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College of Sciences

Isolation and Identification of Agar Degrading Bacteria and Study of the Genetic Nature of this characteristic

A thesis
Submitted to the College of Science of Al-Nahrain University
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By

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بسم الله الرحمن الرحيم

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أقرأ وربكَ الاكرَمُ ٢ الذي ٣ عَلمَ بالقلَمِ ٤ عَلَمَ
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صَدق الله العَظيم

سورة العلق الاية ١–٥

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الى الحنان المتدفق

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اخوتي واخواتي

الى روح من احاطني بحنانه ورعايته

عمي الحبيب رحمه الله

الى من احاطوني بحبهم

هبة

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Committee Certification

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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Supervisor Certification

I certify that this thesis was prepared under my supervision in the College of Science, Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

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Scientific Degree: Assistant Professor

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In review of the available recommendations, I forward this thesis for debate by the examining committee.

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Scientific Degree: Assistant Professor.

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Summary

Forty different samples (water and soil) were collected from different places in Iraq and Syria, 25 isolates were isolated from these samples which have the ability to grow on agar plate (defined media).

These isolates were cultured again on agar plate (defined media) then only 12 isolates showed the ability to degrade agar by growing and forming clearing zone around the bacterial growth. These isolates were identified by morphological and biochemical tests and the results showed that 8 isolates belong to *Pseudomonas* and 4 isolates belong to *Bacillus* and designated as (HA1-HA12).

Screening was done for the 12 isolates that show the ability to grow and degrade agar as a sole carbon source to select the efficient bacterial isolate depending on clearing zone around the bacterial growth and found the (HA1) was the best isolate for *Pseudomonas* and (HA9) for *Bacillus*.

Genetic studies of agar degradation property for choosing isolates of *Pseudomonas* (HA1) and *Bacillus* (HA9) was done by studying the plasmid profiles for both isolates and the result showed that both isolates have a small plasmid DNA bands using salting out method.

Curing experiments by SDS indicated that *pseudomonas* (HA1) had lost their ability to grow on agar as a sole carbon source, while in *Bacillus* (HA9) didn’t loss their ability to degrade agar. These results were confirmed that agar degredative property was plasmid-encoded in *Pseudomonas* (HA1) while in *Bacillus* was chromosomal.

Transfer the agar degradation property from *Pseudomonas* (HA1) to *E. coli* MM294 by transformation was succeeded.

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Chapter One

Introduction

and

Literature Review

1.1 Introduction

Bacteria have long been exploited as a source of natural products for use in medicine, agriculture, and industry. Until recently, most studies utilized microbes that had been isolated from clinical or terrestrial environments. It is now recognized that bacteria living in marine environments provide an abundant and as-yet-untapped source of metabolic properties (Bernan *et al*., 1997).

Agar-decomposing bacteria were first isolated by Gran 1902 (Yaphe, 1957). Consequently, several agarolytic bacterial strains were isolated from marine and other environments (Schroeder *et al*., 2003). These bacteria play an important role in the recycling of organic material at the edges of the seas and oceans where agar forms a significant constituent of the polysaccharides of the red algae (Agbo and Moss, 1979).

Agar is the main matrix polysaccharides of *Ahnfeltiales*, *Gelidiales* and *Gracilariales* (Rhodophyta) (Jam *et al*., 2005), and composed of agarose and agaropectin (Ress, 1969).

Agarase is a biotechnologically important enzyme obtained from agarolytic bacteria mostly found in marine environment. A part from digesting agar-agar, agarose and neoagaro-oligosaccharides, few agarases also act on a range of other substrates such as starch, pectin and other derivatives of polysaccharides.

Hydrolytic enzymes that degrade agarose are classified into two groups Alpha- and beta-agarase, agarolytic enzymes have been purified from various microorganisms, including *Pseudomonas, Cytophaga, Streptomyces, Vibrio, Alteromonas, Pseudoalteromonas, and Alterococcus* (Meškiene *et al*., 2003).

Agarase provide an interesting model system to investigate the molecular bases of the solid phase degradation of sulphated polysaccharides (Jam *et al*., 2005).

1.2 Aims of the Study

- Isolation and identification of bacteria that capable to degrade agar.
- Selection of the efficient bacterial isolates.
- Study of the plasmid profiles of the efficient isolates.
- Determination the role of the plasmid in agar degradation for the efficient isolates by curing and transformation.

1.3 Agarolytic Bacteria

Many agar degrading bacteria have been recognized since 1902 when Gran isolated the first species from waters of the Norwegian coast (Hofsten and Malmqvist, 1975). Agar-degrading bacteria are ubiquitous in marine environments but are found as well as in freshwater, sewage, and soil (Zhong *et al*., 2001). These bacteria belong to divers genera, including *Alterococcus* (Shieh and Jean, 1998), *Bacillus* (Kim *et al*., 1999), *Pseudomonas* (Ha *et al*., 1997) and *Microscilla* (Naganuma *et al*., 1993).

Agarolytic bacteria can be divided into two groups according to their effect on solid agar (Agbo and Moss, 1979). Bacteria in group 1 soften the agar, forming a depression around the colonies, while those in group 2 cause extensive liquefaction of the agar. Although a number of species belong to these two groups have been reported, extensive studies to explain their distinct patterns of agar degradation have not been carried out (Leon *et al*., 1992).

A number of microorganisms have been reported to degrade agarose, including marine bacteria from the genera *Pseudomonas* or *Alteromonas* (Day and Yaphe, 1975; Vera *et al*., 1998), *Vibrio* (Aoki *et al*., 1990; Sugano *et al*., 1993b) and *Cytophaga* (Van der Meulen and Harder, 1975), as well as the land bacterium *Streptomyces coelicolor* (Buttner *et al*., 1987), with the exception of the enzyme from *Alteromonas agarilytica* (Potin *et al*., 1993; Jam *et al*., 2005), Some of these genera are:

1.3.1 Genus: *Alterococcus*

Alterococcus agarolyticus, a halophilic thermophilic bacterium capable of agar degradation, five strains of facultatively anaerobic moderately thermophilic bacteria were isolated from two hot springs in the intertidal zone of Lutao, Taiwan. They produced extracellular agarase on agar medium, yielding reducing sugars and organic acids as the end products under either aerobic or anaerobic conditions.

The growth temperature range was approximately 38-58°C with an optimal temperature of about 48°C. The five strains tolerated a relatively narrow pH rang from 7.0 to 8.5. They were Gram-negative halophiles growing optimally at 2.0-2.5% NaCl. They were capable to grow in anaerobic condition by fermenting glucose and producing various organic acids such as butyrate, propionate, formate, lactate, and acetate. Cells grown in liquid medium were motile monotrichous cocci, normally 0.8-0.9 micron in diameter.

They are the first thermophiles found to degrade agar and also the first halophilic thermophilic bacteria known to be capable of both aerobic and anaerobic fermentative growth. These bacteria are considered to represent a new genus which named *Alterococcus*, and *A. agarolyticus* is the type species (Shieh and Jean, 1998).

