Introduction and Literature Review

1.1 Introduction

Methanol is a valuable raw material used in the manufacture of useful Chemicals as well as a potential source of energy to replace coal and petroleum. Biotechnological interest in the microbial utilization of methanol has increased because it is an ideal carbon source and can be produced from renewable biomass. Formaldehyde, a cytotoxic compound, is a central metabolic intermediate in methanol metabolism. Therefore, microorganisms utilizing methanol have adopted several metabolic strategies to cope with the toxicity of formaldehyde. Formaldehyde is initially detoxified through trapping by some cofactors, such as glutathione, mycothiol, tetrahydrofolate, and tetrahydromethanopterin, before being oxidized to CO2. Alternatively, free formaldehyde can be trapped by sugar phosphates as the first reaction in the C1 assimilation pathways: the xylulose monophosphate pathway for yeasts and the ribulose monophosphate (RuMP) pathway for bacteria. The key enzymes of the RuMP pathway are found in a variety of microorganisms including bacteria and archaea. Regulation of the genes encoding these enzymes and their catalytic mechanisms depend on the physiological traits of these organisms during evolution (Yurimoto *et al.*, 2005).

Methanol is distributed in a wide variety of environments causing contamination, and cleaning such contaminated sites by physio-chemical is expensive and laboratory intensive, and often leads to air pollutions .A better way would be to use biological degradation, such process is usually cheap and environmentally friendly and entails on major technical hurdles. (Atlas and Bartha, 1998).

A problem, however, is the shortage of appropriate microorganisms capable of degrading synthetic chemicals at a reasonably high rate (Chakrabarty, 1996). While microorganisms can degrade most natural compound, they often lack appropriate enzyme to degrade many synthetic compound (Pringnitz and Hartzler, 2001).

To overcome this obstacle ,using molecular and other tools to enhance gene recruitment and metabolic pathway activity in microorganisms, enabling them to degrade these compounds, and also to enhance the activity of these microorganisms and develop means to bring the contaminates into direct contact with the organisms to achieve optimal bioremediation.(Doyle *et al*., 1991; Miyauchi *et al.*,1998).

Several high techniques may form the basis for developing new microbial strains with enhanced appetites for toxic chemicals (Ramos *et al* ., 1994).

According to those mentioned above and because of the limited studies about methanol utilizing bacteria in our country, the aim of this study was to focus on:

1. Isolation and identification of methanol utilizing bacteria and selecting the efficient methanol utilizing isolates.
2. Determination the plasmid profile for the efficient isolates.
3. Determination the role of the plasmids in methanol utilizing for the most efficient isolate by curing and transformation experiments.

Literatures Review

1.2 Methylotrophic Bacteria

Methylotrophic bacteria are these aerobic bacteria that utilize one - carbon compounds as sources of carbon and energy and assimilate formaldehyde as a major source of cellular carbon (Anthony, 1991). Many methylotrophic bacteria have been recognized since 1906, when Sohngen first, reported the isolation of bacteria capable of growing on methane. After that Bassalik observed growth of *Bacillus extorquens* on methanol in 1914.

The need for inexpensive carbon-energy sources for production of single-cell protein has stimulated interest in methane and methanol metabolism (Cooney *et al.*, 1971).

Methylotrophic bacteria utilize a variety of different one-carbon compounds, including methane, methanol, methylated amines, halomethans and methylated compound containing sulfur (Anthony, 1986).

Methylotrophy is wide spread across bacterial groups and found in alpha, beta, and gamma-proteobacteria and in gram positive bacteria. Many representatives of methylotrophic bacteria, such as *Methylobacterium extorquens*, *Methylococcus capsulatus, Paracoccus dentrificans* and *Methylobacillus flagellatus* are readily cultivated in the laboratory, lending themselves to biochemical, physiological, and genetic studies. Whether these and other cultivated methylotrophes fully reflect the makeup of organisms in the environment and where they fit into the evolutionary history of methylotrophy are among the central unanswered questions facing researchers who study these organisms. Recent efforts to use environmental genomics as a mean for analyzing methylotrophs are

providing valuable insights into C_1- cycling populations in natural habitats (Chistoserdova , 2003).

The methylotrophes constitute a diverse group of microorganisms that utilize reduced one-carbon (C_1) compounds, such as methanol. The abundance, purity and low price of methanol compared with sugars, make methylotrophes interesting candidate organisms for production of amino acids, vitamins, cytochromes ,coenzymes, single-cell proteins and recombinant proteins, (Brautaset *et al.*, 2004).

Some of these methylotrophic bacteria are:

1.2.1 Genus : *Bacillus*

Bacillus methanolicus, a thermotolerant soil methylotrophic Gram-positive bacterium, isolated from different locations for its ability to grow on methanol at elevated temperatures.

Bacillus methanolicus can grow on temperatures up to 60ºC, with an optimum between 50-55ºC. With the exception of mannitol, no sugars have been reported to support rapid growth of this organism, which is classified as a restrictive methylotroph.

B. methanolicus uses NAD (P)-dependent methanol dehydrogenase (MDH) to oxidize methanol to formaldehyde (Brautaset *et al.*, 2004).

1.2.2 Genus: *Acidomonas*

Acidomonas methanolica, a gram negative methanol-utilizing bacterium, isolated from activated sludge from three different sewage treatment plants in Tokyo, based on 16S rRNA sequences. All strains formed a single cluster within the Acetobacteriaceae that was clearly different from the genera *Acetobacter, Gluconobacter, Gluconoacetobacter (*Yamashita *et al.*, 2004).

A. methanolica is the type and only species reported so far in this genus. Members of the genus *Acidomonas* are recognized as methanol-utilizing bacteria (Bulygina *et al.*, 1990).

Strain BNS-25 (an acidophilic methanol utilizing bacterium) was one regarded as a promising tool for the production of single-cell proteins (SCPs) from methanol, this strain was later identified as a member of *A. methanolica* (Urakami *et al.*, 1985).

On the other hand, phylogenetic relationship of the genus *Acidomonas* to acetic acid bacteria (the family Acetobacteriaceae), was first suggested by 5S rRNA sequencing (Bulygina *et al.*, 1992) this finding was confirmed by 16S rRNA sequences (Boesh *et al.*, 1998) .

1.2.3 *Paracoccus denitrificans*

Paracoccus denitrificans is a nutritionally versatile bacterium found in soil, sewage, and sludge. The ability of the organism to adapt its metabolism to a variety of carbon and free energy sources may reflect the nature of its natural environment. *P. denitrificans* can grow heterotrophically on a variety of carbon sources and lithoautotrophically using hydrogen, thiosulfate, or reduced C_1 compounds (methanol, methylamine, or formate) as free energy source. Expression of the genes

encoding enzymes involved in C_1 metabolism is tightly regulated. The synthesis of methanol dehydrogenase (MDH) and methylamine dehydrogenase (MADH), the enzymes that catalyze the oxidation of methanol and methylamine to formaldehyde, respectively, is induced when the cells grow on methanol or methylamine as the sole free energy source but is repressed in cells grown on energetically more favorable substrates (De Vries *et al.*, 1988).

The synthesis of glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) and *S*-formylglutathione hydrolase (FGH), the enzymes that catalyze the oxidation of formaldehyde to formate, however, is not fully repressed under these conditions, since low but significant levels of both enzymes can be found in succinate-grown cells (Harms *et al.*, 1996).

These low levels may help ensure a rapid response to small amounts of adventitiously formed formaldehyde or to the formaldehyde first generated during growth with methylotrophic substrates. Low levels of MDH were found in cultures grown on a variety of carbon sources during carbon limitation in a chemostat. Under these conditions, the genes involved in methanol oxidation are expressed at basal levels; maximal expression was observed in cells grown on methanol, methylamine and choline. Oxidation of all these compounds yields formaldehyde, so it has been postulated that formaldehyde is an important trigger in the regulation of expression of gene clusters involved in C_1 metabolism (De Vries *et al.*, 1988).

1.2.4 Genus : *Beijerinckia*

The current definition of the genus *Beijerinckia* characterizes its members as nonsymbiotic, aerobic, chemo-heterotrophic bacteria with the ability to fix atmospheric dinitrogen (Becking, 1999).

Beijerinckia species utilize a wide range of multicarbon compounds. Members of this genus are typical rod-shaped cells with round ends containing polar lipoid bodies. Another distinctive feature of these bacteria is their acid tolerance, which allows them to grow and to fix dinitrogen at pH 3.0 to 4.0. The first isolates of this genus were obtained from acidic soils of tropical regions; later studies revealed that these bacteria are widely distributed in both acidic and neutral soils of different tropical and nontropical regions. One member of this genus is the *Beijerinckia mobilis, which* is capable of growing on methanol as the sole source of carbon and energy. Growth occurred under a wide range of methanol concentrations ranging from 0.01 to 3% (vol/vol). Optimum growth was observed between 0.05 and 0.5% (vol/vol) methanol. *B. mobilis* could be maintained continuously on methanol as the sole carbon and energy source without loss of viability (Dedysh *et al.*, 2005).

1.2.5 Genus : *Methylobacterium*

Methylobacterium is a facultative methylotroph, (meaning it has the ability to grow by reducing carbon compounds with one or more carbon atoms but no carbon-carbon bonds that grows on methylamine, methanol, and C2, C3 and C_4 compounds, including the methanol emitted by the stomata of plants). They are non-motile rod-shaped and are obligately aerobic; they are also called PPFMs-pink-pigmented facultative methylotrophs due to most *Methylobacterium* strains' pink pigmentation. *Methylobacterium* are common in soil and on surfaces of leaves and other plant parts .One of the most widly studied of methanol utilizing species of this genus is the pink-pigmented faculitative methylotroph, *M. extorquens* AM1. This organism is capable of growth on methanol and assimilates it at the level of formaldehyde by serine cycle and has served as the primary model system for the study of methylotrophic metabolism and methylotrophic enzymes (Chistoserdova, 1996).

