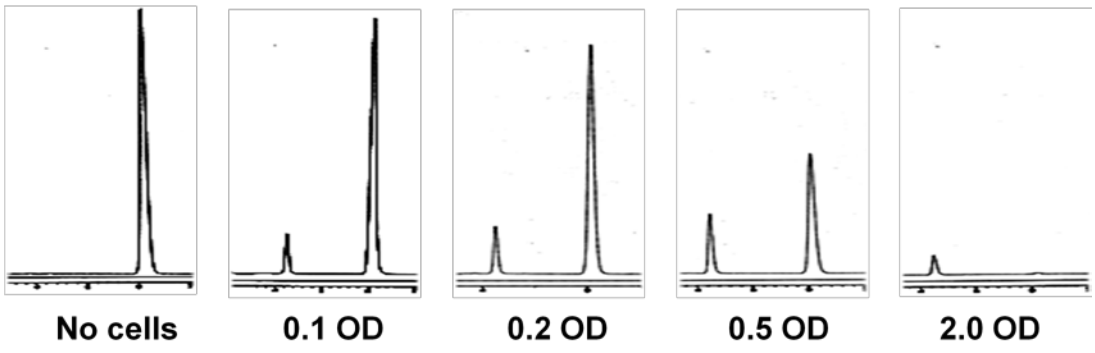


Fig. 4.7. Cell density dependent degradation of acephate. Formation of methamidophos is seen at retention time 4.3 min within 15 min of incubation at 30°C. Progressive disappearance of acephate is seen in liquid chromatograms obtained from the reaction mixtures incubated with different cell densities. Similar pattern is obtained with increase in incubation time.

After detecting disappearance of methamidophos an attempt was also made to detect its metabolites. As methamidophos degradation products lack detectable absorbance in UV range, they were measured in mass spectrometry.

Detection of desmethyl methamidophos

A stated before, either increase of incubation time or increase in cell density has contributed to decrease of acephate concentration and methamidophos formation. When acephate was completely degraded, methamidophos started receding with the formation of its degradation products. A mass peak corresponding to molecular ion [M^+] at m/z 126, which is characteristic pattern of desmethyl methamidophos appeared at retention time 2.8 min (Fig 4.8), suggesting that methamidophos is further dealkylated to generate desmethyl methamidophos.

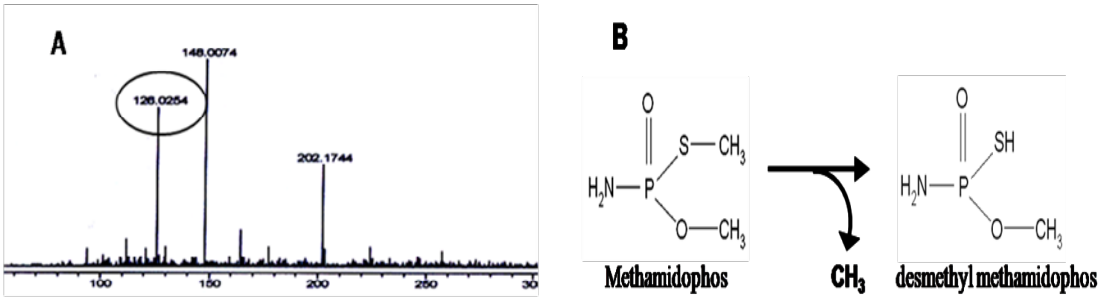


Fig. 4.8. Panel A shows the mass spectrum of desmethyl methamidophos showing the molecular mass 126. The peak in chromatogram appeared at 2.8 min. Panel B shows formation of desmethyl methamidophos from methamidophos through dealkylation during degradation process.

Similarly another characteristic mass peak with a molecular ion [M^+] at m/z 112 corresponding to *o*-methyl phosphoramidate, appeared at retention time 2.62 min (Fig 4.9), which further reiterates existence of typical dealkylation process during stepwise degradation of methamidophos. Further, the formation of phosphoramidate from *o*-

methyl phosphoramidate, is evident from the appearance of the characteristic mass peak at retention time 2.7 min with a molecular ion [M^+] at m/z 98.9 (Fig 4.10).

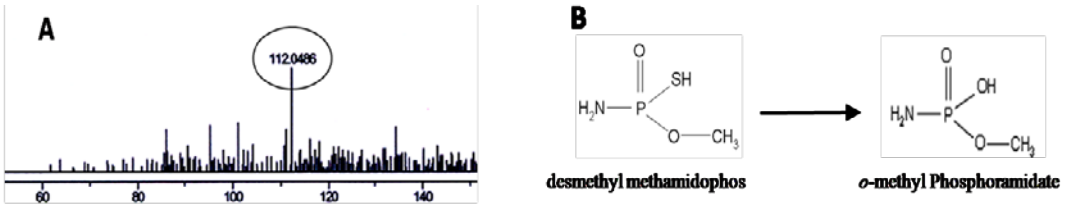


Fig. 4.9. Panel A shows the mass spectrum of *o*-methyl phosphoramidate showing the molecular mass 112. The peak in chromatogram appeared at 2.62 min. Panel B shows the chemical reaction indicating the formation of *o*-methyl phosphoramidate from desmethyl methamidophos through oxidation reaction.

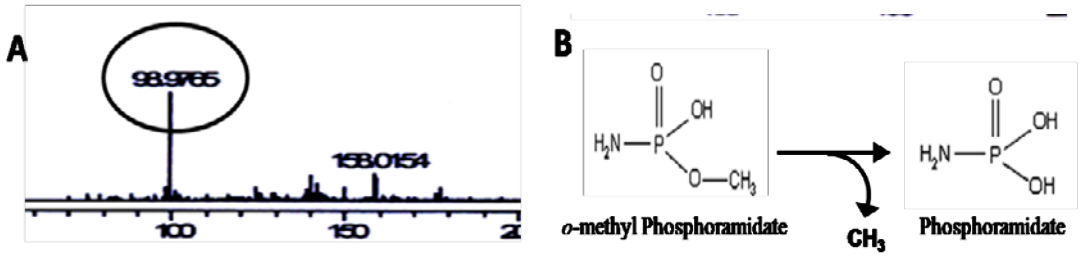


Fig. 4.10. Panel A shows the mass spectrum of phosphoramidate showing the molecular mass 98.9. The phosphoramidate peak appeared at retention time of 2.7 min. Panel B shows chemical reaction indicating formation of phosphoramidate from *o*-methyl phosphoramidate through dealkylation reaction.

Based on the above mass spectrophotometric data a step wise degradation of acephate is shown in Fig. 4.11. If the figure drawn based on the formation of metabolites of acephate and methamidophos is taken into consideration it clearly suggests that the acephate is degraded due to initial hydrolysis of amide linkage leading to the formation of acetate and methamidophos. Methamidophos thus generated was further metabolized by the *P. jinjuensis* biotype *acephalyticus* either through dealkylation or oxidation process. Formation of desmethyl methamidophos

via dealkylation of methamidophos and its oxidation to form *o*-methyl phosphoramidate is indicative of exiting dedicated enzymatic machinery in *P. jinjuensis* biotype *acephalyticus* for mineralization of acephate. Further formation of phosphoramidate from *o*-methyl phosphoramidate is an indication of prominent dealkylation process in mineralization of acephate. Using the mass spectrophotometric data a putative pathway is proposed for degradation of acephate found in *P. jinjuensis* biotype *acephalyticus* (Fig 4.11).

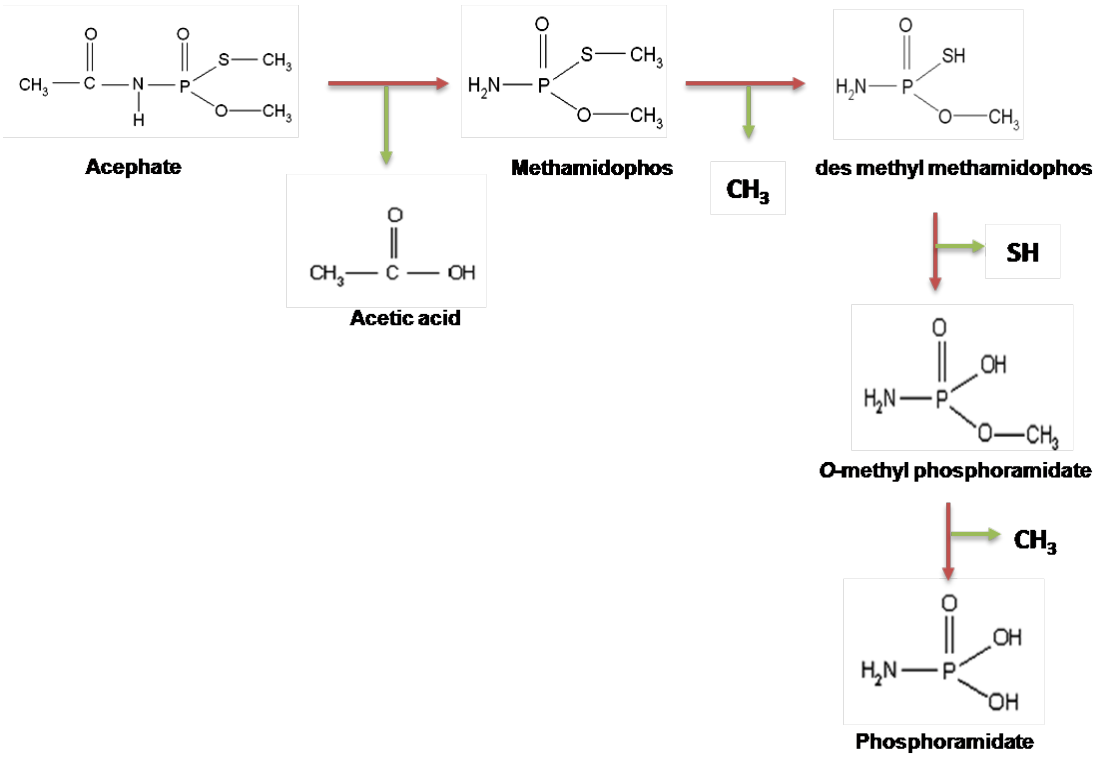


Fig. 4.11. Putative acephate degradation pathway in *Pseudomonas jinjuensis* biotype *acephalyticus*.

4.3. Discussion

Microbes have been shown to degrade a number of recalcitrant and toxic compounds found in the environment as pollutants. While thriving on these toxic

compounds, they clear the environment and hence these eco-friendly bacterial strains are optly called as 'Bioscavengers'. While scavenging the toxic wastes microbes adapt variety of strategies. Based on the degradation mechanism followed, the process of biodegradation is classified into four broad categories (Kumar et al., 1996). Out of these four types, mineralization, a process that leads for complete degradation resulting in mineralization of compound to carbondioxide or methane, is one of the best methods of biodegradation. Microbes that contribute for mineralization of recalcitrant and toxic compounds are the best candidates to be involved in bioremediation process (Singh et al., 1999). In certain cases microbes just degrade toxic residues rendering them non-toxic compounds. But neither parent compounds nor their metabolites are used as carbon source. The process known as detoxification is often useful for converting toxic residues to non-toxic residues (Kumar et al., 1996). Though they are useful for detoxification they are not the preferred candidate organisms for the development of bioremediation tools, as they contribute for accumulation of byproducts. In contrast to these two processes some microorganisms bring about degradation of recalcitrant compounds only while growing on other compounds through a process known as cometabolism (Kumar et al., 1996). They are useful only to certain extent while performing cleanup operation. The fourth category while metabolizing recalcitrant compounds generate and contribute for accumulation of toxic metabolites (Singh et al., 1999). The process, well known as activation, is very dangerous and such microbe have naturally no place while developing bioremediation tools.

A number of biochemical pathways and reactions for microbial degradation of pesticides have been reported. Hydrolases and oxygenases are the two most important

classes of enzyme that are involved in catalyzing the conversion of op-pesticides. Hydrolases include esterases, and amidases. These groups do not require cofactors and are stable in a wide range of pHs and temperatures. Examples are: (1) esterases like parathion hydrolase attack phosphotriester bonds of organophosphates, and (2) amidases hydrolyze amide bonds existing in certain classes of op compounds (Singh et al., 1999). These two classes of microbial enzymes contribute only for detoxification of op compounds by rendering them incapable of inhibiting acetylcholinesterases.