1.3.2 Genus: *Alteromonas*

A marine bacteria strain isolated from the Bay of San Vicente, Chile, was identified as *Alteromonas* sp. strain C-1. In the presence of agar, this strain produced high levels of an extracellular agarase. The production of agarase was repressed by glucose, with a parallel decrease in bacterial growth. The enzyme has a molecular weight of 52,000,d is salt sensitive, and hydrolyzes agar, yielding neoagarotetraose as the main product with an optimum pH of about 6.5 (Leon *et al*., 1992).

1.3.3 Genus: *Cytophaga*

Cytophaga flevensis produced an inducible agarase which was extra cellular under most conditions tested. The effect of cultural conditions on the production of enzyme was studied in batch and continuous culture. In batch culture, production was optimal when *Cytophaga flevensis* was incubated at 20°C in mineral medium with agar as the sole carbon source and ammonium nitrate as the nitrogen source at an initial pH of 6.6-7.0.

The enzyme was appeared to be subject to catabolite repression, since its synthesis was repressed when glucose was added to the medium in batch culture. Furthermore, in continuous culture, enzyme production decreased with increasing growth rate. Extracellular agarase was partially purified and the enzyme preparation obtained was very stable.

The enzyme has a molecular weight of 26,000 daltons. It is a β-agarase which highly specific for polysaccharides containing agarose by the endo-acting enzyme were neoagarotetraose and neoagarobiose. Optimal conditions for its activity were pH 6.3 and 30°C, when agarose was used as a substrate, due to gelling of substrate during the assay procedure (Van der Meulen and Harder, 1975).

1.3.4 Genus: *Vibrio*

β-agarase was purified from the culture fluid of a prophyran-decomposing marine bacterium (strain AP-2) by ammonium sulfate precipitation, successive column chromatography, DNase and RNase treatment. The final enzyme preparation appeared to be homogenous on polyacrylamid gel electrophoresis.

The enzyme had a molecular mass of 20 KDa, a pH optimum of 5.5, and was stable in the pH region 4.0-9.0 and at temperatures below 45°C. The β-agarase was a novel endo-type enzyme which hydrolyzed neoagarotetraose, larger neoagarooligosaccharides and agar to give neoagarobiose [3,6-anhydro-α-L-galactopyranoseyl-(1---3)-D-galactose] as the predominant product.

According to the criteria of Bergey's Manual of Systematic Bacteriology, the strain was assigned to the genus *Vibrio* (Aoki *et al*., 1990).

The DNA G + C content was 44.8 mol %. Phylogenetic analysis based on complete 16s and 23s rDNA sequences revealed that the strains belong to the gamma-Proteobacteria, and are specifically related to *Vibrio* species. Their nearest relatives were species of the *Vibrio fischeri* group, sharing 16s rDNA

sequences similarities below 97% with the agarolytic strains (Macian *et al.*, 2001).

1.3.5 Genus: *Pseudoalteromonas*

The phenotypic and agarolytic features of an unidentified marine bacteria that was isolated from the southern Pacific coast was investigated. The strain was gram negative, obligately aerobic, and polarly flagellated. On the basis of several phenotypic characters and a phylogenetic analysis of the genes coding for the 16s rRNA, this strain was identified as *Pseudoalteromonas antractica* strain N-1.

In solid agar, this isolate produced a diffusible agarase that caused agar softening around the colonies, and it had a molecular mass of 33 kDa. The enzyme hydrolyzed the β-1,4-glycosydic linkages of agar, yielding neoagarotetraose and neoagarohexaose as the main products, and exhibited maximal activity at pH 7. The enzyme was stable at temperatures up to 30°C, and it's activity was not affected by salt concentrations up to 0.5 M NaCl (Vera *et al.*, 1998).

1.3.6 Genus: *Bacillus*

Extracellular agarase of *Bacillus cereus* ASK202 was purified 32-fold, giving a single band on PAGE with activity staining. The M.wt. of purified agarase was determined as 90 KDa by SDS-PAGE. The N-terminal amino acid was sequenced and the sequence did not show homology to any other known agarases. The optimum pH and temperature were 7.0 and 40°C, respectively. This enzyme was found to be a β-agarase which catalyzed the hydrolysis of the beta- 1-4 linkage of agarose to yield neoagarohexaose, neoagarotetraose and neoagarobiose (Kim *et al.*, 1999).

1.4 Important of Agar Degrading Bacteria

Agar-degrading bacteria play an important role in the recycling of organic material at the edges of the seas and oceans where agar forms a significant constituent of the polysaccharides of the red algae. When such organisms have been isolated from non-marine habitats, attempts have been made to rationalize their presence by looking for agar in the environment or by relating the environment to a recent marine origin. Thus, Hofsten and Malmqvist (1975) isolated their strongly agarolytic organism from a sewage treatment plant which they suggest receives quantities of agar laboratory wastes. Although agar as an environmental pollutant could have some selective effect it is probable that agarolytic organisms were present, and had a role to play, in the habitat before the appearance of the pollutant (Agbo and Moss, 1979).

Also the ability of degradation of agar or agarose to oligosaccharides makes it widely used in food, cosmetic and medical industries. The degradation products neoagaro-oligosaccharides inhibit the growth of bacteria and slow the rate of degradation of starch. It is a very good food quality protector or additives for this inhibition of the bacterial growth. Besides, the other degradation product neoagarobiose (NA2) is also a rare reagent with both moisturizing and whitening effects on melanoma cells (Yukari *et al*., 2004).

1.5 Agar and its Structure

Agar is a complex polysaccharide produced by marine red algae from genera such as *Gelidium*, *Gracilaria* and *Ceramium*. It contains a neutral agarose fraction and an ionic agaropectin fraction (Araki, 1937). Agaropectin is believed to be composed of a complex range of polysaccharide chains, but not very much is known about the details of its structure. On other hand agarose is defined as having a linear chain structure composed of alternating residues of 3-O- linked **β**-D-

galactopyranose and 4-O- linked 3,6-anhydro α-L-galactopyranose (Hamer *et al*., 1977).

But both fractions are consist of a linear backbone of alternating L–and D–galactose residues linked by α-(1,3) and β-(1,4) linkages respectively with various substituents such as ester-sulphonic groups, methyl ethers and pyruvic acid (Craigie, 1990).

Substitutions or modifications on the molecular chain, can be occurred like:

A. Methoxylation (-O-CH_3), occurs mainly on C-6 of the D-galactose. Substitution by methyl groups is positively correlated to gelling temperature.

B. Sulphation (-O-SO_3), probably occurs mainly on C-2 of the L-galactose. Agar may contain up to 5-6 % SO_4 Substitution by sulphate esters is negatively correlated to gel strength.

C. Pyruvation (-O-C (CH_3) (COOH)-3, as a ketal coupled onto C-4 and C-6 of the D-galactose. Agar may contain up to 30 % pyruvate and it is not believed to affect gelling properties, where as methoxylation seems to occur regularly on the molecular chain, sulphation and pyruvation are rather irregular (Izumi, 1972).