1.3 Methanol

Methanol (CH_3OH), is the simplest aliphatic alcohol, and is the first member of the homologous series .Methanol is a colorless liquid, completely miscible with water and organic solvents, and is very hydroscopic. It has an agreeable odor, and a burning taste and it is a potent nerve poison .Methanol is made by a method different from that used in the preparation of the other alcohol. The naphtha fraction from the distillation of crude petroleum is used as a raw material for the manufacture of methanol. When naphtha is react with high stream ratio, under pressure and

at high temperature, synthesis gas of low methan content is obtained. Most of the carbon from the naphtha is converted to carbon monooxide and carbon dioxide, which can be removed from the gaseous mixture to leave hydrogen of high purity .When a mixture of hydrogen and carbon monooxide in ratio 2:1 is passed over a catalyst (e.g.: a mixture of zinc at oxide and chromium oxide) under high pressure and at high temperature, methanol is formed (Elevers *et al.*, 1990).

1.4 Properties of Methanol

1.4.1 Physical properties

Methanol is a colorless, volatile, flammable liquid with a mild alcoholic odour when pure. However, the crude product may have a repulsive pungent odour. Methanol is miscible with water, alcohols, esters, ketones and most other solvents and forms many azeotropic mixtures. It is only slightly soluble in fats and oils (Elvers *et al*., 1990).

Important physical constants and properties of methanol are summarized in Table (1-1):

Table (1-1): Some physical properties of methanol, (Haward, 1990)

Physical property	Characteristic
Appearance	clear colorless liquid
Odor	slight alcoholic when pure crude material pungent
Boiling point	64.7°C
Flash point	15.6°C (open cup) 12.2°C (closed cup)
Freezing point	-97.68°C
Specific gravity	0.7915 (20/4°C) 0.7866 (25°C)
Vapour pressure at 20°C at 30°C	 92 mmHg 160 mmHg

1.4.2 Chemical properties

Methanol undergoes reactions that are typical of alcohols as a chemical class. The reactions of particular industrial importance include the following: dehydrogenation and oxidative dehydrogenation over silver or molybdenum-iron oxide to form formaldehyde; the acid-catalyzed reaction with isobutylene to form methyl tertiary butyl ether (MTBE); carbonylation to acetic acid catalysed by cobalt or rhodium; esterification with organic acids and acid derivatives; etherification; addition to unsaturated bonds and replacement of the hydroxyl group (Elvers *et al*., 1990).

1.5 Usage of Methanol

During the 1890s, the market for methanol (then better known as wood alcohol) increased as a commercial product and as a solvent for use in the workplace. It was included in many consumer products such as witch hazel, Jamaica ginger, vanilla extract and perfumes.

Historically, in terms of commercial usage, about half of all methanol produced has been used to produce formaldehyde (Nielsen *et al*., 1993).

1.5.1 Use as Feedstock for chemical Synthesis

Approximately 70% of the methanol produced worldwide is used as feedstock for chemical synthesis. Methyl halides produced from methanol include methyl chloride, methylene chloride and chloroform. Nearly all the formaldehyde manufactured worldwide is produced by oxidation of methanol with atmospheric oxygen. The annual formaldehyde production was projected to increase at a rate of 3%, but because other bulk products have higher growth rates, its relative importance with respect to methanol use has decreased (Elvers *et al*., 1990; Fiedler *et al*., 1990).

Methanol also used in paints, varnishes, antifreeze and gasoline deicers, cleansing solutions, model and hobby glues and adhesivers (ATSDR, 1993).

1.5.2 Use as Fuel

Methanol is a potential substitute for petroleum. It can be directly used in fuel as a replacement for gasoline in gasoline and diesel blends. Methanol is in favour over conventional fuels because of its lower ozone-forming potential, lower emissions of some pollutants, particularly benzene and polycyclic aromatic hydrocarbons and sulfur compounds, and low evaporative emissions. On the other hand, the possibility of higher formaldehyde emissions, its higher conventional fuels (CONCAWE, 1995).

1.5.3 Other Uses

Methanol is used in refrigeration systems, e.g., in ethylene plants, and as an antifreeze in heating and cooling circuits. However, its use as engine antifreeze has been replaced by glycol-based products. Methanol is added to natural gas at the pumping stations of pipelines to prevent formation of gas hydrates at low temperature and can recycled after removal of water. Methanol is also used as an absorption agent in gas scrubbers to remove, for example, carbon dioxide and hydrogen sulfide (Fiedler *et al.,* 1990).

1.6 Methanol in Soil

Methanol is readily degraded in the environment by photo- oxidation and biodegradation processes. Half-lives of 7-18 days have been reported for the atmospheric reaction of methanol with hydroxyl radicals. Many genera and strains of microorganisms are capable of using methanol as a growth substrate. These include *Pseudomonas* sp.; *Methylobacterium rganophilium*; *Desulfovibrio*; *Streptomyces* sp.; *Rhodopseudomonas acidophilia*; *Paracoccus denitrificans; Microcyclus aquaticus*; *Thiobacillus novellus; Micrococcus denitrificans*; *Achromobacter* L1 (isolated from activated sewage sludge) and *Mycobacterium* 50 (isolated from activated sewage sludge). Most microorganisms possess the enzyme alcohol dehydrogenase which is necessary for methanol oxidation.

The toxicity of methanol to each of three bacterial groups, i.e. aerobic heterotrophic, *Nitrosomonas* and *Methanogens* (key agents in recycling of organic material in the environment and in wastewater treatment systems), was described by Blum and Speece (1991).

The following LD_{50} values (mg/litre) (the concentration that inhibited the culture by 50%) compared to the uninhibited controls were reported: *Nitrosomonas* (after 24-h exposure), 880 mg/litre; *Methanogens* (after 48-h exposure), 22000 mg/litre; and aerobic heterotrophs (after 15-h exposure), 20000 mg/litre. Methanol was found to be completely inhibitory to ammonia oxidation by *Nitrosomonas* bacteria at a concentration of 5×10^{-3}M (about 160 mg/litre). (Hooper and Terry, 1973).

Methanol has a fairly low absorptive capacity on soils, The rates of methanol degradation varied considerably between sites, but the soils could be characterized into two general types, namely fast soils, in which degradation rates were high and rates were increased by addition of nitrate and sulfate, and slow soils, in which biodegradation rates were low and

decreased by the addition of nitrate or sulfate and inhibition of sulfate increased degradation rates (Hickman *et al.,* 1998).

1.7 Factors Affecting the Hydrocarbon Biodegradation Process

A number of environmental factors affecting the biodegradation process can be summarized as follow:

- The primary factor limiting biodegradation is the scarcity or abscence of a suitable and available source of energy.

Wherever available energy material is an abundant, microbe capable of using that material are usually abundant (Clark, 1979).

- pH :in general the optimum pH for microbial biodegradation is slightly above 7 (Clark , 1979) .

- Temperature: temperature can have a marked effect on the rate of hydrocarbon degradation.

Zobell found that hydrocarbon degradation was over an order of magnitude faster at 25ºC than at 5ºC (Atlas, 1981).

- There at least 11 essential macronutrient and micronutrient elements that must be present in proper amounts, forms and ratios to sustain the growth of microorganisms.

These include nitrogen, phosphorus, potassium, sodium, sulfur, iron...etc. Therefore, the availability of these elements should be optimized to achieve maximum degradation rates (Reishfeld *et al.*, 1972; Atlas, 1981).

In addition to the above, there are also some factors that may affect the biodegradation rate, these include, the moisture rate in contaminated soil, the exhaustion of dissolved oxygen, salinity and pressure (Atlas and Bartha, 1998).

1.8 Methanol Oxidation in Bacteria

Methanol is oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MDH), in gram negative methylotrophes. MDH is an α_2 β_2 tetramer of large (60-67 KDa) and small (8.5 KDa) subunits fig.(1-1). It is a quinoprotein found in the periplasm of many gram-negative methylotrophic or autotrophic bacteria. Each tetramer contains 2mol of pyrroloquinoline quinone and 1mol of calcium (Anthony, 1992; Frank *et al.*, 1993).

Electrons are transferred from MDH to cytochrom c_L, a typical cytochrom which serves as the specific electron acceptor for MDH, cytochrom c_L is then oxidized by a typical class I cytochrom c (cytochrom c_H), which is also specific for the oxidation of methanol (Anthony, 1992; Dijkhuizen *et al.*, 1992).

The MDH and the two cytochromes are soluble and are located in the periplasm of gram-negative of methylotrophes, these proteins are found in large amounts, and mutants lacking any of them fail to grow on methanol, but facultative methylotrophes can be rescued by growth on heterotrophic substrates or methylamines (Bartha and Hanson, 1993; Harms, 1993).

Methanol is oxidized via an NAD-linked methanol dehydrogenase in gram positive methylotrophes and via a methanol oxidase system in methanol–oxidizing yeast species, but these enzymes have not been detected in gram-negative methanotrophic bacteria (Dijkhuizen *et al.*, 1992).