In the degradation of organophosphates like parathion, methyl parathion and paraoxon the initial reaction is hydrolytic reaction leading to formation of *p*-nitrophenol. To achieve complete mineralization, *p*-nitrophenol has to undergo further degradation. Oxygenases play a prominent role in the degradation of aromatic compounds like *p*-nitrophenol. Hence oxygenases in combination with above mentioned hydrolases are needed to achieve complete mineralization of op compounds. Unlike esterases, oxygenases depend on cofactors and molecular oxygen for activity and hence are less stable than hydrolases. These complex enzymes are further divided into two subgroups: (1) monooxygenases require reduced pyridine nucleotides as cofactors; and (2) dioxygenases do not require a reduced compound as a cofactor. A combined action of these two enzymes is infact essential for complete mineralization of op compounds. In the absence of oxygenases, if only hydrolases are there, they would rather contribute either for detoxification process or for activation.

Several studies have documented involvement of soil microorganisms in the degradation of op compounds. In most of the degradation studies, growth experiments have served as bench marks for generating preliminary data pertaining to the

metabolism of a recalcitrant compound. Usage of organophosphates as source of carbon is not uncommon in literature. Way back in 1973, Sethunathan and Yoshida (1973) have reported isolation of *Flavobacterium* strain ATCC 27551 which utilized diazinon as a sole source of carbon. Further, hydrolysis of parathion and utilization of its hydrolytic product *p*-nitrophenol as a carbon and nitrogen source by a *Pseudomonas* sp. was also reported (Siddaramappa et al., 1973). Rosenberg & Alexander have isolated *Pseudomonas* sp. which used a number of op compounds as source of phosphate (Rosenberg and Alexander, 1979).

Organophosphorus compounds being esters, they have many vulnerable sites and are potentially hydrolyzable. Degradation of organophosphate pesticides is generally faster than that of organochlorines. Microbial degradation through hydrolysis of P-O- alkyl and P-O-aryl bonds is considered the most significant step in the detoxification of organophosphorus compounds. The principal reactions involved in organophosphates metabolism are hydrolysis, oxidation, alkylation, and dealkylation.

Hydrolysis is the important and initial step in the degradation of organophosphates which is catalyzed by a hydrolase enzyme. Hydrolysis of OP compounds reduces their mammalian toxicity by several orders of magnitude (Singh and Walker, 2006). Examples for hydrolysis of organophosphates include hydrolysis of parathion to *p*-nitrophenol and diethylthiophosphoric acid, conversion of methyl parathion to *p*-nitrophenol and dimethylthiosphosphoric acid (Sethunathan and Yoshida, 1973; Siddaramappa et al., 1973; Chaudhry et al., 1988; Pakala et al., 2007). The enzyme catalyzing this hydrolysis step is an esterase known as Organophosphate hydrolase (OPH).

4.3a. Organophosphate degrading enzymes

Several op degrading soil bacteria possess a highly conserved organophosphate degrading gene (*opd*) which encodes organophosphate hydrolase (OPH). As OPH catalyzes hydrolysis of triester linkage present in structurally diverse group of organophosphates is also known as phosphotriesterase (PTE). The OPH is a dimer of two identical subunits that contain 336 amino acid residues and have a molecular mass of ~72 kDa (Raushel, 2002). It has a broad substrate specificity and can hydrolyse P–O, P–F and P–S bonds, but with different efficiencies (Efremenko and Sergeeva, 2001). The OPHs show structural resemblance with members of the amidohydrolase superfamily like urease, dihydroorotase and adenosine deaminase. Alignment and superimposition studies of three-dimensional structures of these enzymes revealed a striking structural similarity consisting of an ellipsoidal $(\beta\alpha)_8$ barrel with a conserved metal binding site at the C-terminal end of strands β1, β5, β6, and β8. The active site residues like four histidine residues, one aspartic acid residue and metal ligands are strictly conserved in the three enzyme families. Though the protein-scaffold and key active-site residues of these enzymes are conserved, the substrate specificity and reaction mechanisms of different family members vary due to differences in the sequence and length of the β- or α-loops, particularly in loops one, seven and eight which contribute for substrate-binding (Raushel, 2000). In view of such structural and catalytic relationship, the members of triesterases such as OPHs have been proposed to have evolved from amidohydrolase superfamily of proteins, through divergent evolution (Holm and Sander, 1997).

Recent reports have shown the isolation of several enzymes that are involved in op degradation but share less or no homology with OPH. These enzymes include

OPDA from *Agrobacterium radiobacter* (Horne et al., 2002), MPH from *Pseudomonas* sp. WBC-3 (Dong et al., 2005) and OPAA from *Alteromonas haloplanktis* (Cheng et al., 1993). Even though OPH and OPDA shares 90% homology they differ with each other due to presence of 20 amino acid tail at the carboxy terminus of OPDA. In addition to these two known classes, a third category of op hydrolyzing enzymes have been discovered in microbes isolated from Chinese agricultural soils. They are named as methyl parathion hydrolases (MPH) and the gene coding the MPH is designated as methyl parathion degrading (*mpd*) gene. The *mpd* gene shown no homology either with the well known *opd* gene or with *opd*A isolated from *Agrobacterium radiobacter* (Horne et al., 2002). Such an observation prompts to propose an independent evolution of op compound degrading triats in bacteria. Supporting this hypothesis, the *mpd*s have been shown to have structural similarities with the members of β-lactamases super family of proteins (Fig. 4.12). The *Ophc2* (Chu et al., 2006) and *ophB* (Tago, 2006) which are isolated from *Pseudomonas pseudoalcaligenes* and *Burkholderia* sp. NF100, despite of showing considerable diversity, have clustered with *mpd* genes. However, the dendrogram constructed for *opd* sequences collected from NCBI database has shown very significant distance between *mpd* and *opd* genes. Interestingly, the *opa*A genes isolated from *Alteromonas haloplanktis* (Cheng et al., 1997) and *Alteromonas* sp. are very distantly related to both *mpd* and *opd* genes. Such diversity among organophosphate hydrolyzing enzymes speaks of divergent evolution.

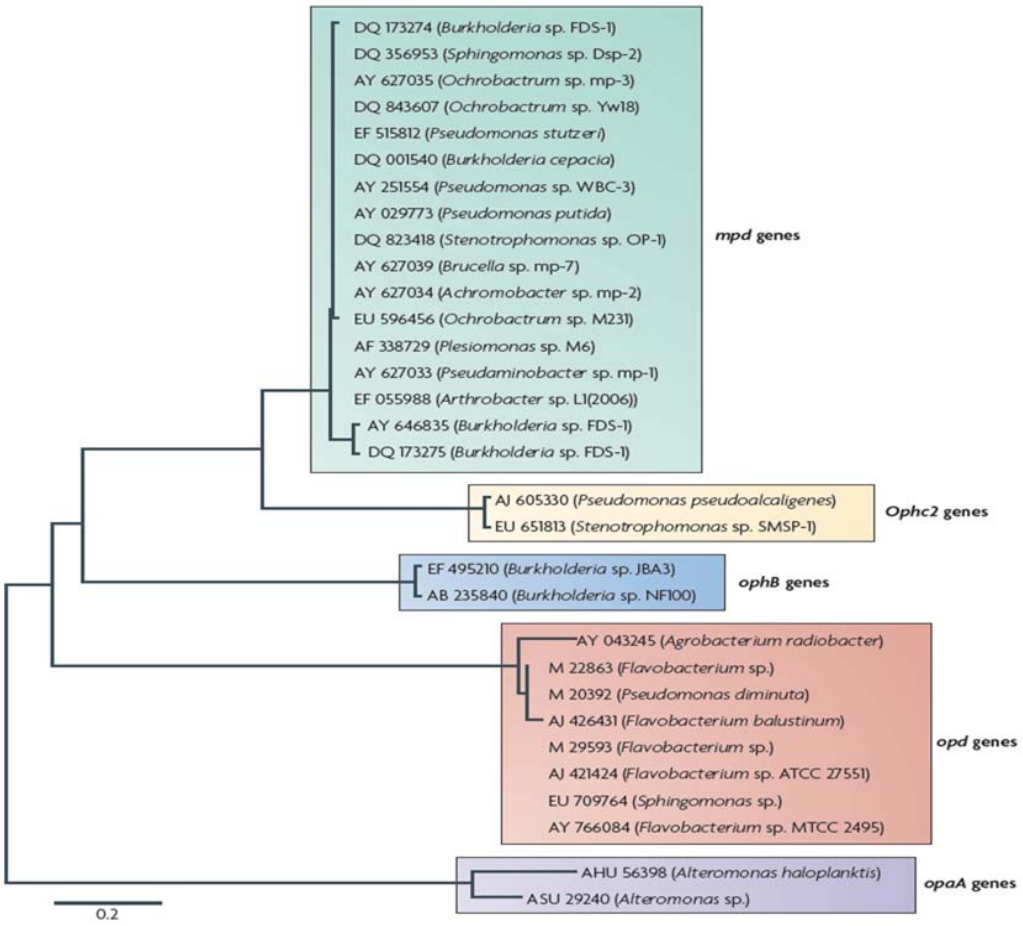


Fig. 4.12. Phylogenetic tree constructed from organophosphorus-degrading gene sequences. The tree was compiled using the sequences that were available in the NCBI database. Distinct groups that correspond to different genes, such as the *mpd* (methyl parathion degrading), *opd* (organophosphorus degrading) and *opaA* (organophosphorus acid anhydrolase) genes are easily delineated (shown in different colours). The tree was constructed using a neighbour-joining method and Mega4 software. All major nodes are supported by a bootstrap value of >50%. The scale bar represents the number of expected changes per site. The locus tags are provided for each gene followed by the species name in brackets (Singh, 2008).

4.3b. Oxygenases

In number of cases, the organophosphate hydrolase mediated hydrolytic cleavage of op compounds generates nitrophenols. Detailed degradation pathways are given in Introduction section. The reviewer may kindly refer the introduction section for gaining more information. The bacterial strains having ability to mineralize these classes of op compounds have been shown to possess several oxygenases, in addition

to the existence of op hydrolyzing enzymes. Degradation of *p*-nitrophenol was catalyzed by either single component monooxygenase (gram-negative bacteria) or two component monooxygenase (gram-positive bacteria) (Kadiyala and Spain, 1998; Kitagawa et al., 2004). In *Bacillus sphaericus* existence of a novel monooxygenase was reported to catalyze the first two steps of *p*-nitrophenol degradation via formation of *p*-nitrocatechol and benzenetriol (Kadiyala and Spain, 1998). This enzyme consists of two components, a reductase and oxygenase, and catalyzes two sequential mono-oxygenation reactions that convert *p*-nitrophenol to benzenetriol. The first oxygenation results in conversion of *p*-nitrophenol to *p*-nitrocatechol while the second removes the nitro group from para positon (Kadiyala & Spain, 1998). The oxygenases invariably contain NADH/ NAD (P) H/ FADH as co-factor (Kadiyala and Spain, 1988; Spain et al., 1979; Mitra and Vidyanathan, 1984). Several other reports have also been shown, the involvement of monooxygenase in the degradation of *p*-nitrophenol (Spain and Gibson, 1991; Jain et al., 1994; Pakala et al., 2007; Lynda and Zylstra, 2007). The benzenetriol formed is directly converted to maleylacetate by means of a dioxygenase, known as benzenetriol dioxygenase (Jain et al., 1994; Bhushan et al., 2000a; Chauhan et al., 2000).

The other principal reaction involved in degradation of organophosphates is dealkylation. The general structure of organophosphate shows two R groups which are substituted with alkyl or aryl groups. Hence in the complete degradation of organophosphates, dealkylation step is necessary. Dealkylation mainly removes the alkyl groups like methyl, ethyl groups present in the OP compounds. The methyl and ethyl groups present in the organophosphates methyl parathion and parathion are

removed by hydrolysis reaction and released as DMTP (dimethyl thiophosphate) and DETP (diethyl thiophosphate).

Acephate is one of the prominent op compound used as insecticide all over the world. Its recalcitrancy is shown to be due to its resistance to OPH hydrolysis. To the best of our knowledge, there are no reports on degradation of acephate. However, few studies are available on microbial degradation of methamidophos, the degradation product of acephate. (Xiao and Wang 1995; Li et al., 1999; Liu and Zhong 1999; Chao et al. 1999, 2000; Liu et al., 2001; Wu et al. 2005; Zheng et al. 2006).