As a simple definition for agar, we would say it is composed by two groups of polysacchrides, agarose and agaropectin.

Agarose, which corresponds to the neutral and linear part of the molecule, which is able to form a strong gel. It is believed that absence of charged groups, as sulphates, enable it to form gel, as well as the molecule neutrality.

Agaropectin, is the charged part of the molecule. The presence of sulphated groups would help to form a more colloidal structure, which would not structure, and not gellify as strong as a linear molecule. Since the molecule is negatively charged, there is repulsion between them, which would contribute to form a soft gel, figure (1-1)

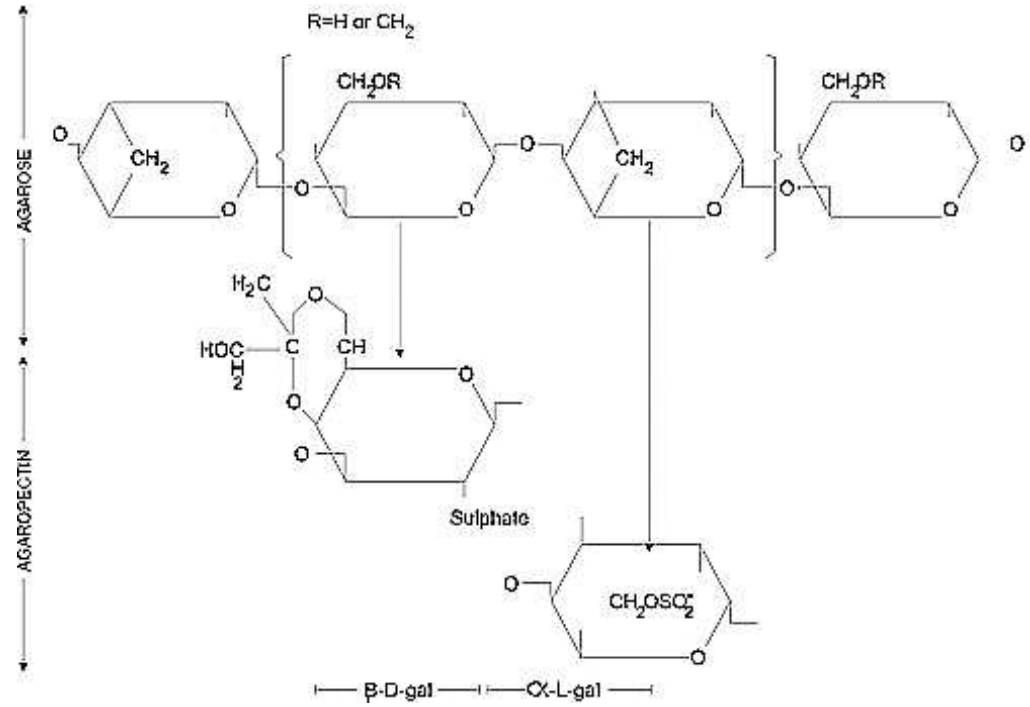

Figure (1.1) Agar Structure (internet 1)

Nevertheless, Agar is now considered to a complex mix of polysaccharides, all on a same structural basis, but with different grade of charged groups substitution (Agbo and Moss, 1979; internet 1).

1.6 Properties of Agar

1.6.1. Solubility

Agar-agar is insoluble in cold water, but it swells considerably, absorbing as much as twenty times its own weight of water. It dissolves readily in boiling water and sets to a firm gel at concentration as low as 0.50 %. Powdered dry agar-agar is soluble in water and other solvents at temperatures between 95C and 100°C .Moistened agar flocculated by ethanol, 2-propanol or acetone, or salted out by high concentrations of electrolytes, are soluble in a variety of solvents at room temperature. (internet 2)

1.6.2 Gelling

The gelling portion of agar-agar has a double helical structure. Double helices aggregate to form a three- dimensional structure framework which holds the water molecules within the interstices of the framework. Thus, thermo-reversible gels are formed. The gelling property of agar-agar is due to three equatorial hydrogen atoms on the 3, 6-anhydro-L-galactose residues, which constrain the molecule to form a helix. The interaction of the helixes causes the formation of the gel. (internet 2)

1.6.3 Viscosity

The viscosity of agar solutions varies widely and is markedly depended upon the raw material source. The viscosity of an agar solution at temperatures above its gelling point is relatively constant at pH 4.5 to 9.0, and is not greatly affected by agar or ionic strength within the pH range 6.0 to 8.0. However, once gelling starts viscosity at constant temperature increases with time. (internet 2)

1.6.4 Stability

An agar-agar solution is slightly negatively charged. Its stability depends upon two factors: hydration and electric charge. The removal of both factors result in flocculation of the agar-agar. Prolonged exposure to high temperatures can degrade solutions of agar-agar, resulting in lower gel strength after temperatures decreased and gel formation. The effect is accelerated by decreasing pH. Therefore, it should be avoided to expose agar-agar solutions to high temperatures and to pH lower than 6.0 for prolonged periods of time. Agar-agar in the dry state is not subject to contamination by microorganisms. However, agar solutions and gels are fertile media for bacteria and / or molds and appropriate precautions should be taken to avoid the growth of microorganisms (internet 2).

1.7 Usage of Agar

Agar-agar has a wide variety of usage. It can be used as gelatin, emulsifier, thickening, and gelling agents, in four fields, these fields are:

Medicine field: It is used as a culture media in plant tissue culture, in pathogenic and non pathogenic bacteria and fungi, it is also used in the preparation of dental casts and production of hollow medicine capsules.

Food field: It has been used as high performance gelling, texturizing, emulsifying, stabilizing, and thickening agents in ice cream, jelly, gummy candy, pork luncheon meat, ham sausage, tomato sauce, coconut sauce, etc. It is also used as clarifying agent in winemaking and brewing. The usage portion of agar-agar is ordinarily between 0.3 and 1.5%.

Chemical field: Agar-agar has been exploited in making high-grade cream for protecting skin, hair emulsion, gel for finalizing hair design, etc.

Building field: Due to stickiness, agar-agar is used in all kinds of paint for water proofing and preventing leaking (internet 3).

1.8 Agar Degrading Enzyme (Agarase)

The research in agarose can be tracked back to almost 50 years ago. (Yaphe, 1957)

Yaphe and co-workers were the first to describe an agar-degrading enzyme system from a marine bacterium (Day and Yaphe, 1975; Groleau and Yaphe, 1977).

Agarase is an enzyme that will completely digest the polysaccharide backbone of molten agarose into alcohol soluble oligosaccharides (internet 4). Specifically digests the agarose Polysaccharide core made up of repeating 1,3-

linked β-D-galactopyranos and 1,4-linked 3,6-anhydro-α-L-galactopyranose into neo-agaro-oligosaccharides (Yaphe, 1957)

The agar-degrading enzymes of both groups *Cytophaga* and *Alteromonas* genes were inducible, not only by agar but also by other galactans and polysaccharides associated with plants (Agbo and Moss, 1979).