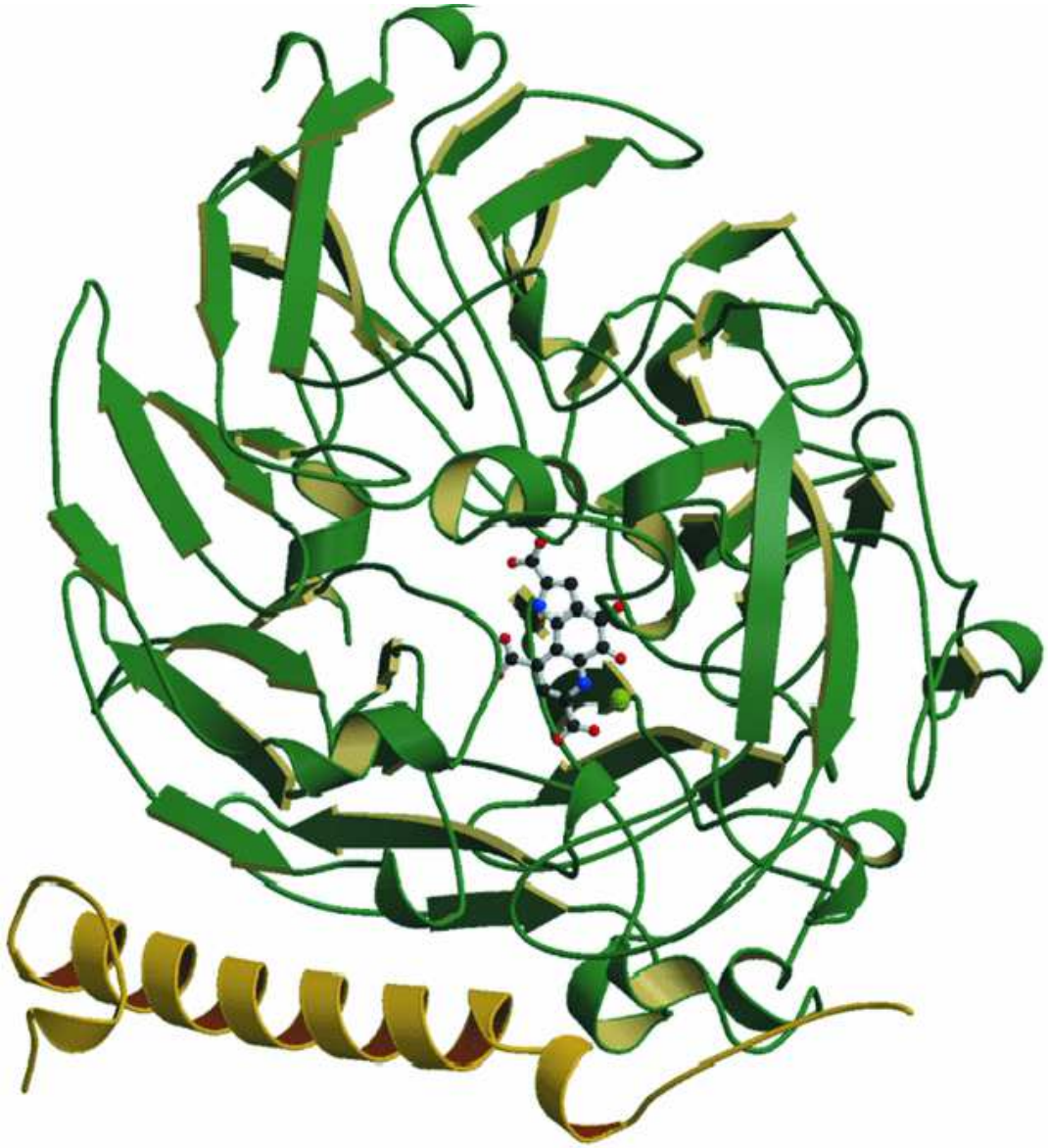

Figure (1-1): An α β unit from methanol dehydrogenase (Williams *et al.*, 2005)

1.9 Genetics of Methylotrophic Bacteria

Bacteria are known to be possesses plasmids, which encode part or all of the degradative pathways of hydrocarbons (Focht, 1988).

Plasmids are extrachromosomal genetic elements which are wide spread in bacteria, they have their origin of replication, and autonomously replicates with respect to chromosomal DNA and stable inherited. Normally, plasmids contain genes that may be essential in certain environments (Friefelder, 1987; Dale, 1998).

Almost all of the plasmids that have genes for hydrocarbons and xenobiotics catabolism are found in Gram-negative bacteria, predominately *Pseudomonas* species (Atlas, 1981; Lindow *et al.*, 1989).

Genetic studies of the biodegradation started in the 70s of the last century. Information obtained about the role of the plasmids, accounted for the evolution of the genetics of biodegradation and molecular organization studies (Franklin *et al.*, 1981).

In 1970, American and British researchers isolated the first degradative plasmid, from the bacteria *Pseudomonas putida*, which is TOL plasmid, that responsible for the utilization of toluene. This plasmid carries genes encode for a set of degradative enzymes (Frantz and Chacrabarty, 1986). Since then, the number of isolated degradative plasmids increased, which enable the bacteria to degrade hydrocarbon compounds and industerial pollutants and convert them to less harmful for the environments (Seeger *et al.*, 1995).

The natural plasmid pBM19 carries the key *mdh* gene needed for the oxidation of methanol into formaldehyde by *Bacillus methanolicus*, there are also five more genes, *glpX, fba, tkt, pfk* and *rpe*, with deduced roles in the cell metabolism, are located on this plasmid.

Two chromosomal genes, *hps,* and *phi,* with the five other genes, all are involved in the fructose biphosphate aldolase/ sedoheptulose biphosphatase variant of the ribulose monophosfate (RuMP) pathway, for formaldehyde assimilation. (Jakobsen *et al.,* 2006).

Three methylotrophic strains were screened for the presence of plasmid DNA, plasmids were detected in three marine isolates, including, *Methanosarcina acetivorans*. All the three plasmids appeared to be similar based on size and restriction site analysis, the plasmid from *M. acetivorans*, designated pC2A, was approximatelly 5.1 kb in size and was estimated to be present in a low copy number of six plasmids per genome (Sowers and Gunsalus, 2001).

The genes enabling methanol oxidation in *M. extorquens* AM1 are found in three different locations on the chromosome. One 12.5-kb cluster (cluster 1) contains 14 genes (*mxaFJGIRSACKLDEHB*), all transcribed in the same direction. These genes encode the structural polypeptides of methanol dehydrogenase, the specific cytochrome *c* that accepts electrons from methanol dehydrogenase, the proteins essential for calcium insertion into the apoprotein, one regulatory protein, and a few proteins whose functions are still unknown (Amaratunga *et al.,* 1997;Lidstorm *et al.,* 1998). One gene (*mxaW*) is located immediately upstream of this cluster, transcribed in the opposite direction by a methanol-inducible promoter, but its function is unknown (Sipringer *et al.,* 1998). One pair of genes involved in transcriptional regulation of MDH (*mxbMD*) are a part of the 60-kb methylotrophy island (cluster 2) (Sipringer *et al.,* 1997), and another pair (*mxcQE*) are located elsewhere on the chromosome. The six genes for PQQ biosynthesis are located in two different clusters: one of them (*pqqABC/DE*) is located in the large methylotrophy island immediately downstream of

mxbMD (Sipringer *et al.,* 1996), while another cluster (*pqqFG*) is not linked to other methylotrophy genes (cluster 2) (Toyama *et al* .,1998) .

1.10 Bacterial Transformation

Transformation was first discovered by Griffith in 1928 in *Streptococcus pneumoniae* (Good enough, 1984) and later discovered in a variety of bacteria including, *Bacillus subtilis, B. Stearothermophilus, Haemophilus influenzae,* and *Acinetobacter* (Rodriguez and Tait, 1983).

A number of bacteria including *Streptococcus pneumoniae, Bacillus subtilis* and *Haemophilus influenzae* are readily induced to undergo transformation in the laboratory, while bacteria such as *E. coli* can undergo transformation only under special laboratory conditions (Good enough, 1984) .Because the early attempts to transform *E. coli* were unsuccessful, it was sugested that this bacterium does not posses a natural transformation system.

Mandel and Higa (1970) found that treatment with $CaCl_2$ allowed *E. coli* cells to take up bacteriophage lambda DNA, while Cohen *et al.,* (1972), showed that $CaCl_2$ treated *E. coli* cells were effective recipients for plasmid DNA. In the naturally occuring transformation systems, bacteria generally enter stage of growth known as competence, during which the cells are capable of taking up exogenous DNA. Competant cells appear to elaborate a surface protein called competence factor, which is involved in an energy independent binding of donar DNA fragments to the cell surface (Good enough, 1984).

In case of abscence of the natural transformation system as in *E. coli,* the competent state could be induced artificially by exposing cells to $CaCl_2$ prior to the addition of DNA (Rodriguez and Tait, 1983).

DNA fragments from the donor cell must also be in a particular stable, they must be large, relatively intact and double–stranded having a molecular weight in the 0.3 to 0.8×10^6 Dalton range. The requierment for duplex DNA presumably relates to the way in which donor DNA enters the cell and intracellular hydrolyze one of its strands (Good enough, 1984). For example, in Gram-positive bacteria the double stranded DNA is degraded by an exonuclease and the remaining strand is transported to the cell, where as Gram-negative bacteria, first take up double stranded DNA in subcellular compartments called transformasomes,the new DNA may not become single stranded untile it enters the cytoplasm (Hanahan, 1983).

In other cases, the special physiological state of competence was required for the introduction of plasmid DNA into host cell, This technique depends on the increase the cell permeability for DNA molecules by cell wall removal .Cell made permeable to DNA by calcium ion treatment, will take up both single-stranded and double-stranded circular plasmid DNA. Therefore, both linear and double-stranded circular plasmid DNAs can be efficiently introduced into chemically treated cells. This fact has made calcium ions-induced competence very useful for cloning and other applications that require the introduction of plasmid and phage DNAs into cells. There are three possible roles for natural competence: A nutritional function allowing competent cells to use DNA as a carbon, energy and nitrogen source;A repair function, in which cells use DNA from neighboring bacteria to repair damage to their own chromosome, thus, ensuring survival of the species; and recombination function, in which bacteria exchange genetic material among members of their species, increasing diversity and accelerating evolution (Snydre and Champness, 1997) .

The extent of studies of gene transfer in natural habitats by conjugation, transformation and transduction is limited. However; many investigations have been performed in studying the possibility of transformation, conjugation and transduction occurrence in soil and marine environments between the predominant bacteria. In 1983 Carlson and his coworkers have reported that natural transformation occured in the soil bacterium *Pseudomonas stutzeri*, so this bacterium aquired the DNA in soil by transformation and the DNA remained intact from digestion by DNases enzymes. In contrast, this bacterium appears to possess a DNA release mechanism dependent on DNA replication in the donor cell, allowing transformation to occure between intact cells of this species (Stewart and Sinigalliano, 1990).

Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments were used in this study:

Equipment	Company (Origin)
Autoclave	Express(West-Germany)
Laminar air flow	Memmert (West Germany)
Compound light microscope	Olympus(Japan)
Distillator	GFL(Germany)
Oven	Memmert(Germany)
Micropipette	Witey(Germany)
Incubator	Termaks(U.K)
pH-meter	Mettler Toledo(U.K)
Portable centrifuge	Hermle labortechnik(Germany)
Refrigerator centrifuge	Harrier(U.K)
Sensitive balance	Delta Rang(Switzerland)
Spectrophotometer	Aurora instruments Ltd.(England)
Vortex	Buchi(Swissrian)
Minigel electrophoresis apparatus	Bio Rad(Italy)
Shaker incubator	GFL(Germany)
UV-transillulminator	Vilber Lourmat(France)

2.1.2 Chemicals

The following chemicals were used in this study:

Material	Company
Chloroform, Iodine, K_2HPO_4, KH_2PO_4, $MgSO_4$, NaCl, NaOH, Glycerol, Sucrose, K_2SO4, $CaCl_2$, Peptone, Urea, Isoamyl alcohol, α -naphthol	BDH-England
Agar,Gelatine	Biolife-Italy
Tris(hydroxyl methyl)aminomethane base (Tris-base), ethylene diamine tetraacetic acid(EDTA), hydrogen peroxide, crystal violet, $CuSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeCl_3.6H_2O$	Fluka-Switzerland
Ethanol, $MgSO_4.7H_2O$, NH_4Cl	Merck-Germany
Ethidium bromide, SDS, Agarose, Lysozyme	Sigma-USA
Methyl red, Safranin	Oxoid
Methanol, P-dimethyl-aminobenzaldehyde	Riedel-DeHaeny (Germany)

2.1.3 Bacterial Strains

Strain	Phenotype	Source
E. coli HB101 harboring pBR322	Amp^r ,Tc^r	Dept.of Biotechnology/ Al-Nahrain University
E.coli DH5α	*lac*Z *rec*$^-$*A1*	Dept. of Biotechnology/ Al-Nahrain University

Amp: ampicilline; Tc: tetracycline; r: resistant; *lac*Z: Gene for lactose utilization as a carbon source; *rec*$^-$*A1*: can not repair DNA radiation damage or recombine.

2.1.4 Media

2.1.4.1 Ready to use Media

These media were prepared according to the manufacturing companies and autoclaved:

1. Triple Sugar Iron Agar (Difco USA).
2. Simmon Citrate Agar (Difco USA).
3. Nutrient Broth (Biolife- Italy).
4. Nutrient Agar (Oxoid).
5. MacConkey Agar (Oxoid).
6. MR-VP Medium (Difco USA).

2.1.4.2 Prepared Media

- **Culture Media (Marshall and White, 2001)**

This defined medium was used for growing of methylotrophic bacteria**,** it was composed of:

K_2HPO_4	3.5g
KH_2PO_4	1.5g
NaCl	0.5g
$MgSO_4$	0.12g
NH_4Cl	2.14g
Trace element	1 ml
Sodium borate	0.57g
$FeCl_3.6H_2O$	0.24g
$CoCl_2.6H_2O$	0.04g
$CuSO_4.5H_2O$	0.06g
$MnCl_2.4H_2O$	0.03g
$ZnSO_4.7H_2O$	0.31g
$NaMoO_4.2H_2O$	0.003g
Distilled water	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving then 1 ml of methanol (sterilized by filtration) was added.

- **King A medium (Starr *et al*., 1981)**

It was composed of:

Peptone	20 g
Glycerol	10 ml
K_2SO_4	10 g
$MgCl_2$	1.4 g
Agar	15 g
D.W.	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving .

- **King B Medium (Starr *et al*., 1981)**

It was composed of:

Peptone	20 g
Glycerol	10 ml
$MgSO_4.7H_2O$	3.5 g
K_2SO_4	1.5 g
Agar	15 g
D.W.	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving .

- **Gelatin Medium (Stolp and Gad Kari , 1984)**

Gelatin 12 %(w/v) was added to nutrient broth and sterilized by autoclaving.

- **Blood Agar Medium (Atlas *et al.*, 1995)**

It was prepared by dissolving 37g of blood agar base in 950 ml of distilled water and autoclaved. After cooling to 50° C, 5 % of blood was added to it, mixed well and distributed into petri-dishes.

- **Cetrimide agar (Stolp and Gad Kari, 1984)**

It was composed of:

Peptone	20 g
$MgCl_2$	4.5 g
K_2SO_4	10 g
Cetrimide	0.3 g
Agar	15 g
D.W.	1000 ml

pH was adjusted to 7.2 and sterilized by autoclaving .

- **Peptone Water Medium (Atlas *et al.*, 1995)**

This medium was composed of the following:

Peptone	20 g
Sodium chloride	5 g
D.W.	1000 ml

pH was adjusted to 7.0, mixed thoroughly and distributed into tubes and sterilized by autoclaving.

- **Semi-Solid Agar Medium (Collee *et al.*, 1996)**

Agar 0.4% (w/v) was added to nutrient broth and sterilized by autoclaving.

- **Urea Agar Medium (Collins and Lyne, 1987)**

It was prepared by dissolving 24g of urea agar base in 950 ml distilled water, pH was adjusted to 7.0 and sterilized by autoclaving, then 50ml of 40%(w/v) urea (sterilized by filtration) was added to the whole media.

2.1.5 Reagents and Stains

- **Catalase Reagent (Atlas *et al.*, 1995)**

This reagent was composed of 3% hydrogen peroxide (H_2O_2).

- **Oxidase Reagent (Atlas *et al.*, 1995)**

This reagent was composed of 1% tetramethyl-*p*-phenylenediamine dihydrochloride (freshly prepared).

- **Methyl Red (MR) Reagent (Cruickshank *et al.*,1975)**

It was prepared by dissolving 0.1g of methyl red in 300ml ethyl alcohol and then 200ml of distilled water was added.

- **Vogas –Proskauer (VP) Reagent (Cruickshank *et al.*,1975)**

It consists of:

Reagent A: 40% potassium hydroxide in distilled water.
Reagent B: 5% alpha naphthol in absolute ethyl alcohol.

- **Kovac's Reagent (Atlas *et al.*, 1995)**

P-Dimethyl-aminobenzaldehyde at 4% in HCl isoamyl alcohol.

- **Crystal Violet Stain (Atlas *et al.*, 1995)**

This stain was prepared by dissolving 2g of crystal violet in 20ml of 95% ethanol and the final volume was completed to 100ml with distilled water, filtered before use.

- **Safranin Counter Stain (Atlas *et al.*, 1995)**

This stain was prepared by dissolving 0.25g of safranin O in 10ml of 95% ethanol and the final volume was completed to 110ml with distilled water, allows standing several days and filtered before use.

2.1.6 Buffers and Solutions

2.1.6.1 Plasmid Extraction Buffers and Solutions

- **SET buffer (Maniatis *et al.*, 1982)**

75 mM NaCl
25 mM EDTA
20 mM Tris-Cl

pH was adjusted to 8.0 and sterilized by autoclave .

- **Lysozyme Solution (Kieser, 1995)**

This was prepared by dissolving 50mg lysozyme in 1ml of sterile distilled water, this prepared freshly.

- **Sodium Dodecyl Sulphate Solution (SDS) (100mg/ml) (Kieser, 1995)**

It was freshly prepared by dissolving 10g SDS in 100ml of sterile distilled water.

- **NaCl Solution (5 M) (Kieser, 1995)**

It was prepared by dissolving 29g NaCl in 100 ml D.W.

- **TE Buffer (Maniatis *et al.*, 1982)**

EDTA 1mM
Tri-HCl 10mM

pH was adjusted to 8.0 and sterilized by autoclave .

- **Solution of Plasmid Isolation by Mini Prep. kit (US Biological)**

It consists of the following:

- Solution No.1(Suspension Buffer) : Tris-HCl, EDTA, pH:8
- Solution No.2(Lysis Buffer) : NaOH, SDS, pH:12
- Solution No.3(Potassium Acetate): Potassium Acetate, pH:5.5
- Solution No.4(Precipitation Buffer) : mineral salt
- Solution No.5(Binding Buffer) : Guanidine and Sorbent
- Solution No.6(Elution Buffer) : Tris-HCl

2.1.6.2 Gel Electrophoresis Buffers

- **5X Tris- Borate- EDTA (TBE) (pH 8.0) (Maniatis *et al.*, 1982)**

Tris- base	54g
Boric-acid	27.5g
EDTA (0.5M)	20ml
Distilled water	1000ml

- **Ethidium Bromide (Maniatis *et al.*, 1982)**

  Prepared in concentration 10mg/ml and stored at 4° C.

- **6X Gel Loading Buffer (Maniatis *et al.*, 1982)**

  Bromophenol blue 0.25% (w/v).

  Sucrose in H_2O 40% (w/v).

2.1.6.3 Curing Solution

It was prepared as stock solution of SDS 10 % (w/v) in distilled water.

2.1.6.4 Transformation Solutions (Sambrook and Russell, 2001)

- $MgCl_2$-$CaCl_2$ Solution ($MgCl_2$ 80mM, $CaCl_2$ 20mM)
- $CaCl_2$ Solution 0 .1M (pH= 8.0)

2.2 Methods

2.2.1 Sterilization Methods (Collins and Lyne,1987)

- Culture media (liquid and solid), and solutions were sterilized by autoclaving at 121°C for 15 minutes.
- Glasswares (cylinders, Petri dishes,etc…) were sterilized in an electric oven at 180-200°C for 2 hours.
- Thermolabile components or materials (such as methanol and urea) were sterilized by filtration through Millipore filter paper (0.22µm).