In the previous chapter, we have reported isolation and characterization of a new isolate *Pseudomonas jinjuensis* biotype *acephalyticus* that thrives on acephate and its principal metabolite methamidophos. The elucidated degradation pathway of acephate in *Pseudomonas jinjuensis* biotype *acephalyticus* clearly shows that acephate degradation is initiated through hydrolysis of amide linkage. This step appears to be catalyzed by an amidohydrolase, as amidohydrolases are known to hydrolyze the amide linkage (CO-NH). Formation of acetate and methamidophos, immediately after the addition of acephate to the resting cells supports the prediction of existence of amidohydrolase in *P.jinjuensis* biotype *acephalyticus*.

In general, microbial amidases are mostly involved in the detoxification of industrial effluents containing toxic amides such as acrylamide or formamide (Andrade et al., 2007). Additionally, immobilized amidase can be used efficiently for production of acrylic acid from acrylamide, thus converting a toxic ambient contaminant into widely used industrial raw material (Babu et al., 1995; Nawaz and Chapatwala, 1991; Nawaz et al., 1991). The unusual catalytic properties of amidases such as thermo stability, catalytic activity, enantioselectivity, and substrate specificity

make them most preferred candidate enzymes in industrial applications. Microbial amidases are a class of enzymes that have potential value for the development of commercial bioprocesses (Banerjee et al., 2002). Further characterization of amidohydrolase involved in the initial hydrolysis of acephate probably contribute for understanding on biodegradation of acephate, besides adding yet another novel amidohydrolase for industrial application. Efforts in this direction are in good progress in our laboratory.

Recently Li and his associates (2009) have reported the complete degradation of methamidophos and the degradation pathway proposed by them is shown in Fig. 4.13. If the proposed pathway is examined, *Hyphomicrobium* species MAP-1 hydrolyzed methamidophos via deamination step generating *O, S* - dimethyl hydrogen thiophosphate. In *Hyphomicrobium* species MAP-1, *O, S* - dimethyl hydrogen thiophosphate proceeded through hydrolytic cleavage generating either *O* – methyl dihydrogen thiophosphate or *S* – methyl dihydrogen thiophosphate. It's not clear which one of these two is formed first. Finally the two metabolites generate inorganic phosphate through one more round of dealkylation process (Fig.4.13, Panel A).

The situation in *Pseudomonas jinjuensis* biotype *acephalyticus* is totally different. After generating methamidophos from acephate it has undergone dealkylation process generating desmethyl methamidophos, *o*-methyl phosphoramidate and phosphoramidate. Further conversion of phosphoramidate into inorganic phosphate and ammonia is not seen though methods employed in this study (Fig. 4.13, Panel B). However, looking at the facts that *P.jinjuensis* biotype *acephalyticus* thrive on acephate using it as source of carbon, nitrogen and sulfur, existence of a final deamination step in this organism cannot be ruled out.

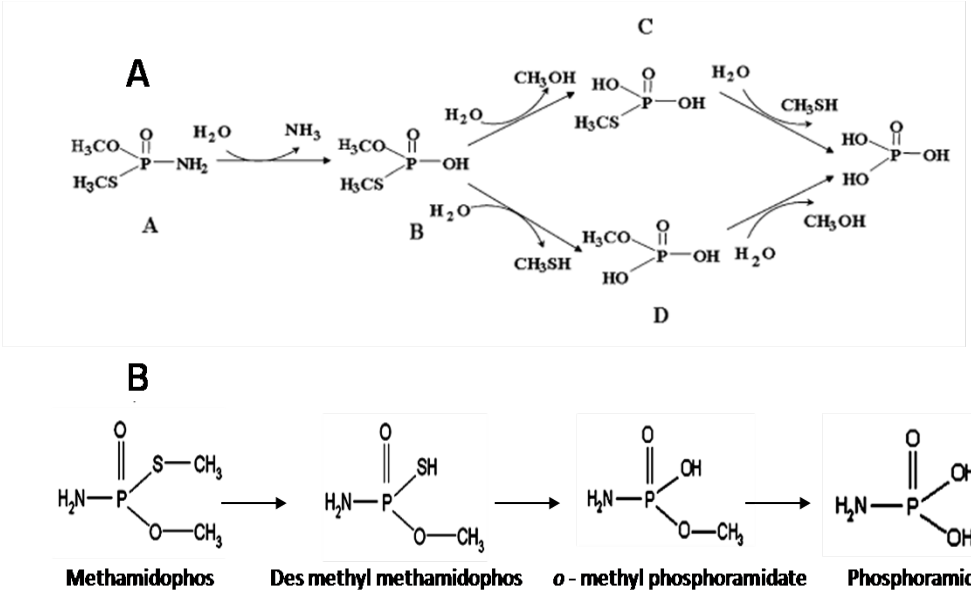


Fig. 4.13. Degradation of methamidophos as reported in *Hyphomicrobium* species MAP-1 (Panel A) and in *P.jinjuensis* biotype *acephalyticus* (Panel B).

Dealkylation of insecticidal organophosphate triesters is a general process of detoxification occurring in mammals, insects, plants and microorganisms. The dealkylation process significantly reduces the toxicity of organophosphates (Hollingworth, 1969). The major enzymes involved in this dealkylation process are cytochrome P450 and GST (Glutathione – S - Transferase). The major site for detoxification in mammals is liver. Glutathione S- transferases (GSTs) belong to a super gene family of enzymes whose main function is to catalyze the conjugation of a diverse array of electrophilic compounds with glutathione (Eaton and Bammler, 1999). Conjugation reactions with glutathione have been suggested as an important pathway of contaminant transformation, particularly in the pesticide literature (Field and Thurman, 1996).The presence of glutathione and glutathione *S*- transferase enzymes in microorganisms suggests that the transformation of electrophilic

chemicals by glutathione conjugation may commonly occur in natural waters and in terrestrial environments. Glutathione reactions with electrophilic chemicals are catalyzed by glutathione *S*-transferase enzymes. Glutathione *S*-transferase enzymes are present in most organisms, including plants, animals, protozoa, fungi, and bacteria (Lamoureux and Bakke, 1984).

In plants the process of dealkylation of methyl parathion proceeds via *N*-dealkylation where as in animals and in microbes it proceeds via *O*-deakylation. In insects, studies suggest that GSTs play an important role in resistance against several classes of insecticides including OPs (Syvanen *et al.*, 1996; Wei *et al.*, 2001). Methyl parathion (MeP), a widely used OP insecticide, is biotransformed via a glutathione dependent pathway in rat and mouse liver fractions (Clark *et al.*, 1973; Benke *et al.*, 1974; Benke and Murphy, 1975; Fukami, 1980).

Recently in our laboratory, we have observed elevation of GST levels when the *Acinetobacter* sp. DS004 was grown in presence of organophosphate methyl parathion. Reasons for such induction and their role in biotransformation of methyl parathion are being examined. As there are number of dealkylation steps in degradation of methamidophos, we suggest existence of novel GSTs in *P.jinjuensis* biotype *acephalyticus* that play a critical role in dealkylation of methamidophos.

4.4. Conclusions

1. Acephate degradation in *Pseudomonas jinjuensis* biotype *acephalyticus* proceeded via hydrolysis of the amide bond to give acetic acid and

methamidophos. This reaction is probably catalyzed by a novel amidohydrolase.

2. GC-MS analysis has clearly shown the formation of acetic acid and methamidophos from acephate.

3. Methamidophos is further degraded through dealkylation and oxidation. LC-MS studies have clearly demonstrated formation of desmethyl methamidophos, *o*-methyl phosphoramidate and phosphoramidate.

4. A novel putative degradation pathway involved in degradation of acephate is proposed in *P.jinjuensis* biotype *acephalyticus*.

Results and Discussion

Chapter III

The basic objective of the study is to isolate a bacterial strain that can mineralize acephate, which is resistant to hydrolytic cleavage of organophosphate hydrolase (OPH). In the first and second chapters the work related to the isolation of acephate degrading bacterium and pathway involved in degradation of acephate has been described. Based on the systematic study described in the first chapter, the new isolate is identified as *Pseudomonas jinjuensis* biotype *acephalyticus*. Degradation of acephate through initial hydrolysis of amide linkage has been established through detection of acephate and methamidophos using GC-MS and LC-MS. Further metabolism of methamidophos is shown through detection of desmethyl methamidophos, *o*-methyl phosphoramidate and phosphoramidate. In a way results presented in chapter II have demonstrated the strains capability to thrive on acephate using as source of carbon and nitrogen and sulfur. Most of organophosphate degrading bacteria, though enriched using a particular type of op compound, have degraded other op compounds especially by hydrolyzing ester linkages. This has been possible due to existence of phosphotriesterases, coded by structurally similar *opd* genes among these bacterial strains. *Pseudomonas jinjuensis* biotype *acephalyticus* is isolated from the activated sludge collected from a pesticide manufacturing company. This company makes variety of op compounds and hence their residues are expected to exist in the sludge. A bacterial strain isolated from such environment is also expected to have innate ability to degrade or to thrive on other op compounds. It is therefore tested to know if *Pseudomonas jinjuensis* biotype *acephalyticus* can use other op compounds as source of carbon or possess the ability to hydrolyze prominent op compounds. The current chapter describes experimental strategies used to test the

hypothesis and to manipulate *Pseudomonas jinjuensis* biotype *acephalyticus* for degradation of wider range of op compounds.

5.1. Organophosphates other than acephate as carbon source

The minimal media prepared as described in materials and methods section was supplemented with prominently used organophosphate insecticides, methyl parathion, monocrotophos, phosalone and ethion respectively and inoculated with the overnight cultures of acephate grown *Pseudomonas jinjuensis* biotype *acephalyticus* culture. The inoculated flasks were incubated at 30^{o}C and the growth of the bacterium was observed for 24 hours. No increase in the cell density was seen even after incubating the culture for more than 48 hours. This clearly shows that the *P. jinjuensis* biotype *acephalyticus* has no capability to utilize organophosphates other than acephate as carbon sources. As stated in earlier sections a number of bacterial isolates that are involved in degradation of organophosphate compounds possess a highly conserved plasmid borne organophosphate degrading gene (*opd*) (Serdar et al., 1982; Mulbry et al., 1987). The organophosphate hydrolase (OPH), the product of *opd* gene is found to be responsible for degradation of op compounds. In number of cases, the op compound degrading bacterial strains failed to grow using them as sole source of carbon suggesting that the mere degradation capability doesn't confirm the strains ability to grow on op compounds. In such a situation assaying for OPH activity and looking for existence of *opd* gene is a better and viable option. Hence *Pseudomonas jinjuensis* biotype *acephalyticus* is used to test OPH activity and presence of *opd* gene.

5.2. Amplification of *opd* gene from *Pseudomonas jinjuensis* biotype *acephalyticus*

Based on the published sequences of *opd* gene from different sources (Serdar and Gibson, 1985; Mulbry et al., 1986; McDaniel et al., 1988; Mulbry and Karns, 1989), oligonucleotides were designed taking the conserved internal sequences of *opd* gene and used as primers. The *opd* gene was amplified using genomic DNA isolated from *E. coli*, *B.diminuta* and *Pseudomonas jinjuensis* biotype *acephalyticus*. A 20 mer oligo with a sequence of 5' GAAGCGGGTGCCACACTGAC 3', was used as forward primer. Similarly a 25 mer oligo with a sequence of 5' GTCGGGGTTCACAGAATCCATCACG 3' has served as reverse primer. After completion of the PCR reaction, the reaction mixture was analyzed by agarose gel electrophoresis (Fig.5.1).

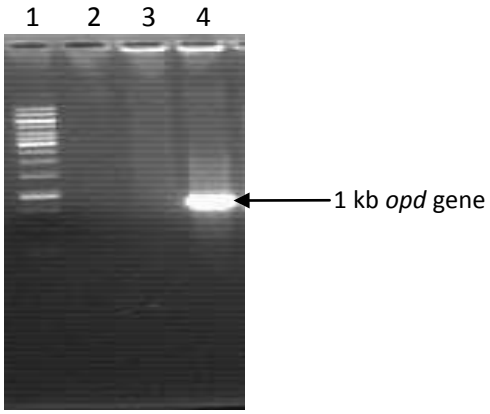


Fig. 5.1. Lane 1 represents 1 Kb DNA ladder; lane 2, 3 and 4 represents PCR reaction mix containing genomic DNA of *E.coli*, *Pseudomonas jinjuensis biotype acephalyticus* and *Brevundimonas diminuta*. Amplification of 1.0 Kb *opd* gene is shown with arrow mark.