1.8.1 Types of Agarase Enzyme

Hydrolytic enzymes which degrade agar are classified into two groups in terms of their mode of action on agar (Sugano *et al*., 1993a).

Those that enable to cleave the α-1,3-anhydro-L-galactosidic bonds are named α-agarase. Likewise those that enable to cleave the **β**-1,4-galactosidic bonds are called **β**-agarase. In fact since the great difference of amino acid sequences between **β**- and α-agarase, it is possible to determine the type of the agarase (i.e. α- or **β**-) just based on the comparison of the amino acid sequences with the known agarase sequences. (internet 5)

Agarase which was secreted from marine bacterium *Zobellia galactanivorans* Dsij or derived from *Pseudomonas atlantica*, were believed to selectively cut the α-1,3-and **β**-1,4-linkage of agarose's galactose-anhydrogalatose repeat unit (Julie *et al*., 2003; 2004).

1.8.2 Alpha and Beta Agarase Enzyme

Alpha Agarase would exist in gram negative bacteria, but non has yet been purified or characterized (Young *et al*., 1978). The bacterium was assigned to the genus *Alteromonas* and the new combination *A. agarlyticus* (cataldi) is proposed. An α-agarase, i.e. specific for α (1 -3) linkages presents in agarose (Potin *et al*., 1993)

Beta-agarase is one member of the GH-16s (Glycoside Hydrolase 16) (Buttner *et al*., 1987; Voget *et al*., 2003), with occurrences of the new glycoside hydrolase, GH family has been growing steadily from 85 in 2001 to 97 now.

Previous researches demonstrate that β-agarase has a specific sub domain that distinguishes from the other members by binding the specific substrate (internet 5).

All of the bacterial strains e.g. *Alteromonas* (Day and Yaphe, 1975; Vera *et al*., 1998), *Cytophaga* (Van der Meulen and Harder, 1975), *Streptomyses* (Buttner *et al*., 1987), *Vibiro* (Aoki *et al*., 1990; Sugano *et al*., 1993b), *Pseudomonas atlantica* (Julie *et al*., 2004) and *Zobellia galactnivorans Dsji* (Julie *et al*., 2003; Julie *et al*., 2004) contain this glycoside hydrolase's β-agarase

Several types of **β**- agarase have been found in *Pseudomonas* spp. (Malmqvist, 1978; Morrice *et al*., 1983c).

However, biochemical and genetic studies have shown that there appears to be diversity among **β**- agarase proteins and genes, even though the enzymes are functionally similar in catalysis of the agar (1-4)-**β**-D backbone. The enzymes are widely varying in the sizes for example, *C. flevensis* is about 26,000 daltons, *S. coelicolor* is about 28,000 daltons, and *P. atlantica* is about 49,000 daltons (Belas, 1989).

Reported a new β-agarase, which called agarase 0107, this enzyme is secreted by the marine bacterium *Vibrio* sp. strain JT0107 and has a Mwt. of 107,000 (Sugano *et al*,. 1993b). Its distinguishing characteristics are its optimum pH (pH 8) and its ability to decompose even gelled agarose, which may have practical application in gene technology for isolation of DNA fragments from agarose gels after electrophoresis (Sugano *et al*., 1993a). Of three principal genera characterized as agar-digesting bacteria, most research to date has focused on the agarolytic properties of the marine bacterium, *Pseudomonas atlantica* (Belas *et al*., 1988; Belas, 1989).

Since the nucleic acid sequences of the agarases from different bacterium species are different, the encoding amino acids of the respective gene also show great variables.

Despite of that, the function domains are well conserved and have high homologies. *Pseudomonas* sp. SK38 shows 58% similarity to the beta- agarase from *P. atlantica* and 57%, 54 % homologies with *Aeromonas* sp. and *Z. galactanivorans* respectively (kang *et al*.**,** 2003; Yukarei *et al*., 2004).

The β-agarase cleave the β-D-(1, 4) linkages and the α-agarase cleave the α-L-(1,3) linkages. These enzymes are very interesting because agar is an additive in many foods and feed products, as well as an important compound for a large number of molecular biology applications, but **β**-agarase shows no homology with α-agarase (Kang *et al*., 2003; Voget *et al*., 2003).

1.9 Mechanism of Agar Degradation

Previous studies shown that agar degradation can occur by two mechanisms that depend on the specifity of the cleaving enzymes. The first pathway for agar breakdown comes from studies on *P. atlantica* ATCC 19292 (Morrice *et al*., 1983a; 1983b), and relies on extra cellular β-agarase. In this bacterium, an endo β-agarase I cleave the β-(1-4) linkages of large agar polymers to a mixture of oligosaccharides with neoagarotetraose as the final product. These oligosaccharides are then hydrolyzed by the cell-bound exo β-agarase II, yielding neoagarobiose. Finally, neoagarobiose is hydrolyzed to 3, 6-anhydro-L-galactose in the cell cytoplasm by neoagarobiose hydrolase (Day and Yaphe, 1975).

The second lytic mechanism involves the cleavage of α-(1-3) linkages on agarose by extracellular α-agarase (Young *et al*., 1971; 1978; Potin *et al*., 1993) yielding oligosaccharides from agarobiose series, which contain D-galactose at the non-reducing end. The agarolytic system of *A. agarolyticus* strain GJIB consist of two enzymes : an α-agarase that cleaves the α-(1-3) linkages and a β-galactosidase specific for the presence of 3,6-anhydro-L-galactose units at the reducing end (Potin *et al*.,1993), Agarotriose was the smallest product detected in this system (Vera *et al*., 1998).

1.10 Genetic of Agar Degradation

Plasmids are extrachromosomal genetic elements, 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).

An agar degrading marine bacterium identified as *Microscilla* species was isolated from coastal California marine sediment. This organism harbored a single 101-Kb circular DNA plasmid designated pSD15. The complete nucleotide sequence of pSD15 was obtained, and sequence analysis indicated a number of genes putatively encoding a variety of enzymes involved in polysaccharide utilization. The most striking feature was the occurrence of five putative agarase genes, loss of the plasmid, which occurred at a surprisingly high frequency, was associated with loss of agarase activity, supporting the sequence analysis result. It is possible that this high loss rate is a result of culturing the strain in the laboratory as opposed to growth in the natural environment; where the ability to degrade agar (or any other traits conferred on the host by the plasmid) may provide enough of a selective pressure to maintain the plasmid (Zhong *et al.,* 2001).

P. antarctica strain N-I is one agarolytic bacterial strain, it can isolated from decomposing algae in Niebla, Chile (Jorge *et al*., 1998). Recently the *Z. galactanivorans* Dsij was found to be one member of these bacterial strains (Julie *et al*., 2003; 2004).