2.2.2 Sample Collection

Fifty samples of soil (contaminated and uncontaminated with hydrocarbons) were collected from different places in Iraq, from Baghdad 34 samples, Karbala 3 samples, Shahraban 2 samples, Khanaqin 6 samples and 5 samples from Sulaimania. Samples were taken two inches under the soil surface and they were wet.

2.2.3 Bacterial Isolation

Fifty ml of sterile nutrient broth was inoculated with soil sample 1% (w/v) and incubated at 30°C for 24 hrs.

One hundred ml of defined medium (2.1.4.2), was dispensed in flasks (250ml) and sterilized by autoclave, after cooling 0.1% (v/v) of methanol (sterilized by filtration) was added for each flask, then each flask was inoculated with 1ml of the culture above, controls were made by inoculating flasks containing the same defined medium but without any source of carbon (methanol), flasks were incubated with shaking 150 rpm at 37°C for 2-3 days. After that serial dilutions were made from

these cultures and spreaded on nutrient agar plates in order to obtain pure single colonies.

2.2.4 Maintenance of bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al.,* (1982) and Atlas *et al.,* (1995) as following:

2.2.4.1 Short Term Storage

Isolates of bacteria were maintained for one or two weeks on the surface of nutrient agar plates. The plates were tightly wrapped with parafilm and stored at 4°C.

2.2.4.2 Medium Term Storage

Strains of bacteria were maintained in stab culture for long periods of a few months. Such culture was prepared in small screw capped tubes containing 2-3ml of agar medium. The cultures were incubated using a sterile straight platinum wire that is dipped into dense liquid culture of bacteria and then stabbed deep into the agar medium. After proper incubation, the tube was wrapped tightly with parafilm to prevent desiccation of the medium, and kept at 4° C.

2.2.4.3 Long Term Storage

Bacteria can be stored for many years in nutrient broth medium containing 15% glycerol at low temperature without significant lose of

viability. This was done by adding 1.5ml of sterile glycerol to an exponential growth of bacteria in small screw-capped vials with final volume of 10ml and stored at -20°C.

2.2.4.4 Measurement of Bacterial Growth

Growth of bacteria was monitored by measuring the optical density (O.D) of liquid culture in spectrophotometer (spectronic20) at wave length of 600nm.

2.2.5 Identification of Bacterial Isolates

Bacterial isolates that were well grown on methanol as sole source of carbon and energy are identified as follow:

2.2.5.1 Cultural and Morphological Tests

- **Morphology of Colonies**

Shape, color and viscosity of the colonies were studied on plates of nutrient agar media, after incubation of the isolates on these plates at 37° C for 24 hrs.

- **Gram's Stain (Atlas *et al.*,1995)**

A single colony was transferred by a loop to a clean slide. The smear was stained with crystal violet, treated with iodine, decolorized

with ethanol alcohol (95%), and counter stained with safranin, then examined under a microscope.

- **Motility Test(Collee *et al*.,1996)**

In semi-solid agar medium, motile bacteria (swarm) give a diffuse spreading that is easily recognized by naked eye. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. Ten ml amounts was dispensed in test tubes and left to set in vertical position, tubes was inoculated with bacteria by making a single stab down the center of the tube to about half depth of the medium, incubated at 37°Cfor 24-48 hrs.

2.2.5.2 Biochemical Test

- **Catalase Production Test (Maza *et al.*, 1997)**

This test was performed by adding drops of 3% hydrogen peroxide (H_2O_2) on a single colony grown on nutrient agar .The production of gaseous bubbles indicate a positive result**.**

- **Oxidase Production Test (Harely and Prescott, 1996)**

Filter paper was saturated with the substrate (tetramethyl-p-phenylenediamine dihydrochloride); colony of suspected bacterial isolates to be tested was transferred on the filter paper with sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result**.**

- **Gelatin Hydrolysis Test (Harely and Prescott, 1996)**

Inoculate the tubes of gelatin medium with bacteria by stabbing and then the tubes were incubated at 30°C for 5 days. Liquification of the media indicates a positive result.

- **Growth on MacConkey Agar (Atlas *et al*., 1995)**

MacConkey agar is a differential plating medium for the selection and recovery of Enterobacteriaceae and related enteric gram negative rods. Lactose is the sole carbohydrate. Lactose-fermenting bacteria produce colonies that are varying shades of red because of the conversion of neutral indicator dye (red below pH 8.0) from the production of mixed acids. Colonies of non-lactose fermenting bacteria appear colorless or transparent on this medium.

- **Growth on King A Medium (Cruickshank *et al.*, 1975)**

This test was performed to study the production of the characteristic pigment, (pyocyanine). Inoculate the bacteria on the plates of King A medium by streaking and incubate at 37°C for 24 hrs.

- **Growth on King B Medium(Cruickshank *et al.*,1975)**

This test was used to study the production of the characteristic pigment, (fluorescine). Inoculate the bacteria on the plates of the king B medium by streaking and incubate at 37°C for 24 hrs. Then the plates were exposed to U.V. light to detect the presence of fluorescine.

- **Triple Sugar Iron Test (TSI) (Atlas *et al* ., 1995)**

Isolates were cultured on the TSI agar slant by stabbing and streaking on surface, and then incubate at 37°C for 24 hrs. When the color of medium was changed from red to yellow, it is an indication of acid production, while appearance of precipitate indicated ferric sulfate formation, pushing the agar to the top indicates CO_2 formation.

- **Citrate Utilization Test (Atlas *et al*., 1995)**

Slant of Simmon's citrate was inoculated with a single colony, incubated at 37° C over night**.** The appearance of blue color indicates the presence of alkaline end products and a positive citrate test.
This test was used to examine the ability of bacteria to utilize citrate as a sole source of carbon.

- **Indole Test (Collee *et al*., 1996)**

Peptone water was inoculated and incubated at 37°C for 48 hrs. A quantity of 0.05ml of Kovac's reagent was added and mixed gently. Positive result was recorded by the appearance of a red ring on the surface.

- **Methyl red and Voges Proskauer (MR-VP)Test (Maza *et al*.,1997)**

The methyl red test was used to determine the pH of the end products of glucose fermentation. While Voges Proskauer test detects

acetoen production. The tubes of MR-VP liquid media were inoculated and incubated at 37°C for 48 hrs.Both tests were performed from the same culture, which was dividing for testing. The MR test was performed after adding about five drops of MR reagent (2.1.5), positive test is bright red in color and negative test is yellow, while the VP test was performed after adding the VP reagent (2.1.5), and the positive test is pink color.

- **Urease Test (Atlas *et al.*, 1995)**

Urease activity was detected by inoculating the surface of christensen urea agar slants with the bacterial growth and incubated at 37° C for 24 hrs. The appearance of a red-violet color indicates a positive result, while a yellow-orange color indicates a negative result.

2.2.5 Plasmid Extraction

Plasmid DNA was isolated by plasmid isolation mini prep. kit (US Biological), and the salting-out method which described by Kieser, (1995).

❖ Salting –out Procedure

- Culture of bacteria grown in nutrient broth, was pelleted from 20ml by centrifugation at 6000rpm for 15 min.
- The pellet washed with 3ml SET buffer and resuspended the cells with 1.6ml of SET buffer, and then freshly prepared lysozyme (final concentration 2mg/ml) was added and incubated at 37°C for 30min.
- One ml of 10% SDS was added and mixed by inversion then incubated at room temperature for 15 min.

- Two ml of 5M NaCl was added and mixed by inversion at room temperature.
- An equal volume of chloroform was added, mixed by inversion for 15 min., then centrifuged (6000rpm at 4°C) for 20 min.
- The aqueous phase (upper) was transferred to another sterile tube, and 0.6 volume of isopropanol was added, mixed by inversion and kept at room temperature for 5 min.
- Centrifuged at 13000rpm for 15min at 4°C.
- The isopropanol was discarded and the precipitated DNA dissolved in 100 µl of TE buffer and stored at -20°C.

❖ Plasmid Isolation by Mini Prep. Kit (US Biological)

- Two ml of bacterial culture was pelleted by 6000rpm for 1min., and the suspension discarded.
- One hundred µl solution No.1 was added and vortexing vigorously (up to cell homogenization).
- One hundred µl solution No.2 was added, the tube was inverted five times, incubated at room temperature for 3min.
- One hundred µl solution No.3 was added, the tubes was inverted five times then mixed and incubated at room temperature for 3min.
- Two hundred µl solution No.4 was added and centrifuged in 12000rpm for 3min.
- Four hundred µl aqueous phase was transferred to a new 1.5ml tube, 800 µl solution No.5 was added. Vortexed in 3-5 second and then incubated at room temperature for 3min., vortexed 3-5 second and centrifuged it for 1 min. in 6000rpm.
- The supernatant was discarded by tube inversion.

- One ml of 70% ethanol was added. Vortexed for 5-10sec pelleted it in 6000rpm for 1 min.
- Supernatant was discarded by tube inversion.
- The tube was inverted on tissue paper for 1 sec.
- The pellet was dried, 3min in 45ºC or by speed Vacuum.
- The pellet was suspended in 30µl of solution No.6, and the tube was stored in 45ºC for 5 min. centrifuged for 1min of 12000rpm and then gently, supernatant was transferred to a new tube.

2.2.6 Agarose Gel Electrophoresis (Sambrook and Russell,2001)

Agarose gels (0.7%) were run horizontally in Trise-borate–EDTA (TBE 1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on the gel.

Generally, gel was run for 2-3 hrs at 5v/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gel was stained with ethidium bromide by immersing it in distilled water containing the dye at a final concentration of 0.5µg/ml for 30-45min.

DNA bands were visualized by U.V. illumination at 302nm on an U.V. transilluminator. Gels were destained in distilled water for 30-60 min. to remove access staining before photographs were taken.