As shown in Fig. 5.1 the 1.0 kb PCR product is seen only in the lane loaded with the PCR mixture obtained using genomic DNA of *Brevundimonas diminuta* which served as positive control. There was no such amplicon in the reaction mixture containing genomic DNAs of *E. coli* and *Pseudomonas jinjuensis* biotype

acephalyticus. This result clearly demonstrates that the *opd* gene is not present in *Pseudomonas jinjuensis* biotype *acephalyticus*.

5.3. OPH assay

A number of microorganisms have been reported for microbial degradation of organophosphates especially parathion and methyl parathion (Sethunathan and Yoshida, 1973; Serdar et al., 1982; Chaudhry et al., 1988; Singh, 2008). In most of the cases, the initial hydrolytic step that inactivates op compounds was catalyzed by a triesterase also known an as organophosphorus hydrolase (OPH) or parathion hydrolase (Brown, 1980; Serdar et al., 1982; Mulbry and Karns, 1989). However, recently a number of reports have come showing organophosphate hydrolyzing (OPH) enzymes that are coded by *opd* genes that share no homology with the well characterized *opd* gene (Cheng et al., 1996, 1997; Zhongli et al., 2001). Based on these observations an independent evolution of *opd* gene is proposed. If less conserved or altogether new *opd* gene is present in *P. jinjuensis* biotype *acephalyticus,* PCR amplification, using primers designed based on the *P. diminuta opd* gene, is not a correct approach. Therefore, OPH activity was assayed using methyl parathion as substrate. The cell lysate prepared from *P. jinjuensis* biotype *acephalyticus*, *Brevundimonas diminuta* and *E.coli* were used as enzyme source. The OPH assay was performed by following the protocols described in materials and methods section. The enzyme activity was observed only in reaction tube where cell lysate of *Brevundimonas diminuta* was used as enzyme source, which apparently served as positive control. No OPH activity was observed with the cell lysate prepared from *E.coli* and *Pseudomonas jinjuensis* biotype *acephalyticus* which served as

negative control and test respectively (Fig. 5.2). If PCR data is seen together with OPH activity, it clearly shows non-existence of organophosphate degrading (*opd*) gene in *Pseudomonas jinjuensis* biotype *acephalyticus.*

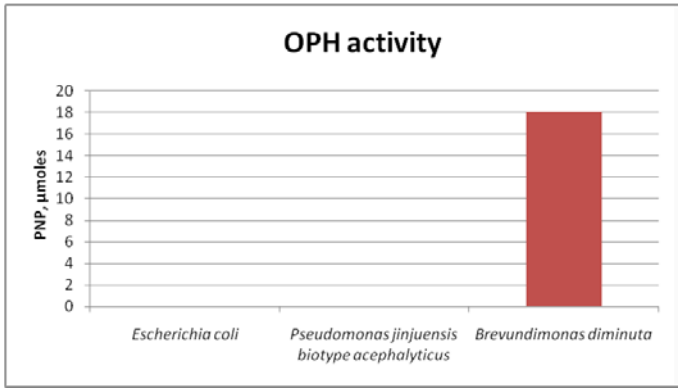


Fig. 5.2. OPH activity in cell lysates of *E.coli, P.jinjuensis* biotype *acephalyticus* and *Brevundimonas diminuta*. OPH activity is expressed as µmoles of PNP produced per minute/mg of protein.

OPH hydrolyses structurally diverse organophosphorus compounds including those present in nerve gas agents such as sarin and soman (Benning et al., 1994; Cho et al., 2004). The OPH is a homodimer having monomer with a binuclear metal center weighing approximately 36 kDa (Donarski et al., 1989). Though OPH is capable of hydrolyzing P-O, P-F, P-CN and P-S bonds, existing in structurally diverse organophosphates, it shows very weak activity towards P-S bond hydrolysis. Hence the op compounds like acephate, which contains P-S bond, will act as poor substrates for OPH. It is precisely for this reason an attempt is made to manipulate, *P. jinjuensis* biotype *acephalyticus* which has innate capability to thrive on acephate, by expressing OPH both in membrane and cytoplasmic fractions. Such manipulated strain is expected to acquire the ability to degrade wide range of op compounds besides its ability to thrive on acephate.

While attempting to perform genetic manipulation of *P. jinjuensis* biotype *acephalyticus* three strategies were followed. In the first strategy the *opd* gene was cloned with its native promoter to know if its native promoter is recognized by *P. jinjuensis* biotype *acephalyticus*. The second strategy is to direct the OPH to the periplasmic face of *P. jinjuensis* biotype *acephalyticus* inner membrane by expressing it with its signal peptide. The third strategy is designed to clone the *opd* gene without its signal coding region. Such *opd* variant is expected to code for a mature form of OPH (mOPH), which is expected to stay in cytoplasm. All three strategies used in the present study are shown in Fig.5.3.

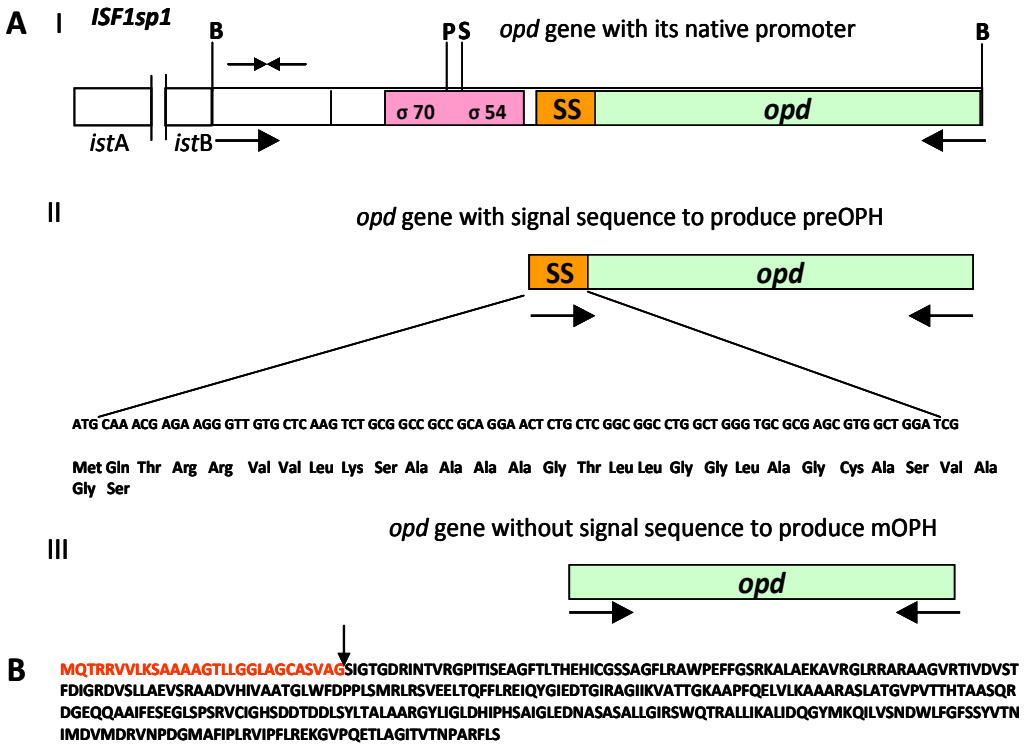


Fig. 5.3. Pane A shows strategies used to clone the *opd* gene in to *P. jinjuensis* biotype *acephalyticus.* Panel B represents amino acid sequence of OPH. Signal peptide sequence was shown in red letters. Signal peptide cleavage site is shown with arrow mark.

5.4. Construction of expression plasmids

Based on the aforementioned experiments, the absence of OPH in *Pseudomonas jinjuensis* biotype *acephalyticus* is evident. If OPH is successfully expressed in *Pseudomonas jinjuensis* biotype *acephalyticus* it would add hydrolyzing ability of structurally diverse group of op compounds to the strain's innate ability of mineralizing acephate. The present chapter describes strategies used to express OPH in *Pseudomonas jinjuensis* biotype *acephalyticus*. To express *opd* gene in *P. jinjuensis* biotype *acephalyticus* conventional vectors used to achieve heterologous expression of cloned genes in *E. coli* are not suitable. Therefore a broad host range mobilizable expression vector pMMB206 (Morales et al., 1991) is selected for expressing *opd* gene in *P. jinjuensis* biotype *acephalyticus*. However, pMMB 206 has chloramphenicol resistance marker and it can't be used to mobilize into chloramphenicol resistant *P. jinjuensis* biotype *acephalyticus*. Therefore the pMMB206 backbone was manipulated by replacing the chloramphenicol resistant gene with a kanamycin resistant marker taken from pUC4 (Mazodier et al., 1985). The chloramphenicol resistant marker from pMMB206 was excised from pMMB206 by digesting the vector with *Dra*I and a kanamycin marker from pUC4, obtained as *Sma*I fragment was cloned in pMMB206. The resulting expression vector is designated as pMMBKM206 and used as vector to clone and express *opd* gene and its variants in *P. jinjuensis* biotype *acephalyticus*. (Fig. 5. 4)

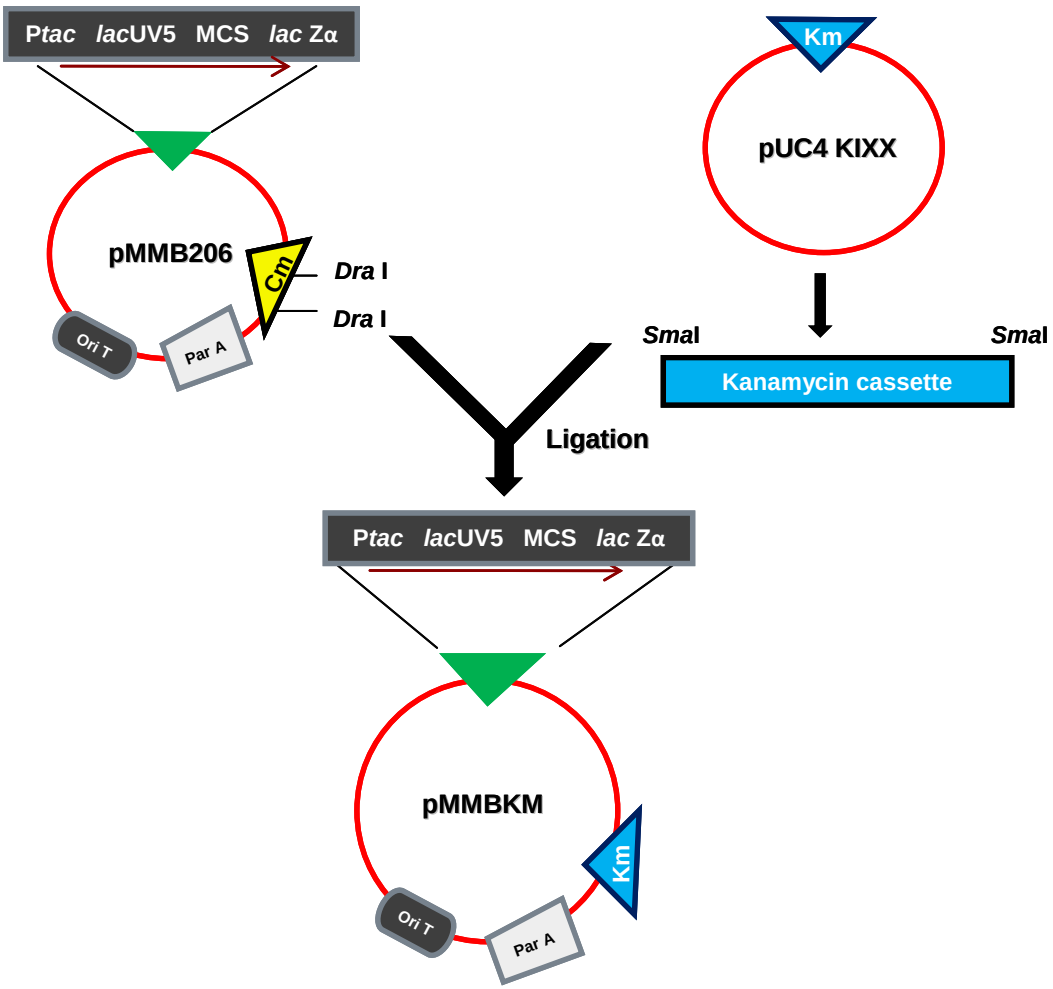


Fig. 5.4. Construction of expression vector pMMBKM.