Although the genes that encode the agarases are quite different, the similar biotechniques are applied here to isolate the plasmid that encodes the β-agarase. The green spot rot in Porphyra dentate causing by the marine bacterium, *pseudomonas* sp. SK38 contains the enzymatic β-agarase. The *Pseudomonas* sp. SK38 was isolated in Zobell medium pH 7.5. Artificial sea water was used to grow the Porphyra dentata culture. The porphyra dentata culture was then infected with appropriate bacterial strains (e.g. *E. coli*) (Kang *et al.,* 2003).

Other study indicates the involvement of chromosomal genes in agar degradation by *Vibrio* sp. strain JT0107 (Sugano *et al*., 1994).

The recombinant plasmid pDA1, isolated from a *P. gracilis* B9 genomic library, was responsible for the agarolytic activity exhibited by *E. coli* transformants, when grown on solid medium.

A BLAST search of the GenBank database showed that an 873 bp ORF (*aag A*) located on pDA1 had 85 % identity to the β-agarase (*dag A*) from *P. atlantica* ATCC 19262 at the amino acid level. AagA was purified from the extracellular medium of an *E. coli* transformant harboring pDA1 by using a combination of gel filtration and ion-exchange chromatography (Schroeder *et al*., 2003).

The *pjaA* gene of *pseudomonas* sp.W7 consist of an open reading frame of 1926 bp encoding β-agarase, a protein of 642 amino acids and a molecular weight of 69,540 Da. The expressed protein of plasmid pEAG3-3, in which 259 amino acid residues from C-terminus of the over expression plasmid (pEAG3) were eliminated, led to the complete loss of agarolytic activity (Lee, *et al*., 2000).

An extracellular agarase gene was cloned from *S. coelicolor* A3 (2). Strain M130 into *S. lividan*s 66 using the multicopy plasmid vector pIJ702. Various deletion derivatives of the initial clone (pMT605) were obtained by in-vitro and in-vivo methods. This allowed the gene to be localised to a 1.9-Kb segment of DNA. The agarase enzyme was overproduced (up to 500 times) and exported efficiently into the medium (Kendall and Cullum 1984; Bibb *et al.*, 1987)

An agarase gene (*agaA*) was cloned from genomic DNA of *Vibrio* sp. strain JT0107 an open reading frame of 2.985 nucleotides gave a primary translation product composed of the mature protein, agarase 0107 (975 amino acid residues, with a molecular weight of 105,271) and a signal peptide of 20 amino acid residues at the N-terminus.

The AgaA protein which was expressed in *E. coli* had the agarase activity. Agarase 0107 hydrolyzes not only agarose but also neoagarotetraose [O-3,6-anhydro-α-L-galactopyranosyl (1-3)-O-β-D-galactopyranosyl(1-4)-O-3,6 anhydro-α-L-galactopyranosyl (1-3)-D-galactose] to yield neoagarobiose O-3,6-anhydro-α-L-galactopyuranosyl (1-3)-D-galactose]. This is a quite unique characteristic for β-agarase (Sugano *et al*., 1993a).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments were used in this study:

Autoclave	Express (West-Germany)
Balance	Ohans (France)
Compound light microscope	Olympus(Japan)
Distillator	GFL(Germany)
Electrical oven	Memmert (Germany)
Glass Pasture pipette	Johan poulten Ltd (England)
Hot plate with magnetic Stirrer	GallenKamp (England)
Incubator	Termaks (U.K)
Micropipette	Witey (Germany)
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 (Swissrain)
Water bath	GFL (Germany)
Minimal 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:

Chloroform, Glucose, Iodine, K_2HPO_4, $MgSO_4$, KI, HCl, NaCl, NaOH, Glycerol, Sucrose, K_2SO_4	BDH-England
Agar, Gelatin	Biolife-Italy
Starch, Skim milk	Difco-USA
Tris (hydroxyl mehthyl) aminomethane base (Tris-base), Ethylenediaminetetraacetic acid (EDTA), Hydrogen peroxide, Crystal violet, $FeSO_4.7H_2O$, $CuSO_4.7H_2O$, and $ZnSO_4.7H_2O$	Fluka-Switzerland
Boric acid, Cetrimide, Calcium Chlorid hydrate, Sodium acitate, Bromo phenol blue, Methanol	Riedel-deHaeny Germany
Ethanol, MgSO4.$7H_2O$	Merk-Germany
Ethedium bromide, SDS, agarose, Lysozyme	Sigma-USA
Methyl red, Safranine	Oxoid

2.1.3 Bacterial Strains

Bacterial Strains	Phenotype	Source
E. coli HB101Harboring pBR322	Ap^r, Tc^r	Department of Biotechnology / Baghdad University
E. coli MM 294	Rif^r	Department of Biotechnology / Al-Nahrain University
S. aureus ATCC 8625		Department of Biotechnology / Baghdad University

2.1.4 Media

2.1.4.1 Culture Media

The defined medium which used for growing of the agarolytic bacteria was prepared according to a method of Voget *et al*., (2003).

It was composed of (per litter):

$NaNO_3.3H_2O$	1g
$K_3HPO_4.3H_2O$	1g
$MgSO_4.7H_2O$	0.25g
$CaCl_2.7H_2O$	0.015g
$FeSO_4.7H_2O$	0.14g
EDTA	0.02g
Trace element	Per litter
$Co(NO_3)_2.6H_2O$	15 mg
H_3BO_4	60 mg
$MnCl_2.4H_2O$	200 mg
$ZnSO_4.7H_2O$	60 mg
$NiCl_2.H_2O$	50 mg
$CuSO_4.5H_2O$	2.5 mg
Na_2MoO_4	2.5 mg
$CaCl_2.5H_2O$	4 mg
agar	1.5%
glucose	0.2%

Ten ml of a trace element solution was added to the whole media, pH was adjusted to 7 before sterilizing by autoclaving at 121°C for 15 min.

2.1.4.2 Differential Media

- **These media were ready made media prepared according to the manufacture's instructions:**

  1-Triple sugar Iron (Difco USA)

  2-Simmon Citrate agar (Difco USA)

  3-Nutrient broth (Biolif-Italy)

  4-Nutrient agar (Oxoid)

  5-MacConkey agar (Oxoid)

  6-MR-VR medium (Difco USA)

- **Cetrimide agar (Stolp and Gadkari, 1984)**

It is composed of:

Peptone	20g
$MgCl_2$	4.5g
K_2SO_4	10g
Cetrimide	0.3g
agar	15g
Distilled water	1000 ml

pH was adjusted to 7.2 before sterilizing by autoclaving at 121°C for 15 min.

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

  It is composed of

Peptone	20 g
glycerol	10 ml
K_2SO_4	10 g
$MgCl_2$	1.4 g
agar	15 g
Distilled water	1000 ml

pH was adjusted to 7.2 before sterilizing by autoclaving at 121°C for 15 min.

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

It is composed of:

Peptone	20g
glycerol	10 ml
$MgSO_4.7H_2O$	3.5g
K_2SO_4	1.5g
agar	15g
Distilled Water	1000 ml

pH adjusted to 7.2 before sterilizing by autoclaving at 121°C for 15 min.