2.2.7 The Role of Plasmid in Methanol Utilization

2.2.7.1 Curing of Plasmid DNA

Curing experiments were performed on *P. aeruginosa* HK8 by using curing agent (SDS) and depending on Trevors, (1986). Cells of this isolate were grown in 5ml of nutrient broth to mid log phase. Then 0.05 ml inoculum of the culture was inoculated in series of 5ml fresh nutrient broth tubes containing various concentration of SDS (0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). All tubes were incubated at 37° C for 24-48 hrs. The growth density of different tubes was observed by naked eye and compared with control to determine the effect of SDS on bacterial growth. The lowest concentration of SDS that inhibited the growth of the bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of SDS that allow bacteria to grow, and diluted appropriately, then (0.1ml) samples from proper dilution were spreaded on nutrient agar plates and incubated overnight at 37°C to score the survived colonies. One hundred of these colonies were picked and inoculated into vials containing 10 ml of the defined medium supplemented with methanol as a sole carbon source. If a colony was not able to grow in the defined medium compared with the control (W.T), this means the cells of this colony were cured cells that lost their ability to utilize methanol as a sole source of carbon and energy.

2.2.7.2 Transformation

E. coli $DH_5\alpha$ was transformed with plasmid DNA of *P. aeruginosa* HK8 according to the procedure of Sambrook and Russell, (2001).

One ml of an overnight *E. coli* $DH_5\alpha$ culture was inoculated in 100ml of nutrient broth and incubated at 37° C for 3hrs.with vigorous agitation. Bacterial cells (OD_{600} = 0.4) was transferred to sterile, disposable, ice-cold 50ml tubes.

The culture was cooled to freeze point by storing the tubes on ice for 10min. After centrifugation at 6000rpm at 4° C for 10min., the medium was decanted and the tubes standed in an inverted position on a filter paper for 1min.to allow the last traces of media to drain away. Each pellet was resuspended by gentle vortexing in 30ml of ice-cold $MgCl_2$-$CaCl_2$ solution, and then the cells were recovered by centrifugation at 6000rpm at 4° C for 10min.

The medium was decanted and the tubes standed in an inverted position for 1min., then the pellet was resuspended by gentle vortexing in 2ml of ice-cold 0.1M $CaCl_2$ for each 50ml of original culture. 200µl of each suspension of competent cells was transferred to sterile, chilled tubes using chilled micropipette tips, then 10µl of DNA was added to each tube and the contents of the tubes mixed by swirling gently, the tubes stored on ice for 30min.

The tubes were transferred to a rack placed in a preheated 42°C circulating water bath and stored for exactly 90 seconds without shaking, the tubes were transferred rapidly to an ice bath to allow the cells to chill for 1-2min. then 800µl of nutrient broth was added to each tube and the culture incubated for 45min. at 37°C, this period allow the bacteria to recover and begin to express. 100µl of transformation mixture was transferred to vials containing defined medium

(10ml), supplemented with methanol as a sole carbon source, the vials incubated at 37°C for 2-3 days.

Two controls were made, the first, 100µl of TE buffer was added to 10µl DNA, the second control, 100µl of transformation mixture was added to 10µl of TE buffer, the two controls were inoculated in vials containing 10ml of the defined medium and incubated as the transformation mixture. After that 0.1ml was transferred from the vials to MacConky agar plates and incubated at 37°C for 24hrs.

Frequency of Transformants % = (No. of transformants /Total viable count) ×100.

Results and Discussion

3.1 Isolation and Identification of Methylotrophic Bacteria

3.1.1 Isolation

Fifty soil samples were collected from different hydrocarbon contaminated and uncontaminated areas in Iraq during the period from 20 November 2005 to 5 January 2006.

Twenty five isolates were recognized by their ability to grow on methanol as a sole source of carbon and energy. These isolates were repeatedly tested in the defined medium supplemented with methanol ,in order to ensure their utilizing ability, then only 12 isolates that showed the ability to grow in defined medium were chosen and signed as (HK1,HK2,.....,HK12) table (3-1).

Results indicated that there was, in general, a lag phase before active growth ensured, as was noticed by an increased in the turbidity of the culture, and that may be attributed to the various molecular mechanisms enable microorganisms to recruit genes and modify nucleotide sequences in the structural and regulatory genes to enhance expression and use synthetic compounds as substrates (Chacrabarty, 1996).

Most methylotrophic bacteria were isolated from soil .Hanson *et al.*, (1993), reported that these bacteria can also be isolated from a wide variety of environments including, ground water, sea water and sediments.

These microorganisms were distributed especially in polluted environments. The number of microbial community that degrades hydrocarbons is proportionally increased when the degree of pollution increase. The percentage of hydrocarbon-degrading bacteria isolated from unpolluted area is 0.01%, while the percentage reaches 60-80% in polluted area (Azoulag *et al.*, 1983).

Failure of 13 isolates to utilize methanol for their growth as a source for carbon and energy, may be attributed to these isolates can benefit the metabolites of other bacteria that utilize and degrade them to less complex compounds (Snedecor and Cooney, 1974).

Table (3-1): The growth density of the bacterial isolates in mineral salt medium supplemented with methanol along 3 days of incubation at 37ºC.

Isolates	Incubation Time(Days)		
	1	2	3
HK1	-	+	+
HK2	-	+	+
HK3	-	±	+
HK4	-	+	+
HK5	-	+	+
HK6	-	±	+
HK7	-	+	+
HK8	-	+	++
HK9	-	+	++
HK10	-	+	+
HK11	-	+	++
HK12	-	-	±

(-): No growth

(±): Slightly growth (Absorbance at 600nm = 0.1- 0.2)

(+): Good growth (Absorbance at 600nm= 0.21- 0.3)

(++): Very good growth (Absorbance at 600nm > 0.31)

3.1.2 Identification of Bacterial isolates

The efficient isolates (HK8, HK9, and HK11) in utilizing methanol were selected and identified depending on morphological and biochemical characteristics. Bacterial isolates reveals different colonial appearance depending on the selective or differential media used.

Colonies of the isolates (HK8, HK9) appeared in medium size, low convex and yellow color on nutrient agar plates, while the isolate (HK11) appeared in medium size, mucoid, cream-white color on the same media.

Microscopic examination of the bacterial isolates (HK8, HK9) showed that their cells occurred singly, non-spore forming, gram negative rod shape, while (HK11) isolate cells occurred singly or in chain, non-spore former, gram positive cocci shape.

Several biochemical tests were made to identify bacterial isolates. Results as pointed out in table (3-2), showed that (HK8,HK9) isolates gave a positive results for oxidase, catalase, king A media, king B media, cetrimide media and gelatin hydrolysis, while(HK11) isolate gave positive results for catalase, oxidase and motility test. The isolates do not produce CO_2 and H_2O on TSI medium, but they gave variable results for Simmon citrate and urea hydrolysis, all these results indicated that (HK8, HK9) isolates were belong to *Pseudomonas aeruginosa* and the isolate (HK11) belongs to the genus *Micrococcus* spp.

The morphological and biochemical test results for these isolates were in agreement with Holt *et al.*, (1994).

Pseudomonas is capable of rapid growth on methanol as a sole carbon source, its doubling time is about 100 min. at 32º to 37ºC, and it grow well at methanol concentration up to 2% (Chalfan and Mateles, 1972).

A methanol dehydrogenase mutant, *Pseudomonas* M15A, was unable to take up methanol; it's proposed that methanol diffuses into the cell where it is rapidly oxidized by methanol dehydrogenase (Bellion *et al.*, 1983).

One of the widely distributed genera that have been identified as capable of using methanol as a growth substrate is *Micrococcus* sp. (Nielsen *et al*., 1993).

Micrococcus was found to grow aerobically on methanol as sole source of carbon and energy. It was decided to determine whether this organism assimilates methanol carbon at the oxidation level of formaldehyde via a reduced C_1-fixation pathway such as the ribulose monophosphate cycle or serine pathway or whether it oxidizes methanol completely to CO2 and uses its capability to operate the ribulose bisphosphate cycle for carbon assimilation. (Cox and Quayle, 1975).

Table (3-2): Morphological and Biochemical Tests of Methanol Utilizing Isolates

Characteristics		Isolates		
		HK8	HK9	HK11
Colony color		Yellow	Yellow	Cream-white
Cell shape		Rod	Rod	Cocci
Gram stain		-	-	+
Catalase production		+	+	+
Oxidase production		+	+	+
Growth on king A		+	+	N.D.
Growth on king B		+	+	N.D.
Growth on Cetrimide		+	+	N.D.
Growth on MacConkey		+	+	+
Growth on Blood agar		α	α	α
Citrate Utilization		+	-	-
Gelatine hydrolysis		+	+	-
Urea hydrolysis		-	+	-
Motility		+	+	+
Voges Proskauer		+	+	N.D.
Methyl red		-	-	N.D.
Indole production		-	-	N.D.
Triple Sugar Iron	Slant/Butt	K/A	K/A	K/A
	H_2S	-	-	-
	CO_2	-	-	-

(K): Alkaline, (A): Acid, (N.D.): Not determined, (+): Positive, (-): Negative, α: blood hemolysis

3.2 Ability of Bacterial Isolates to Utilize Methanol

The efficient isolates (HK8, HK9, and HK11) were grown on gradual concentrations of methanol, in order to choose the best isolate that is able to survive at high concentration of methanol.

As illustrated in table (3-3) the isolate (HK8), which belongs to *P. aeroginosa*, was the most efficient one for its ability to utilize high concentration of methanol (0.8%) v/v, while the other isolates failed to grow on this concentration .This may be attributed to the physiological and genetic properties of this isolate, or this bacterium was isolated from soil contaminated with hydrocarbons as mentioned before, and that leads to increase the adaptation of soil bacteria to this compound.

Jakobsen *et al.,* (2006) indicated that there were evidences for the role of Ribulose monophosphate (RuMP) pathway and the methanol dehydrogenase gene (*mdh*), in methanol utilizing in *Bacillus methanolicus* MGA3.