5.41. Expression of *opd* gene with its native promoter

The expression plasmid pMMBKMOPD was constructed by cloning the *opd* gene taken as *Bam*HI fragment available in pSS15 (Siddavattam et al., 2006) in pMMBKM206. A detailed strategy used to construct pMMBKMOPD is shown in Fig.5.5. In the expression plasmid pMMBKMOPD, the *opd* gene contains its native promoter. The 1.5 kb *Bam*HI fragment not only contain *opd* gene it also contains transcriptional terminator sequence of *ist*B gene upstream of the *opd* promoter. Therefore the transcription initiated from vector specific *lac*UV5 promoter is

terminated before reaching the *opd* gene and hence if expression of OPH is seen it got to be solely from the native promoter of *opd* gene (Fig.5.5).

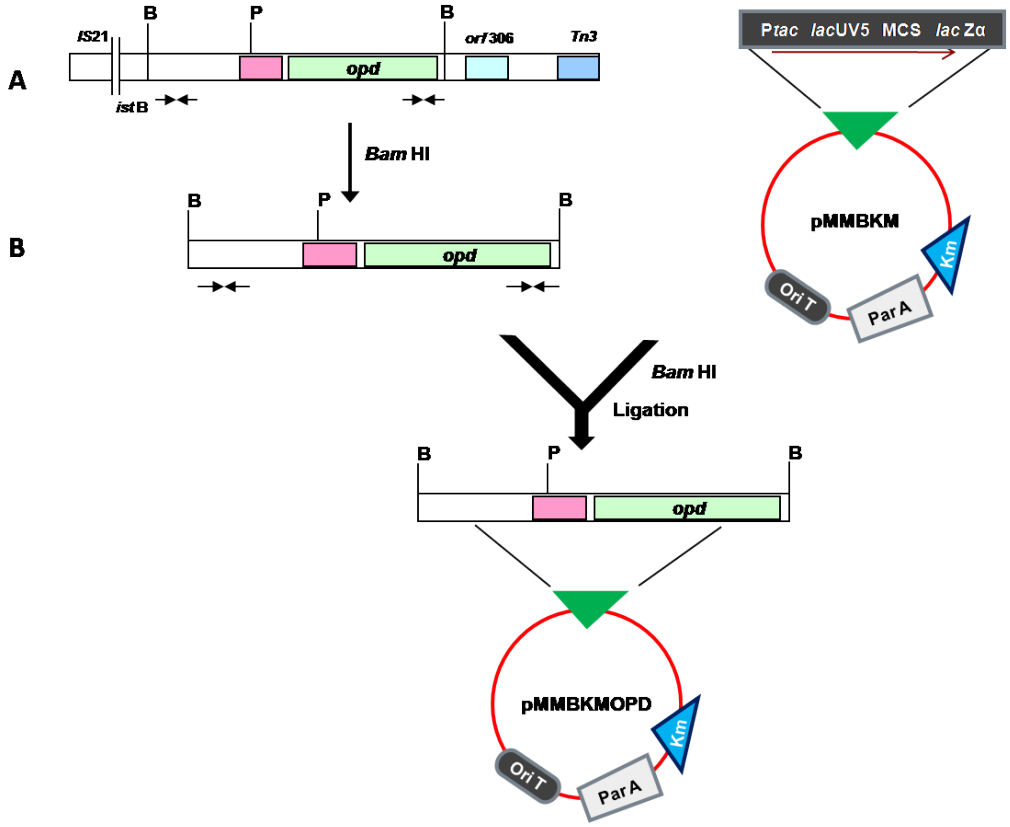


Fig. 5.5. Cloning strategy used to clone *opd* with its native promoter. Panel A indicates organization of *opd* gene cluster. The terminator sequences of *ist* B and *opd* genes are shown with inverted arrows. Panel B shows cloning of *opd* gene as *Bam* HI fragment.

5.42. Expression of OPH in the inner membrane of *P. jinjuensis* biotype *acephalyticus*

In our previous studies our lab has shown that OPH is a Tat substrate and it targets the periplasmic space of the inner membrane in a prefolded state (Gorla et al, 2009). If *opd* gene is cloned with its signal sequence, it's expected to direct the OPH to the periplasmic space. Our lab has generated expression plasmid pHYS400 to

express precursor form of OPH in *E.coli* with C-terminal his tag (Siddavattam et al., 2006). This plasmid is used as source and *opd* gene is amplified by designing vector specific primers taking the sequences found upstream of ribosomal binding site and downstream of transcriptional terminator sequence. An *Eco*RI site was included in the forward primer (5'GGGAGACCACAAC**GAA TTC**CCTCTAGAAA3'), whereas a *Bam*HI is included in the reverse primer (5'GCTCAGC**GGATCC**AGCAGCCAACTCAGC3'). Inclusion of these restriction sites facilitates cloning of the ORF of *opd* gene containing vector pET23b specific ribosomal binding site and transcription terminator sequence under the control of p*tac* *lac*UV5 promoter of expression vector pMMBKM. The cloning strategy followed to generate pHLCKMOPD is shown in Fig. 5.6. As the expression plasmid pHLCKMOPD contains pMMB206 vector backbone, it can be mobilized into *Pseudomonas jinjuensis* biotype *acephalyticus* by following conventional conjugation protocols (Simon et al., 1983). Further the localization of OPH can be monitored by performing anti-His antibodies as it has C-terminal his-tag.

5.43. Expression of cytoplasmic OPH in *Pseudomonas jinjuensis* biotype *acephalyticus*

An identical strategy followed to construct pHLCKMOPD was used for constructing expression plasmid coding for OPH without signal peptide (mOPH), except that the plasmid pHNS400 (Pandey et al., 2009) was used as a template. In the plasmid pHNS400, the *opd* gene without signal sequence was cloned as *Nde*I and *Xho*I fragment. The 3' end of the *opd* gene was altered in such a way that the stop codon was mutated to fuse with the vector encoded sequences that ensures addition of

a 6x His-tag immediately after *opd* coding sequences. This pHNS400 plasmid was used as a template to amplify *opd* gene along with ribosomal binding site and the region that specifies his-tag by using the vector specific forward (5'GGGAGACCACAAC**GAATTC**CCTCTAGAAA3') and reverse primer (5'GCTCAGC**GGATCC**AG CAGCCAACTCAGC3') having *EcoRI* and *BamHI* respectively. The amplicon was then cloned as *Eco*RI and *Bam*HI fragment in pMMBKM to keep the *opd* gene under the control of *lac*UV5 promoter and the resulting plasmid was designated as pHNSKMOPD (Fig.5.6).

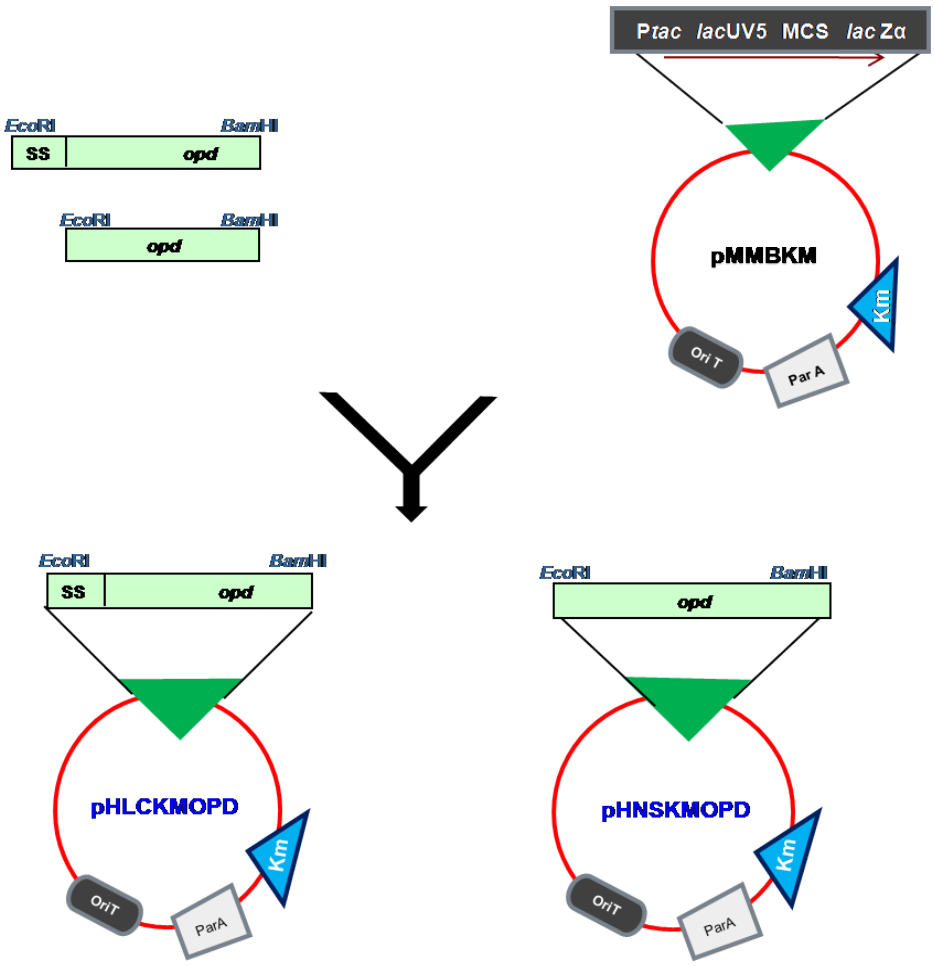


Fig. 5.6. Strategy used for construction of expression plasmids coding precursor (preOPH) and mature (mOPH) forms of OPH with C-terminal his-tags.

5.5. Mobilization of expression plasmids into *Pseudomonas jinjuensis* biotype *acephalyticus*

The expression plasmids pMMBKMOPD, pHLCKMOPD and pHNSKMOPD were mobilized into *Pseudomonas jinjuensis* biotype *acephalyticus* by conjugation (Simon et al., 1983) experiments. The expression plasmids pMMBKMOPD, pHLCKMOPD and pHNSKMOPD were first independently transformed into *E.coli* S17.1 which serves as conjugal donors for the transfer of the plasmids to the recipient *Pseudomonas jinjuensis* biotype *acephalyticus*. The conjugation experiments were performed following the protocols described in materials and methods section. The exconjugants were selected on chloramphenicol (30 µg/ml) and kanamycin (100 µg/ml) plates. Strategy used for mobilization of expression plasmids is shown in Fig.5.7.

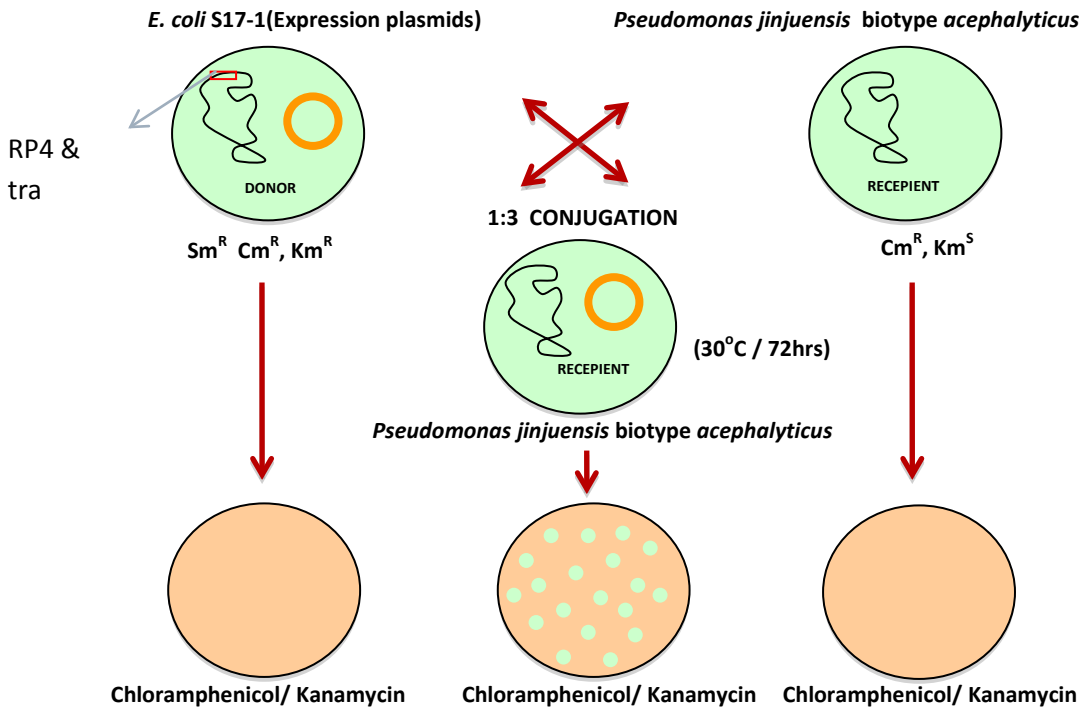


Fig. 5.7. Mobilization of expression plasmids into *P.jinjuensis* biotype *acephalyticus* by conjugation.