- **Starch agar (Atlas *et al*., 1995)**

Starch agar was used to test the ability of bacteria to hydrolyze starch, and produce amylase enzyme, it was prepared by:

Soluble Starch	10 g
Nutrient agar	23 g
Distilled water	1000 ml

pH was adjusted to 7.2 before sterilizing by autoclaving at 121°C for 15 min.

- **Gelatin Medium (Stolp and Gadkari, 1984)**

Gelatin (12% W/V) was adding to nutrient broth and sterilized by autoclaving at 121°C for 15 min.

- **Casein Medium (Collee *et al*., 1996)**

It was used to test the ability of bacteria to produce proteinase enzyme and casein hydrolysis, it composed of:

Nutrient agar, Sterile	87.5 ml
Skimmed milk, Sterile	12.5 ml

Melted the agar, cool to 50°C, add the milk and pour plate

- **DNase Medium (Collee *et al*., 1996)**

DNase agar	42 g
Toludine blue	0.1 g
Distilled water	1000 ml

pH was adjusted to 7.2 before sterilizing by autoclaving at 121°C for 15 min.

2.1.5 Reagents and Stains

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

This reagent was composed of 3% hydrogen peroxide.

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

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

-Starch Hydrolysis Reagent (Atlas *et al*., 1995)

The starch hydrolysis reagent was Lugol's iodine or (Gram's iodine) which was prepared by mixing 2 grams of potassium iodide with 1 gram of iodine in 300 ml of distilled water.

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

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

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

It consist of:

Reagent A: 40% potassium hydroxide in distilled water.

Reagent B: 5% alpha-naphthol in absolute ethyl alcohol.

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

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

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

This stain was prepared by dissolving 0.25 gram of safranin O in 10 ml of 95% ethanol and the final volume was completed to 110 ml with distilled water allow to stand several days and filter before use.

2.1.6 Buffers and Solutions

2.1.6.1 Plasmid Extraction Buffers and Solutions

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

  75mM NaCl

  25mM EDTA

  20mM Tris-Cl

pH was adjusted to 8 and sterilized by autoclave

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

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

- **Sodium Dodecyl Sulphate Solution (SDS) (Kieser, 1995)**

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

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

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

EDTA 1 mM

Tris-Cl 10 mM

pH was adjusted to 8 and sterilized by autoclave.

2.1.6.2 Gel Electrophoresis Buffers

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

Tris-base 54 g

Boric-acid 27.5 g

EDTA (0.5 M) 20 ml

Distilled water to 1000 ml

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

Prepared in concentration 10 mg/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 Solution (Maniatis *et al*., 1982)

$CaCl_2$ Tris-Cl (pH 8.0)

$CaCl_2$ 50 mM

Tris-Cl 10 mM

2.2 Methods

2.2.1 Samples Collection

Forty samples of soil and water were collected from different places in Iraq and Syria, as following:

A. Iraq

1. Baghdad (15 soil samples)
2. Dijla river (1 soil and 1 water samples)
3. Furat river (1soil and 1 water samples)
4. Aumara (3 soil and 3 water samples)
5. Kute (2 soil and 1water samples)
6. Bechal (1 soil and 1 water samples)
7. Dukan (1 soil and 1water samples)
9. Bhasra (1 soil and 1 water samples)
10. Shalal Kali Ali beg (1 soil and 2 water samples)

B. Syria - Tartous (3 soil samples)

2.2.2 Bacterial Isolation

To screen bacteria for its ability to degrade agar

One hundred ml of sterilizing nutrient broth was inoculated with (soil or water samples) and incubated at 30°C for 24 hr., then streak a sample from nutrient broth into an agar plate (defined media 2.1.4.1) incubated at 30°C for (2-7) days.

2.2.3 Maintenance of Bacterial Isolates

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

2.2.3.1 Short Term Storage:

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

2.2.3.2 Medium Term Storage:

Isolates of bacteria were maintained by streaking on slants of nutrient agar medium for period of few months. Such medium was prepared in screw-capped vials containing 10-15 ml of the medium. The isolates were streaked on these slant media and incubated at 37°C for 24 hr. After that the slant were taken and wrapped with parafilm and stored at 4°C.

2.2.3.3 Long Term Storage:

Bacteria can be stored for many years in nutrient broth medium containing 15% glycerol at low temperature with out significant loss viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria in small screw-capped vials with final volume of 10 ml and stored at -20°C.

2.2.4 Identification of Bacterial Isolates

Bacterial isolates that were well grown on agar plate (defined media) and showed agarolytic activities were identified as follow:

2.2.4.1 Cultural and Morphological Study

- **Morphology of Colonies**

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

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

The specimen was applied to a clean slide, then the specimen was fixed by heat, crystal violet was applied (for 1 minute) and excess stain was washed then Gram's iodine was applied for (1 minute) and the excess was washed with

Distilled water. Alcohol decolorizing agent was applied and the excess was washed, after that safranin was applied (for 30 second) and the excess was washed, and finally the slide was dried for examination under the microscope.

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

In semisolid agar media, motile bacteria (swarm) and give a diffuse spreading that is easily recognized by the naked eye. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. 0.1% agar dissolved in nutrient broth. It is important that the final medium should be quite clear and transparent. Dispense 10 ml amounts in test tubes and leave to set in the vertical position. Inoculate with a straight wire, making a single stab down the centre of the tube to about half depth of the medium incubated at 37°C for 24-48 hrs.

2.2.4.2 Biochemical Test

- **Catalase 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 indicates the ability of bacteria to produce catalase enzyme.

- **Oxidase 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 rubbed on the filter paper with sterile wooden applicator stick. An immediate color change to a deep blue indicates the ability of bacteria to produce oxidase enzyme.

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

Inoculate the tubes of gelatin medium with bacteria by stabbing, and then the tubes were incubated at 30°C for five days. This test was performed to demonstrate the ability of bacteria to hydrolyze gelatin.

- **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 blew pH 6.8) from the production of mixed acids. Colonies of non-lactose fermenting bacteria appear colorless or transparent on this medium.

- **Growth on Cetrimide Agar (Stolp and Gadkari, 1981)**

This medium was used as a selective medium for *Pseudomonas* spp. the plates of this medium were inoculated with bacteria by streaking and incubated at 37°C for 24 hrs.

- **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 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, fluorescin. 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 florescin.

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

The bacteria was inoculated on the plate by making a single streak of bacteria and incubated at 37°C for 48 hrs. After incubation the plates were flooded with iodine solution. Development of yellow color around the colonies indicates the hydrolysis of starch. This test was used to examine the extracellular enzyme alpha-amylase that catalyzes that breakdown of starch to maltose.

- **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 incubated for 24 hrs at 30°C. When the color of medium was changed from red to yellow it is indication of acid formed, 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 appearances of blue color indicate 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.