Table (3-3): Growth Density of the Isolates Grown in Defined Medium Supplemented with Gradual Concentration of Methanol Along 3 days of incubation

Isolates	Methanol Concentration V/V (%)									
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
HK8	++	++	+	+	+	+	+	±	-	-
HK9	++	+	+	±	-	-	-	-	-	-
HK11	++	++	+	+	+	±	-	-	-	-

(-): No growth

(±): Slightly growth (Absorbance at 600nm =0.1-0.2)

(+): Good growth (Absorbance at 600nm =0.21-0.3)

(++): Very good growth (Absorbance at 600nm > 0.31)

3.3 Plasmid Isolation

To determine the plasmid profile of the efficient isolates, (HK8, HK9, HK11), the Plasmid Isolation by Mini Prep. Kit (US Biological), and the salting out method (Kieser, 1995), were used, the last method gave an accepted results.

The results in figure (3-1), indicated that the isolates HK8 and HK9, which belongs to *P. aeruginosa*, containing two small plasmid DNA bands, and these plasmid bands approximately in the same size compared with each other, and with pBR322 plasmid (4.363Kb).

The results in figure (3-1) showed that *Micrococcus* isolate (HK11), has two small plasmid bands in the same size compared with pBR322 plasmid.The bacterial isolates tested in this study may be containing another plasmids does not detected (may be because of its large size).

As illustrated in figure (3-1), the plasmid bands were common in the two isolates (HK8, HK9). It can be concluded that they are belonged to related strains, since the related strains often contain the same number of plasmids with the same molecular weight and similar phenotype (Prescott *et al.*, 1990).

Many studies also indicated that a different *Pseudomonas* isolates containing plasmid with the same size but have different set of genes (Bayly and Barbour, 1984) or the plasmids are the same plasmids but they were transfer between the soil population of bacteria by conjugation and transformation (Devereux and Sizemore, 1982; Trevors, 1998).

Brautaset *et al* .,(2004) , found that the sequence analysis of 19167bp circular plasmid (pBM19), isolated from *Bacillus methanolicus* MGA3,demonstrated the presence of the methanol dehydrogenase gene , *mdh*, which is crucial for methanol consumption in this bacterium .

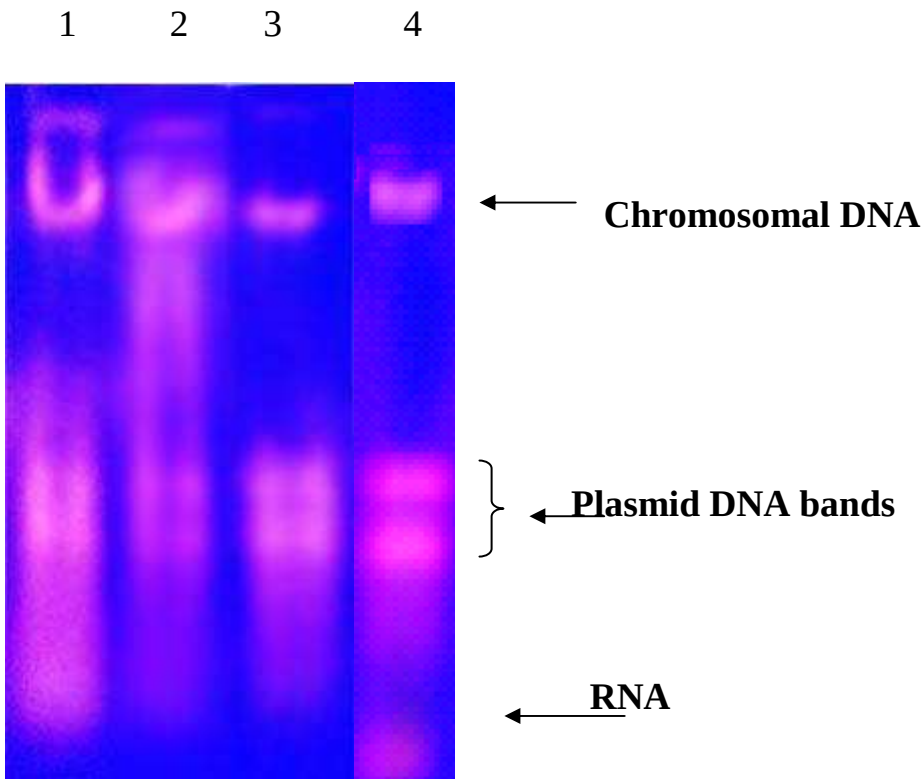

Figure (3-1): Gel Electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.7%) in TBE buffer at (5V/cm) for 2hrs.

1. ***Pseudomonas aeruginosa* HK9**
2. ***Pseudomonas aeruginosa* HK8**
3. ***E. coli* HB101 harboring pBR322**
4. ***Micrococcus* spp.**

3.4 Role of Plasmid in Methanol Utilization

The isolate (HK8) which belongs to *P. aeruginosa*, was candidate for study the role of plasmid in methanol utilization because this isolate gave a good growth rate, growth yield and can utilize higher concentration of methanol than the other isolates along three days of incubation on methanol as a sole source of carbon and energy, for that, curing and transformation experiments were performed on this bacterium.

3.4.1 Curing of Plasmid DNA

Plasmid curing of bacterial isolate was used to determine whether the gene(s) responsible for methanol utilization is located on the plasmid or not, for that, many attempts were made in order to cure *P. aeruginosa (*HK8) plasmids by using SDS.

Results in table (3-4), indicated that the highest concentration of SDS that allows the growth of *P. aeruginosa (*HK8) was 4%. From this treatment, appropriate dilutions were made and spreaded on nutrient agar plates .One hundred colonies were selected and each one of these colonies was grown in the defined medium supplemented with methanol as a sole carbon source in order to determine the cured colonies, which can not grow in this defined medium, the obtained cured colonies were retested in this medium to ensure loosing their ability to grow.

Results indicated that the rate of curing was (9%) i.e., nine colonies failed to grow in this defined medium .In order to prove the results of curing experiments, plasmid DNA was isolated from one of the cured colonies, and compared with plasmid content of the original isolate.

Results in figure (3-2), showed that the cured colony has lost its plasmid DNA bands. This result indicated that gene(s) responsible for methanol utilization is located on plasmid, Brautaset *et al.,* (2004) found that methanol dehydrogenase gene, *mdh,* is located on plasmid of *Bacillus methanolicus* MGA3.

The use of SDS as a curing agent in this study showed that SDS is a powerful agent in eliminating plasmids; hence, the curing percentage for *P. aeruginosa* (HK8) was 9%. This result was in agreement with Trevors, (1998) who found that SDS was used successfully as a curing agent .Ramose *et al.,* (1994) indicated that SDS was used successfully in curing of hydrocarbon degradative plasmids

Table (3-4): Effect of SDS on the growth of *P. aeruginosa* HK8

SDS Concentration	**0%**	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**10%**
Bacterial growth(HK8)	+++	+++	++	+	±	-	-	-	-	-	-

(-) : No growth , (±) :Slightly growth, (+) :Moderate growth , (++) :Good growth, (+++) :Very good growth

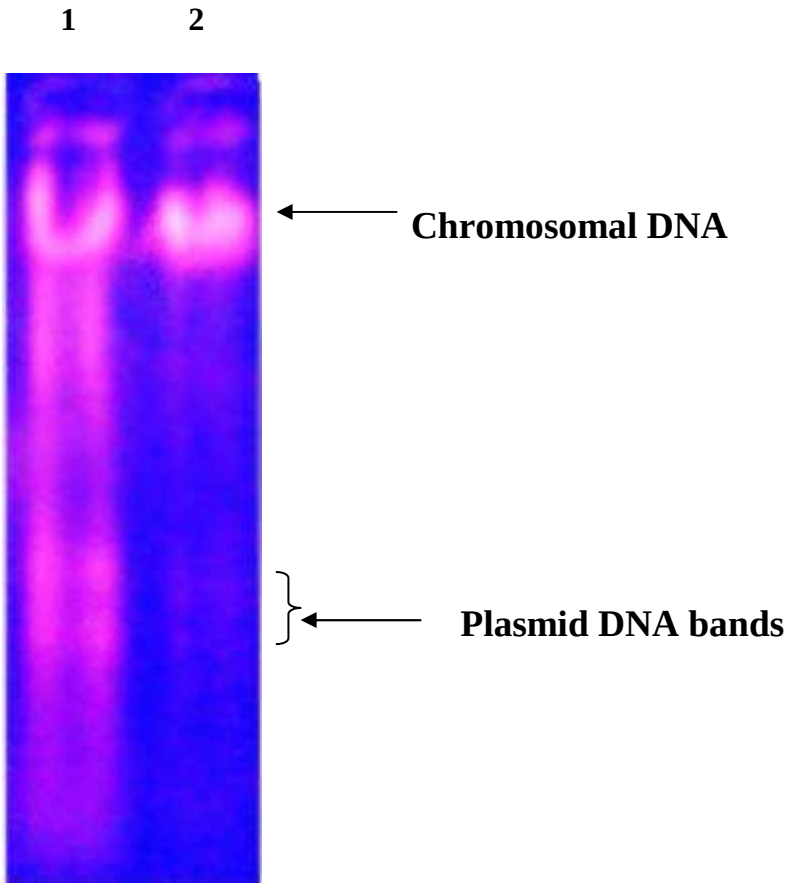

Figure (3-2): Gel electrophoresis of plasmids content of *P.aeruginosa* HK8 Before and after treatment with SDS on agarose gel (0.7%) in TBE buffer At 5V/cm for 2hrs.

1. **Plasmid content of the *P.aeruginosa* (HK8)**
2. **Plasmid content of the *P.aeruginosa* (HK8) (cured)**

3.4.2 Transformation

In order to investigate the transfer of methanol utilization trait from one bacterium to another, attempts were made to transform *E.coli* DH5α with plasmid DNA of *P. aeruginosa*(HK8).