5.6. Screening of exconjugants for the presence of expression plasmids

5.61. Growth on acephate kanamycin plates: The purity of the exconjugants was checked by growing the cells on acephate, kanamycin plates. All the three exconjugants have grown very well on both acephate/kanamycin plates indicating presence of expression plasmid in *P .jinjuensis* biotype *acephalyticus.* No such growth was recorded in the wild type *Pseudomonas jinjuensis* biotype *acephalyticus.* (Fig.5.8).

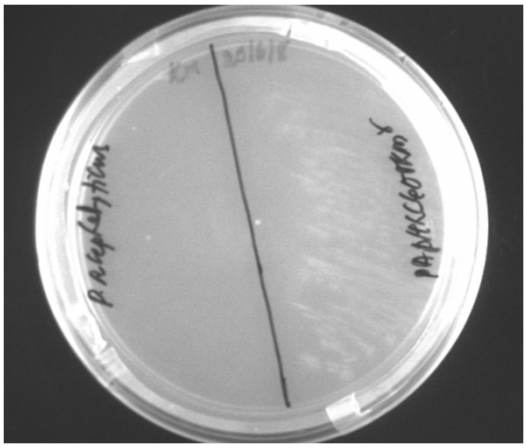


Fig. 5.8. Growth of exconjugants on acephate kanamycin plate. The wild type cells, sensitive to kanamycin failed to grow on acephate kanamycin plates.

5.62. Colony PCR using *opd* specific primers: After establishing growth properties of exconjugants single colonies developed on kanamycin and acephate plates were used to perform colony PCR using *opd* specific primers. There was a clear amplification of *opd* gene in exconjugants and no such amplification is seen in wild type cells. These results reinforce the growth behaviour of exconjugants and confirm existence of expression plasmids containing *opd* variants in *P.jinjuensis* biotype *acephalyticus* (Fig.5.9).

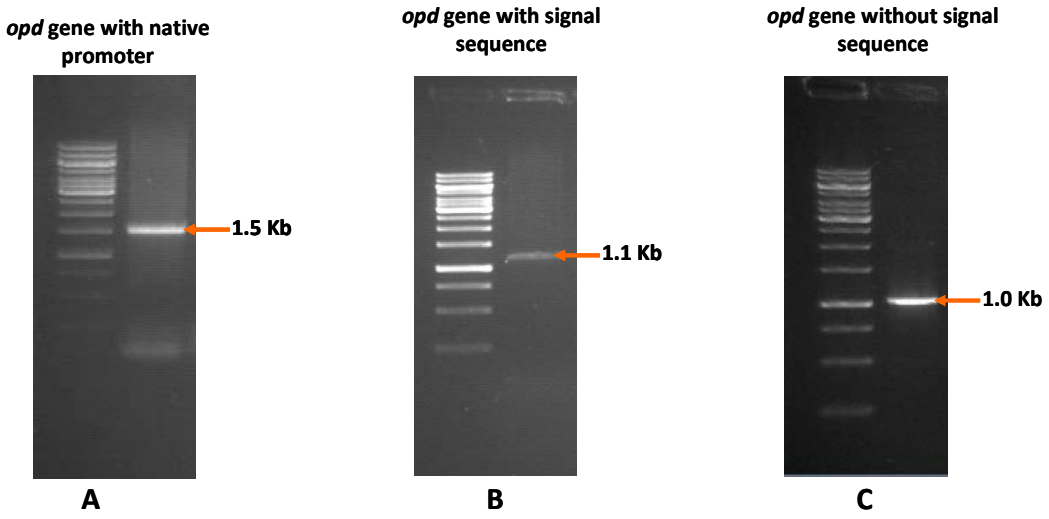


Fig. 5.9. Colony PCR showing the existence and stable maintenance of expression plasmids pMMBKMOPD, pHLCKMOPD and pHNSKMOPD in *P.jinjuensis* biotype *acephalyticus.* In Panel A Lane 1 kb ladder, lanes 2 represent exconjugants of *P.jinjuensis* biotype *acephalyticus* containing pMMBKMOPD. Panel B, Lane 1 kb ladder, lane 2 represent exconjugants containing expression plasmid pHLCKMOPD. Panel C, Lane 1 kb ladder, lane 2 represents exconjugants containing expression plasmid pHNSKMOPD.

5.7. Expression of OPH in *Pseudomonas jinjuensis* biotype *acephalyticus*

The exconjugants containing pMMBKMOPD contain *opd* gene with its native promoter. Therefore there is no need to induce the cultures following conventional methods. The mid log phase cultures of *P.jinjuensis* biotype *acephalyticus* (pMMBKMOPD) were directly used to fractionate the cells and to obtain membrane and cytoplasmic fractions. However, the cells containing pHLCKMOPD and PHLNSKMOPD were induced by conventional methods described in materials and methods section. The cultures expressing *opd* variants were taken to prepare cytoplasmic and membrane fractions and these fractions were further used to assay for OPH activity and to analyze for existence of precursor and mature forms of OPH through western blots.

5.71. Expression of *opd* gene with native promoter

While assessing the expression of *opd* gene using its own promoter two strategies was followed. Fist strategy was to assay OPH activity in whole cell extracts and sub-cellular fractions. As shown in Fig. 5.10 there was no OPH activity in exconjugants containing pMMBKMOPD suggesting that the *opd* gene when cloned with its native promoter is not expressed in *P.jinjuensis* biotype *acephalyticus* (Fig.5.10). To rule out the possibility of having inactive OPH, protein gels were prepared for the subcellular extracts and western blots performed using anti-OPH antibodies. Western blots failed to give OPH specific signals confirming the lack of expression of OPH in exconjugants having expression plasmid pMMBKMOPD (Fig.5.11).

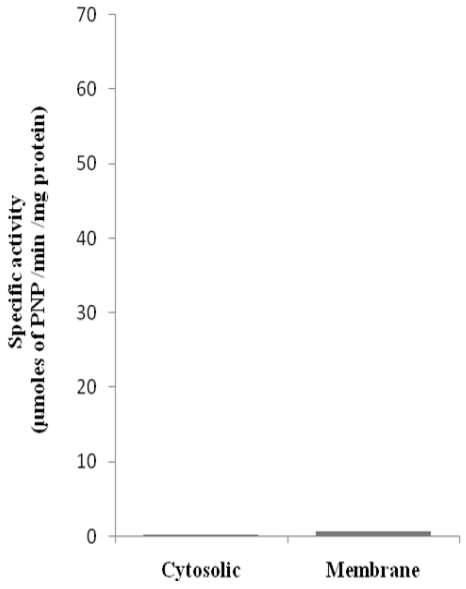


Fig. 5.10. OPH activity in subcellular fractions. The bar diagram represents OPH activity obtained in cytoplasmic and membrane fractions prepared from *P.jinjuensis* biotype *acephalyticus* (pMMBKMOPD).

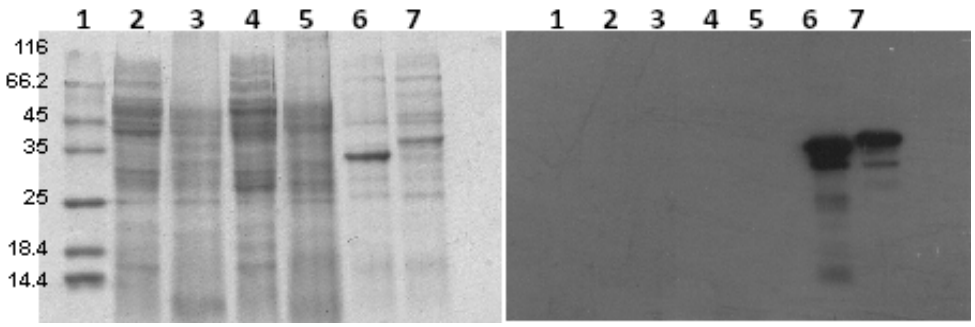


Fig. 5.11. Subcellular localization of OPH in *P.jinjuensis* biotype *acephalyticus* (pMMBKMOPD). Panel A) SDS-PAGE analysis of subcellular fractions. Panel B represents corresponding western blots performed using OPH antibodies to detect OPH. Lanes 2 and 3 represent protein extracts prepared from cytoplasmic and membrane fractions of wild type *P.jinjuensis* biotype *acephalyticus.* Lanes 4 and 5 represent similar extracts prepared from strains containing expression plasmid pMMBKMOPD. Partially purified mOPH and preOPH used as size markers are loaded in lanes 6 and 7. The OPH specific signal is not detected in any subcellular fractions.

5.72. Expression and sub cellular localization of preOPH in *Pseudomonas jinjuensis* biotype *acephalyticus*

The subcellular fractions of induced cultures of P. *jinjuensis* biotype *acephalyticus* (pHLCKMOPD) were used for subcellular localization of OPH. When OPH activity was assayed most of it was found in the membrane fractions. Its quantity was very negligible in cytoplasmic fraction (Fig.5.12). In support of this result, even the western blots performed to detect OPH using anti-His antibodies gave very prominent signal only in lanes loaded with proteins extracted from the membrane fractions (Fig.5.12). The size of the signal has perfectly matched with the signal obtained for mOPH used as marker protein, implicating that the expressed preOPH is processed in *P.jinjuensis* biotype *acephalyticus* (Fig.5.13, Lane 5).

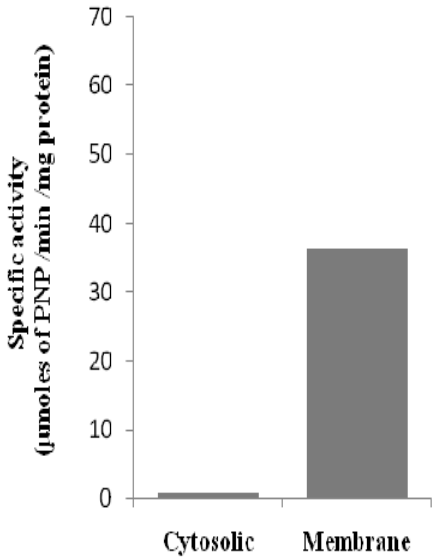


Fig. 5.12. OPH activity in subcellular fractions of *P.jinjuensis* biotype *acephalyticus* (pHLCKMOPD). The bar diagram represents OPH activity obtained in cytoplasmic and membrane fractions.