- **Methyl-Red and Voges-Proskaure (Maza *et al*., 1997)**

Inoculated the liquid medium (MR-VP) tubes and incubated at 37°C for 48 hrs. Both tests are performed from the same culture, which was divided for testing. The Methyl-red test was performed after adding about five drops of methyl-red reagent positive test is bright red and negative test is yellow, while the Voges-Proskaure test was performed after adding the Voges-Proskaure reagent positive test has pink color. This test was used to determine the pH of the end products of glucose fermentation, while Voges-Proskaure test detects acetone.

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

DNase agar plate was inoculated with bacteria, incubated at 37°C for 24-48 hrs. Development of rose pink halo around the area of the growth indicates the hydrolysis of DNA.

- **Casein Hydrolysis Test (Collee *et al*., 1996)**

Casein medium was inoculated with bacteria by making a single streak of bacteria and incubated at 30°C for 24-48 hrs. Casein hydrolyzing bacteria show clear zone around the streak of growth. This test was used to examine the production of proteinase enzyme which hydrolyze casein protein.

2.2.5 Plasmid Extraction

Plasmid DNA was isolated by salting-out method which described by Kieser, (1995).

- Culture of bacteria grown in nutrient broth, was pelleted from 20 ml by centrifugation at 6000 rpm for 15 min.
- The pellet washed with 3 ml of SET buffer and resuspended the cells with 1.6 ml of SET buffer, and then freshly prepared lysozyme (final concentration 2 mg) was added and incubated at 37°C for 30 min.
- One ml of 10% SDS was added and mixed by inversion then incubated at room temperature for 15 min.
- 2 ml of 5 M NaCl was added, mixed thoroughly by inversion.
- An equal volume of chloroform was added, mixed by inversion for 15 min. Then centrifuged (6000 rpm 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 13000 rpm for 15 min. at 4°C.
- The isopropanol was discarded and the precipitated DNA dissolved in 100µl TE buffer and stored at -20°C.

2.2.6 Agarose Gel Electrophoresis (Maniatis *et al*., 1982)

Agarose gels (0.7%) were run horizontally in Tris-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 gels were stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 ug /ml for 30-45 min. DNA bands were visualized by U.V.

Illumination at 302 nm on a U.V. tranilluminator. Gels were destained in distilled water for 30-60 min to get ride of background before photographs were taken.

2.2.7 Role of the Plasmid in Agar Degradation

2.2.7.1 Curing of Plasmid DNA

Curing experiments were performed on *Pseudomonas* HA1 and *Bacillus* HA9 by using curing agent (SDS) depending on Trevors *et al*., (1986).

Bacterium was grown in 5 ml of nutrient broth to mid log phase. Then 0.05ml inoculums of the culture were inoculated in a series of 5ml fresh nutrient broth tubes containing various concentration of SDS (1%, 2%, 3%, 4%, 5%, 6%, 7% , 8%, 9%, 10%) for *pseudomonas* (HA1) and (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%) for *Bacillus* (HA9). All tubes were incubated at 30°C for 24-48 hrs.

The growth density of different tubes was observed by nicked 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 strain considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of SDS that still allow bacterial growth , and diluted appropriately, then 0.1 ml from proper dilution were spreaded on nutrient agar plates and incubated

overnight at 30°C to score the survived colonies. Those colonies were replica plated (using tooth pick) on nutrient agar and on agar plate (defined media) containing agar as a sole carbon source. If a colony was able to grow on the master plate but not on the selective medium agar plate (defined media), It means that the cells of this colony were cured cells that lost the ability to the utilize agar as a sole carbon source and energy. The percentage of cured cells was determined.

2.2.7.2 Transformation

E. coli MM294 was transformed with plasmid DNA according to the procedure of Maniatis *et al*., (1982)

1 ml of an overnight bacterial culture was inoculated in 100 ml of nutrient broth and incubated at 37°C with shacking. 3 ml of exponential culture (O.D550 = 0.2) was taken, this usually takes 2-4 hrs. The cultured was chilled on ice for 10 min. Then centrifuge at 6000 rpm for 10 min at 4°C.

The supernatant was discarded and resuspend the cells in a half of the original culture volume of an ice cold, sterile solution of 50 mM $CaCl_2$ and 10 mM of Tris-Cl (pH 8.0), cells suspension was placed in an ice bath for 15 min. and centrifuge at 6000 rpm for 5 min at 4°C. Supernatant was discarded and resuspend the cells in 1/15 of the original volume of an ice-cold sterile solution of 50 mM $CaCl_2$ and 10 mM of Tris-Cl (pH 8.0) dispense 0.2 ml aliquots into pre chilled tubes store the cells at 4°C for 12-24 hrs. plasmid extraction was added and mix with cells suspension and store on ice for 30 min. transfer to a water bath, pre heated to 42°C for 2 min. 1 ml of nutrient broth was added to each tubes and incubated at 37°C for 30 min. or 1 hr without shacking this period allows the bacteria to recover and begin to express. Samples were diluted appropriately and spread onto selective media agar plate (defined media), plates were incubated overnight at 37°C.

Chapter Four

Conclusions

and

Recommendations

4.1 Conclusions

1. *Pseudomonas* isolate HA1 was the most efficient isolate in degradation of agar-agar.

2. SDS was affected as curing agent for the plasmid encoding agar degradation.

3. In *Pseudomonas* plasmid responsible for agar degradation while in *Bacillus* the feature of agar degradation is chromosomal.

4. Transfer of plasmid harboring agar degradation from *Pseudomonas* isolate HA1 to *E. coli* MM294 by transformation was succeeded.

4.2 Recommendations

1. It is interesting to do research on another microbial genera have the ability to degrade agar-agar.

2. It is important to isolate bacterial strains capable to degrade another type of polysaccharides.

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

4. Study the genetic property of agarase i.e. sequencing of genes coding for agarase enzyme.

Chapter Three

Results

and

Discussion

3.1 Isolation and Screening of Agarolytic Bacteria

Forty aquatic and soils samples collected from different places in Iraq and some of them from Syria, 10 of aquatic samples and 30 of soils samples were used in order to isolate agar degrading bacteria.

Agar degradation were made in a (defined medium) containing agar as a sole carbon source using streaking method, and incubated at 30°C for 7 days.

Twenty five isolates were appeared have the ability to grow on agar as a sole carbon source, these isolates were repeatedly tested on agar plate (defined media) in order to ensure the degradative ability, then only 12 isolates that appeared really have ability to degrade agar and utilized it as a sole carbon source and still keep their ability to grow at the same density while the other 13 some of them lost their ability to grow on agar, and other have grow weakly, and to be sure about the ability of degradation to these 12 isolates, it will be compared with another type of bacteria (known bacteria) used as control, and the result improved the ability of agar degradation to these 12 isolates while the known bacteria couldn't grow and degrade agar as in figure (3-1).

These (12) isolates named HA1, HA2, HA3, HA4, HA5, HA6, HA7, HA8, HA9, HA10, HA11, HA12. Eight of these 12 isolates were isolated from soil and 4 isolates from aquatic environment as in table (3-1).