The mixture of transformation was inoculated in vials containing 10ml of the defined medium and supplemented with methanol (0.8%)ml (a concentration which *P.aeruginosa*(HK8) can grow on, while *E.coli*DH5α can not).The vials incubated at 37ºC for 2-3days.After incubation, serial dilutions were made from these cultures and spread on MacConky agar plates.

High frequency of transformants (17.5×10^{-3}) were obtained, then the plasmid isolation for one of the transformants colonies was made and compared with the W.T. isolates (controls) (*P. aeruginosa* HK8 and *E. coli*DH5α).

As shown in fig. (3-3), the transformant colony contain plasmid bands which is similar to *P.aeruginosa* HK8 plasmid bands, this indicated that the plasmids of *P.aeruginosa* HK8 was transferred to *E.coli*DH5α which was free of plasmids (control).

Transformation results confirmed that gene(s) responsible for utilization of methanol as a sole source of carbon and energy, is located on plasmids (Brautaset *et al.*, 2004) and that confirmed the curing experiments.

*Pseudomonas*AM1 DNA was ligated with plasmid R1162 and transferred to *E.coli,* a number of colonies were obtained which can grow on methanol, the subsequent studies and plasmid analysis suggest that *Pseudomonas* methanol dehydrogenase gene has been cloned in this vector (Gautier and Bonewald, 1980).

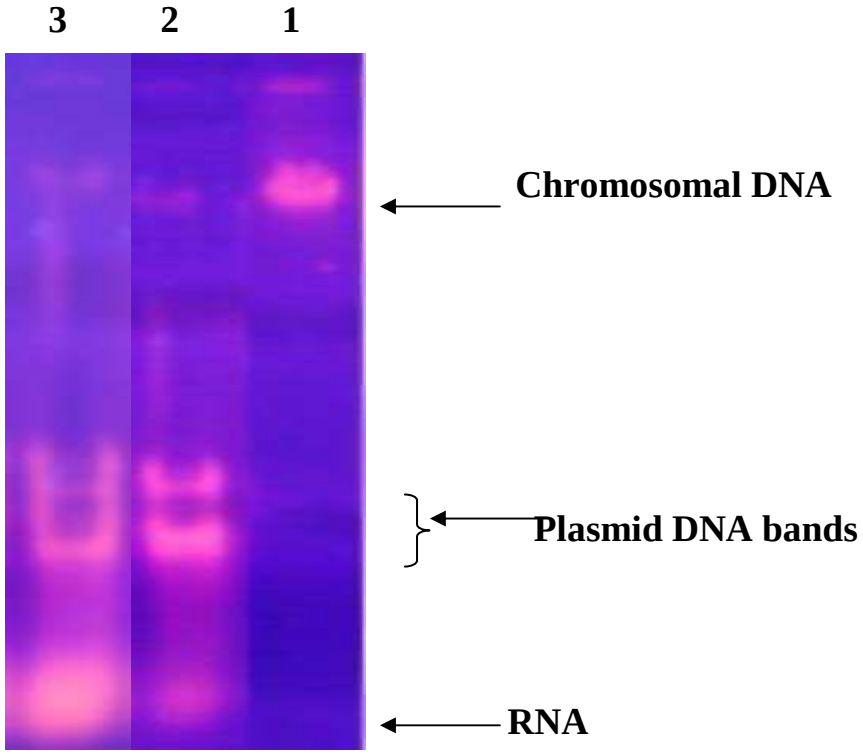

Figure (3-3): Gel electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.7%) in TBE buffer at (5V/cm) for2hrs.

1. ***E. coli*** **DH5α**
2. ***E .coli*** **DH5α (transformant)**
3. ***P. aeruginosa*** **HK8**

4.1 Conclusions

1. *Pseudomonas aeruginosa* HK8 was the most efficient isolate in utilizing methanol as a sole source of carbon and energy.

2. SDS was affected as curing agent for the plasmid encoding methanol utilization trait.

3.Transfer of plasmid harboring methanol utilization trait from *Pseudomonas aeruginosa* HK8 to *E.coli* DH5α was succeeded.

4.Gene(s) responsible for methanol utilization trait was located on plasmid in *Pseudomonas aeruginosa* HK8 .

4.2 Recommendations

1. It is interesting to do research on another microbial genera have the ability to utilize methanol as a sole source of carbon and energy.

2. It is important to isolate bacterial strains capable of utilizing another type of alcohol.

3. Characterize, purify and study the best conditions for methanol dehydrogenase enzyme activity and production.

4. Studying the genetic property of the enzyme i.e. sequencing of genes coding for methanol dehydrogenase enzyme.

Chapter Three

Results and Discussion

Chapter Four

Conclusions
and
Recommendations

Chapter Two

Materials and Methods

Chapter One

Introduction
and
Literature Review

References

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We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Dedication...

To The Memory of Those Who Made The Ultimate Sacrifice So That Others Could Reach For The Stars....

Hadeel

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Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

Genetic Study on Methanol Utilizing P. aeruginosa and Micrococcus sp.

A thesis
Submitted to the College of Science of AL-Nahrain University
in partial Fulfillment of the Requirements for the Degree of
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By

Hadeel Kadir Rustam

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Muharram 1428

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

دراسه وراثيه على بكتريا
Micrococcus sp. و P. aeruginosa

المستهلكه للميثانول

رسالة
مقدمه إلى كلية العلوم جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانه الاحيائيه

من قبل

هديل قادر رستم

بكالوريوس تقانه إحيائية

٢٠٠٤

محرم ١٤٢٨

كانون الأول ٢٠٠٧

Summary

Fifty different soil samples (contaminated and uncontaminated with hydrocarbons compounds) were collected from different places in Iraq.

Twenty five isolates showed the ability to utilize methanol as a sole source of carbon and energy. These isolates were repeatedly tested in the defined medium supplemented with methanol to ensure methanol utilizing ability, then only 12 isolates that appeared, really have the ability to utilize methanol and were named as (HK1,HK2,……,HK12), it was found that these isolates were varied in their growth density in utilizing methanol. Three isolates (HK8, HK9, and HK11) were selected as the efficient isolates.

The efficient isolates were identified by morphological cultural characterization and biochemical tests, the results showed that two isolates belong to *Pseudomonas aeruginosa* (HK8, HK9) while the other one belongs to *Micrococcus* (HK11).

Ability of bacterial isolates to utilize high concentration of methanol was done by growing these isolates on serial concentration of methanol to choose the most efficient isolate which is able to survive at the highest concentration, results indicated that *P. aeruginosa* HK8 was the most efficient isolate in utilizing 0.8% (v/v) methanol.

Agarose gel electrophoresis of whole DNA of the three efficient isolates showed that these isolates harbored two plasmid bands.

Curing experiments by SDS for the chosen isolate (*P. aeruginosa* HK8) showed that some colonies have lost their ability to utilize methanol as a sole carbon source .Plasmid DNA extraction from these colonies indicated the loss of plasmids.

Transformation experiments for this isolate indicated that the gene(s) responsible for methanol utilization were located on plasmids after their expression in *E. coli* DH5α ,these results were confirmed by data obtained from curing experiments as mentioned above.

الـخلاصة

تم جمع خمسين عينة تربه (ملوثه وغير ملوثه بالمركبات الهيدروكاربونيه) من مناطق مختلفة من العراق، أظهرت 25 عزله قابليتها على النمو على وسط الأملاح المعدنية المضاف له الميثانول كمصدر وحيد للكاربون والطاقة، أخذت هذه العزلات وأعيد زراعتها على نفس الوسط للتأكد من قابليتها على النمو واستهلاك الميثانول ،أظهرت النتائج أن 12 عزلة فقط لها القابلية على النمو واستهلاك الميثانول كمصدر وحيد للكربون وبكثافة نمو مختلفة، انتخبت 3 عزلات كفؤه في استهلاكها لهذا المركب.

أثبتت نتائج التشخيص المظهرية والكيموحيويه لهذه العزلات عائدية عزلتين للنوع *Pseudomonas aeruginosa* (HK8,HK9) بينما العزلة الأخرى تعود لجنس *Micrococcus* (HK11).

درست قابلية هذه العزلات على استهلاك تراكيز عالية من الميثانول، تم اختيار العزلة *Pseudomonas aeruginosa* (HK8) التي أظهرت قابليه على استهلاك أعلى تركيز من الميثانول هو (8٠0.%) حجم/حجم.

درس المحتوى البلازميدي للعزلات الكفؤه (HK8,HK9,HK11) لمعرفة مدى العلاقة بينه وبين الجينات المشفرة لصفة استهلاك الميثانول كمصدر وحيد للكاربون والطاقة وأظهرت نتائج الترحيل الكهربائي احتواء هذه العزلات على حزمتين بلازميديتين.

أظهرت نتائج التحييد باستخدام SDS والتي أجريت على العزله المختاره(HK8) *P. aeruginosa*، الحصول على بعض المستعمرات الفاقدة لصفة استهلاك الميثانول كمصدر وحيد للكاربون والطاقة.وعند محاولة استخلاص الدنا البلازميدي لتلك المستعمرات تبين إنها فاقده للبلازميدات.

أظهرت نتائج تجارب التحول للبكتريا المختاره قابلية بلازميدات هذه العزلات على التعبير المظهري في بكتريا *E. coli* DH5α ،وان الجينات الوراثية المسؤولة عن صفة استهلاك الميثانول كمصدر وحيد للكاربون والطاقة كانت بلازميدية الموقع وهذا ما أكدته نتائج تجارب التحييد السابقة.

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيم

وَاللهُ أَخْرَجَكُمْ مِّنْ بُطُونِ أُمَّهَاتِكُمْ لا
تَعْلَمُونَ شَيئًا وَجَعَلَ لَكُمُ السَّمعَ
وَالأَبْصَارَ وَالأَفْئِدَةَ لعَلَّكُمْ تشْكُرُونَ

صَدَقَ اللهُ العَظيم

سورة النحل
الآية:٧٨

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