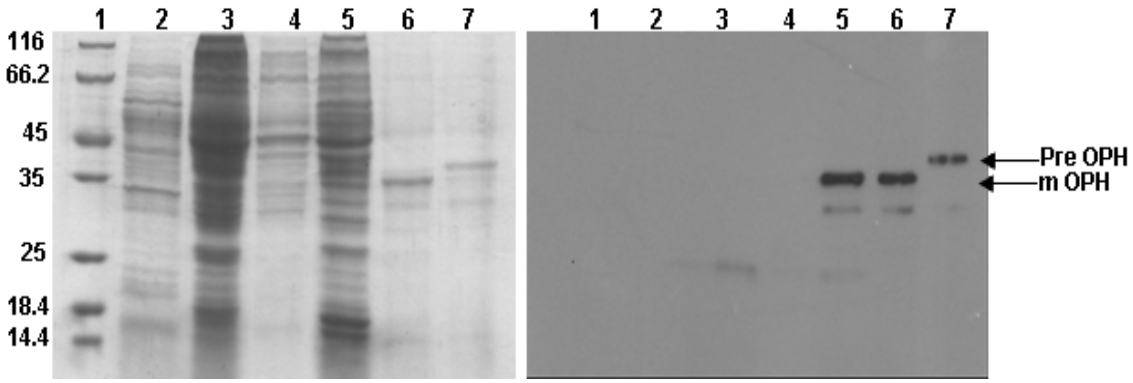


Fig. 5.13. Subcellular localization of preOPH in *P.jinjuensis* biotype *acephalyticus* (pHLCKMOPD). Panel A) SDS-PAGE analysis of subcellular fractions. Panel B represents corresponding western blots performed using anti-His antibody to detect OPH-6His. Lane 2 and 3 represent protein extracts prepared from cytoplasmic and membrane fractions prepared from wild type *P.jinjuensis* biotype *acephalyticus*. Lane 4 and 5 represent similar extracts prepared from strains containing expression plasmid pHLCKMOPD. Partially purified mOPH and preOPH used as size markers are loaded in lanes 6 and 7. The OPH specific signal corresponding to the size of mOPH is seen in lane 5.

5.73. Expression and subcellular localization of mOPH in *Pseudomonas jinjuensis* biotype *acephalyticus*

Experiments related to expression of mOPH are done to obtain *P.jinjuensis* biotype *acephalyticus* cells with cytoplasmically located OPH. The logic behind conducting such experiments is discussed in detail in subsequent sections of this chapter. As described in the preceding sections the sub-cellular fractions prepared from *P.jinjuensis* biotype *acephalyticus* (pHNSKMOPD) were used to assay for OPH activity and detect mOPH. As expected, the OPH activity was found only in cytoplasm. There was no OPH activity in the membrane fraction (Fig.5.14). In consistent of this observation, the OPH specific western signal is seen only in cytoplasmic fraction. (Fig.5.15).

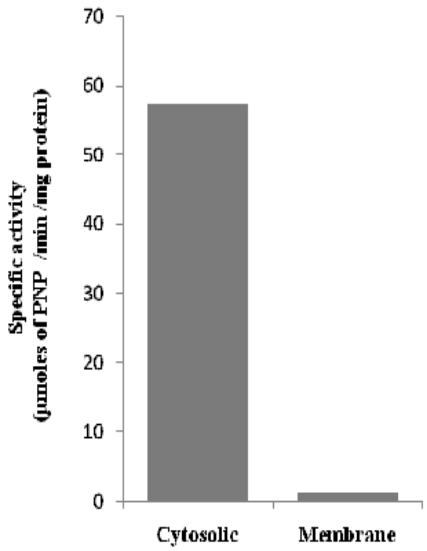


Fig. 5.14. OPH activity in subcellular fractions. The bar diagram represents OPH activity obtained in cytoplasmic and membrane fractions prepared from *P.jinjuensis* biotype *acephalyticus* (pHNSKMOPD).

Such observations testify the OPH ability to fold prior to targeting the membrane and confirm that the cytoplasmically located OPH is active in *P.jinjuensis* biotype *acephalyticus*. (Fig.5.15, Lane 4).

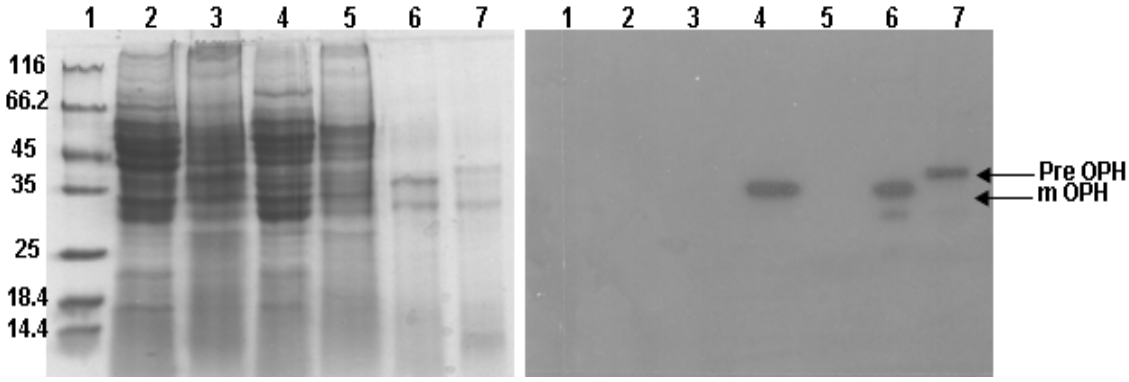


Fig. 5.15. Subcellular localization of mOPH in *P.jinjuensis biotype acephalyticus* (pHNSKMOPD). Panel A) SDS-PAGE analysis of subcellular fractions. Panel B represents corresponding western blots performed using anti-His antibody to detect OPH-6His. Lanes 2 and 3 represent protein extracts prepared from cytoplasmic and membrane fractions prepared from wild type *P.jinjuensis biotype acephalyticus*. Lanes 4 and 5 represent similar extracts prepared from strains containing expression plasmid pHNSKMOPD. Partially purified mOPH and preOPH used as size markers are loaded in lanes 6 and 7. The OPH specific signal corresponding to the size of mOPH is seen in lane 4.

5.8. Degradation of organophosphates by engineered strains of *Pseudomonas jinjuensis* biotype *acephalyticus*

After ascertaining the expression and subcellular localization of OPH the manipulated strains expressing membrane and cytoplasmic OPH were tested for their ability to degrade op compounds other than acephate. Further, the influence of OPH expression was also tested on the strains innate ability to use acephate as carbon source.

The induced cultures of *P.jinjuensis* biotype *acephalyticus* expressing either preOPH or mOPH were supplemented with 100 µmole of methyl parathion. The

concentration of the methyl parathion and formation of *p*-nitrophenol due to the OPH mediated hydrolytic cleavage of substrate were periodically tested by taking absorbance at 270 and 410 nm. As reported in table 1 the methyl parathion added to the induced culture has quickly disappeared leading to the accumulation of PNP in the culture medium.

Table.5.1. Degradation of organophosphates methyl parathion and paraoxon by engineered *P.jinjuensis* biotype *acephalyticus* in culture medium.

Time in min	**Methyl parathion**	***p*-Nitrophenol**
0 min	100 µmole	0
15 min	45.5 µmole	54.5 µmole
30 min	36.4 µmole	63.6 µmole

Time in min	**Paraoxon**	***p*-Nitrophenol**
0 min	100 µmole	0
15 min	39.4 µmole	60.6 µmole
30 min	2.3 µmole	97.7 µmole

Even the spectra (200nm to 600nm) obtained at different time intervals showed time dependent increase in peak height at 410 nm suggesting that *p*-nitrophenol accumulation in the culture media. Such increase was not seen in the uninduced and wild type cultures (Fig.5.16). In fact the cultures expressing both preOPH and mOPH have shown more or less similar degradation efficiency. Similar results were obtained when experiments were conducted using paraoxon instead of

methyl parathion (Fig.5.17) reassuring the strains ability to degrade wide range of op compounds due to existence of *opd* gene.

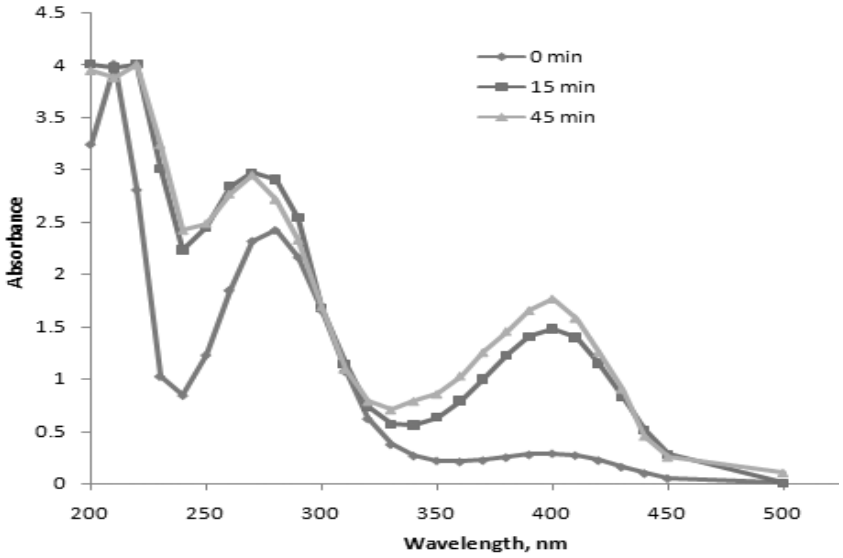


Fig. 5.16. Degradation of Parathion by *Pseudomonas jinjuensis* biotype *acephalyticus* expressing mOPH.

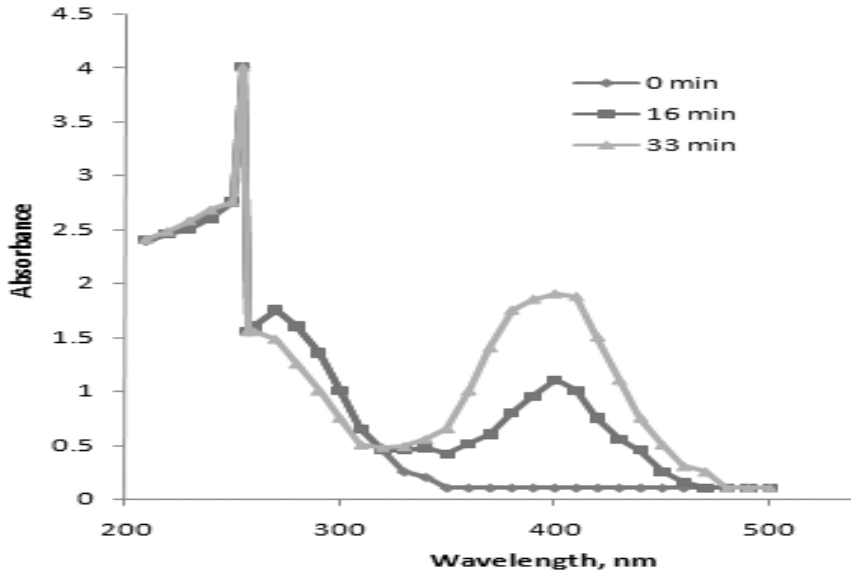


Fig. 5.17. Degradation of Paraoxon by *Pseudomonas jinjuensis* biotype *acephalyticus* expressing mOPH.

5.9. Influence of OPH expression on strains acephate mineralization ability

After establishing the manipulated strains ability to successfully degrade wide range of op compounds, the manipulated strain was again tested to know if the expressed OPH has any influence on its acephate degradation ability. While trying to gain evidence on this point, the strain was grown in minimal medium having acephate as carbon source and the growth properties and OPH mediated methyl parathion hydrolysis was tested. The cells have successfully grown in acephate medium and quickly hydrolyzed methyl parathion due to successful expression of OPH. In the spent medium methamidophos peak is also seen indicating active acephate metabolism in the manipulated strains of *P.jinjuensis* biotype *acephalyticus* (Fig.5.18). Such observations rather suggest possible usage of manipulated strains for successful elimination of op residues from the contaminated strains.

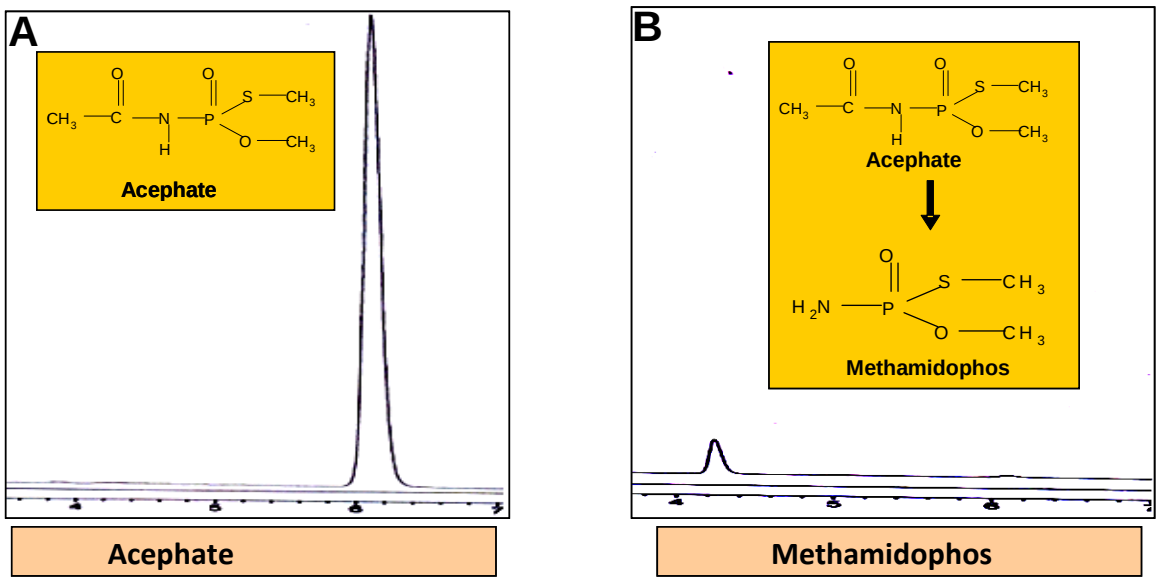


Fig. 5.18. Degradation of acephate by *P.jinjuensis* biotype *acephalyticus* expressing mOPH. Detection of acephate (Panel A) and its degradation product methamidophos (Panel B) is seen in the chromatogram obtained by performing LC-MS.

5.10. Discussion

Ever since, the extraordinary power of OPH to degrade structurally diverse group of op compounds is discovered, a number of investigators have attempted to achieve heterologous expression of *opd* gene (Mulbry and Karns, 1989). The studies on heterologous expression of *opd* gene, for simplicity and understanding, can be divided in to three categories. The first category attempted to express *opd* gene from its own promoter, which have given mixed results. When expressed in *E.coli* by cloning *opd* gene with indigenous promoter, no OPH activity was seen (Mulbry and Karns, 1989). The active OPH is produced only when the 5' region of *opd* gene specifying signal peptide coding region is removed and fused to the *lac* promoter (Mulbry and Karns, 1989). However, in contrast to these experiments, the OPH is expressed as an extracellular protein, when *opd* gene cloned in *Streptomyces lividans* using its indigenous promoter (Rowland et al., 1991; Steiert et al., 1989). The *Streptomyces lividans* transcriptional machinery successfully recognized *opd* promoter and facilitated production of an active OPH. Further, the protein secretion machinery of *Streptomyces lividans* successfully directed the OPH in to extracellular environment. This is rather a very unusual observation. There is no experimental evidence till date to explain, how a signal peptide that facilitates membrane targeting of OPH in native species, is directing OPH to the extracellular environment. In fact the OPH expressed in *Streptomyces lividans* served as good source for producing active OPH to be used for cleaning the containers used to preserve op compounds.

The second category expression strategies exploited availability of second generation expression vectors. Most of the expression plasmids used to achieve

heterologous expression of OPH have cloned *opd* gene by eliminating signal peptide coding sequence. Deletion of 5' region of *opd* gene while achieving heterologous expression was found to be advantageous in number of ways. Initially, the signal peptide unlike in other cases, is not required for OPH to acquire active confirmation (Pandey et al., 2009). Expression of OPH without signal peptide helped in producing cytoplasmic OPH and thus contributed for reducing cumbersomeness associated with purification of OPH. As shown from the studies of our own laboratory, the signal peptide coding region of OPH has inverted repeated sequence with a potential to form stable stem loop structure. Destabilization or elimination of stem loop has significantly elevated the quantity of OPH produced in *E.coli* cells (Pandey et al., 2009). This has really contributed for achieving large scale production of OPH.

The third category of OPH expression studies are based on manipulation of signal peptide. Though OPH is an excellent enzyme for the detoxification of organophosphates, its accessibility to substrate will be limited due to outer membrane permeability. The outer membrane acts as permeability barrier and prevents the pesticides from interacting with the OPH, anchored to the inner membrane of the cells (Richins et al., 1997). Hence expression of OPH on cell surface is considered to be more advantageous than expressing it in either cytoplasm or in membrane. This can be achieved by the fusion of *opd* gene with various surface-anchoring signal peptides that possess the potential to cross both the cytoplasmic and outer membranes. For example fusing OPH to the lipoprotein-outer membrane protein A (Lpp-OmpA), ice nucleation protein (INP) (Samuelson et al., 2002; Lee et al., 2003) take OPH on the surface of the cells. Recently, active OPH has been successfully expressed on the cell surface of *Escherichia coli* by using either the Lpp-OmpA fusion system (Richins et

al., 1997) or the truncated ice nucleation protein (INPNC) anchor (Shimazu et al., 2001). Cultures with surface-expressed OPH degraded parathion and paraoxon very effectively without the transport limitation observed in cells expressing OPH intracellularly (Shimazu et al., 2001).

While planning to express OPH in *Pseudomonas jinjuensis* biotype *acephalyticus* we have followed first two strategies i.e. expression of *opd* gene using its native promoter, the second being the one which expressed OPH from inducible *lac*UV5 promoter. The first attempt has met with a failure. As seen in case of *E.coli* the *opd* gene is not expressed when cloned with its indigenous promoter. However, the expression of OPH is successfully seen when *opd* gene is cloned under the control of inducible *lac*UV5 promoter. Interestingly, the precursor form of OPH (preOPH) which contains signal peptide successfully targeted to the membrane in *P. jinjuensis* biotype *acephalyticus*. The results presented in this study gave clear indication to claim that the preOPH expressed is processed and successfully targeted to the membrane. The size of the OPH associated with the membrane of *P. jinjuensis* biotype *acephalyticus* clearly matches with the size of recombinant mature OPH (mOPH) expressed in *E.coli* to use as molecular size marker.

In our previous studies our lab has shown existence of twin arginine transport motif (T-R-R-V-V-L-K) in the signal peptide of OPH (Gorla et al., 2009). Existence of twin arginine motif in the N-terminal signal peptides of precursor proteins targets to the Tat (twin-arginine translocation) protein export pathway (Berks et al., 2000). The energy for this transport comes from transmembrane proton electrochemical gradient (Hulford et al., 1994; Yahr and Wickner, 2001). The genetics of bacterial Tat

pathway is well studied in *E.coli*. The Tat pathway mainly comprises of *tatA*, *tatB*, *tatC* and *tatE* genes (Bogsch et al. 1998; Sargent et al. 1998, 1999; Weiner et al. 1998). The *tatA*, *tatB*, and *tatE* gene products are sequence-related proteins that are each predicted to comprise a transmembrane N-terminal α-helix followed by an amphipathic α-helix at the cytoplasmic side of the membrane. The *tatC* gene product is predicted to be a polytopic membrane protein with six transmembrane helices. Genetic experiments have shown TatA and TatE to have overlapping functions on the Tat pathway. Of the three Tat proteins, TatBC forms a complex and interacts with Tat signal peptides (Alami et al., 2003) where as TatA forms a transport channel which takes folded proteins across the membrane in the presence of transmembrane proton electrochemical gradient (Leake et al., 2008) (Fig.5.19). As stated before, in our previous studies we have shown OPH as a Tat substrate and essentiality of twin arginine motif (T-R-R-V-V-L-K) for membrane targeting of OPH. OPH has utilized the Tat specific translocases that exists in the wild type *Brevundimonas diminuta* and is transported to the periplasmic face of the inner membrane. If the results generated in the present study are taken into consideration, the preOPH expressed *P.jinjuensis* biotype *acephalyticus* is processed and correctly targeted to the membrane (Fig 5.13). When OPH was expressed without its signal peptide the OPH was retained in cytoplasm suggesting that signal peptide is prerequisite for membrane targeting. It appears that in *Pseudomonas jinjuensis* biotype *acephalyticus*, the OPH processed using similar processing mechanism followed in wild type *B.diminuta*.

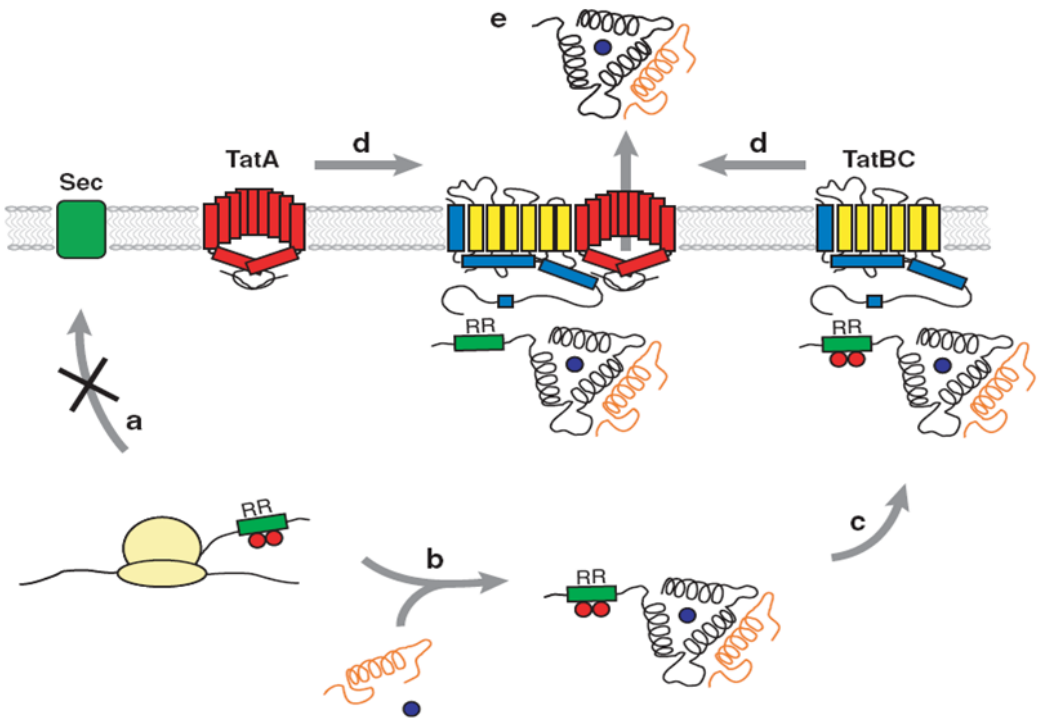


Fig. 5.19. Model of Tat targeting and transport. In the membrane TatA is depicted in red, TatB in blue, and TatC in yellow. (*a*) Upon emerging from the ribosome the preprotein must avoid targeting to other pathways such as Sec, which is aided by the characteristics of the signal peptide and mature protein and/or the binding of Tat-specific chaperones (red circles). (*b*) After folding, any cofactors and/or additional subunits are added prior to targeting to the TatBC receptor complex. (*d*) The proton motive force drives the formation of an active translocase and the substrate is transported through a pore consisting mainly of TatA. (*e*) Upon removal of the signal peptide the mature protein is released on the periplasmic side of the membrane (Lee et al., 2006).

Justifying our previous claim, the mOPH expressed in the cytoplasm of *P.jinjuensis* biotype *acephalyticus* acquired folded confirmation and didn't depend on signal sequence. Even the OPH produced without signal peptide is seen more in quantity (Fig. 5.15, Lane 4). This is infact supportive of our study where we have shown that the secondary structure of *opd* specific mRNA dependent down regulation of OPH (Pandey et al., 2009).

The engineered strain expressing OPH degraded methyl parathion, parathion and paraoxon. There was no influence of OPH expression on the strains innate ability of using acephate as source of carbon, nitrogen and sulfur. Such properties of manipulated *P. jinjuensis* biotype *acephalyticus* puts it ahead of all other bioremediation strategies being used for elimination of op compounds.

5.11. Conclusions

1. Mobilizable expression vector is constructed for achieving heterologous expression of OPH.
2. The preOPH expressed in *Pseudomonas jinjuensis* biotype *acephalyticus* successfully processed and targeted to the membrane.
3. The mOPH expressed in *P. jinjuensis* biotype *acephalyticus* is active and localized in cytoplasm.
4. The manipulated *P. jinjuensis* biotype *acephalyticus* degraded number of op compounds added to the culture medium. Expression of OPH showed no influence on strains ability to thrive on acephate.

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