Although most agarolytic bacteria described have been isolated from marine sources, there are reports that they also occur in soil and fresh water, e.s *Cytophaga flevensis* from water and *Bacillus* from soil (Agbo and Moss, 1979).

Indeed, Nicol (1931), having noticed the presence of agar-softing, yellow-pigmented bacteria from soil in Palmer's Green, London, observed that it seemed likely that the discovery of such bacteria would be more wide spread if they were looked for, and that mean not only the marine bacteria have the ability to degrade agar. The agarase enzyme hydrolyzed a wide range of plant-derived polysaccharides, including some associated with terrestrial and freshwater plants rather than with marine algae. These results suggest that there is no special

ecological reason for the presence of agarolytic bacteria in freshwater but that their activity reflects the wide substrate spectrum of the polysaccharides of such organisms (Agbo and Moss, 1979).

Failure of 28 of the isolates to degrade agar may be attributed to these isolates can benefit the metabolites of the other bacteria that utilize and degrade them to less complex compounds.

Table (3-1) The isolates that have the ability to degrade agar their region and type of samples.

Isolate	Region	Type of Samples
HA1	Syria- Tartous	Sea sand
HA2	Syria- Tartous	Sea sand
HA3	Dijla- Baghdad	Soil
HA4	Aumara- Mashrah	Water
HA5	Aumara- Mashrah	Soil
HA6	Baghdad- Yarmok	Soil
HA7	Mashrah	Soil
HA8	Faluga- Furat	Water
HA9	Baghdad- Yarmok	Soil
HA10	Baghdad- Yarmok	Soil
HA11	Kute	Soil
HA12	Baghdad- Jadrea	Soil

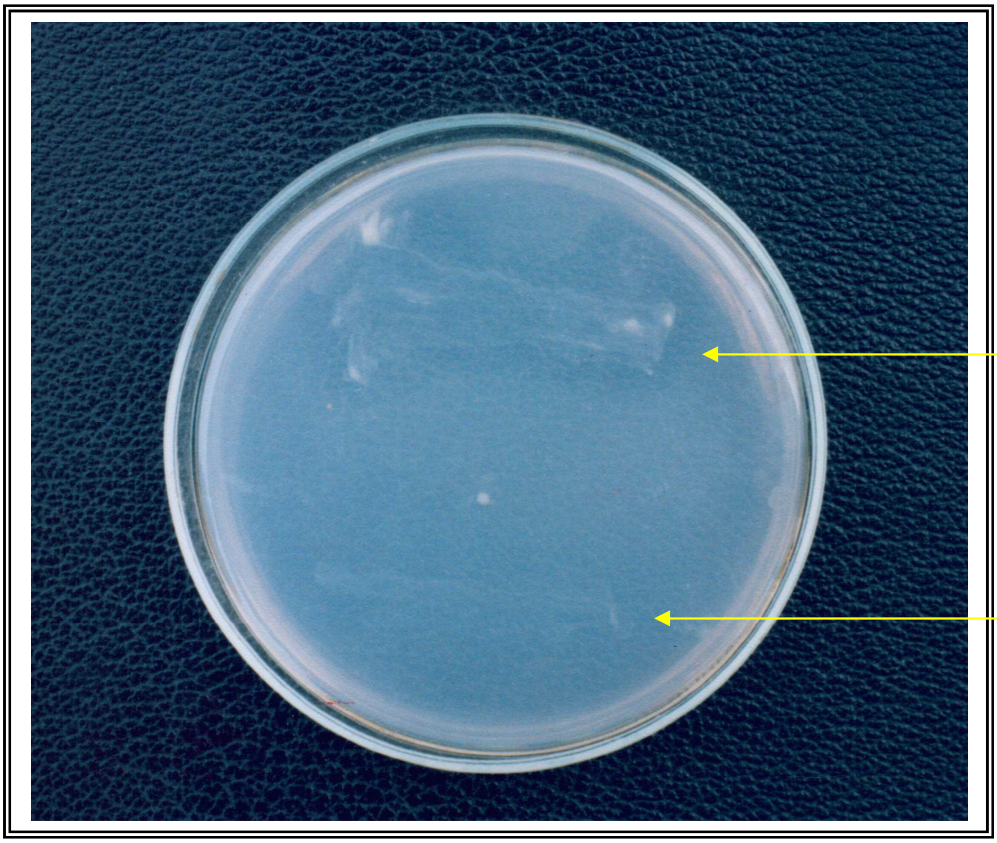

Figure (3-1) The ability of HA1 isolate to grow and degrade agar while the *S. aureus* ATCC 8625 (control) couldn't grow and degrade agar.

3.2 Identification of Bacterial Isolates

The bacterial isolates that isolated from deferent habitats **(**12 isolates**)** and have the ability to degrade agar were identified, depending on morphological and biochemical tests. The results were agreement with Holt *et a*l., (1994), and showed that these bacteria belong to 2 genus; *Pseudomonas* and *Bacillus*, as in table (3-2), (3-3) and figure (3-2).

Many agar-degrading bacteria have been recognized since 1902. Most strains are marine and require relatively high salt concentrations for growth, but several non-marine species have also been isolated (Hofsten and Malmqvist, 1975).

Results indicated that 66.6% of identified bacteria belonged to *Pseudomonas*, 33.3% belong to *Bacillus*. *Pseudomonas* strains are known to exhibit a wide range of metabolic activities against a most of natural and xenobiotic because of extreme nutritional (Chakrabarty, 1976; Holloway *et al*., 1979; Ramos *et al*., 1994).

Agar is polysaccharide can be degrading by several bacterial strains from marine environments and other source. Some of the bacterial isolates have been assigned to the genera *Pseudomonas* (Ha *et al*., 1997; Lee *et al*., 2000), and *Bacillus* (Kim *et al*., 1999).

Table (3-2) Morphological and biochemical tests of agarolytic bacterial isolates of (*Pseudomonas*)

Tests	**HA1**	**HA2**	**HA3**	**HA4**	**HA5**	**HA6**	**HA7**	**HA8**
Colony color	yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow
Cell shape	rod	rod	rod	rod	rod	rod	rod	rod
Gram stain	–	–	–	–	–	–	–	–
Catalase production	+	+	+	+	+	+	+	+
Oxidase production	+	+	+	+	+	+	+	+
Growth on King A	+	+	+	+	+	+	+	+
Growth on King B	+	+	+	+	+	+	+	+
Growth on Cetrimide	+	+	+	+	+	+	+	+
Growth on MacConky	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	–	+
DNase	+	+	+	+	+	+	–	+
Gelatin hydrolysis	+	+	+	+	+	+	–	+
Citrate utilization	+	+	+	+	+	+	+	–
Motility	+	+	+	+	+	+	+	+
Voges-Proskauer	–	–	+	+	+	+	+	+
Methyl-Red	+	+	–	–	–	–	–	–
Pyocyanine production	–	–	+	–	+	+	+	+
Flourescence under UV-transilluminator	+	+	–	+	–	–	–	–

		HA1	HA2	HA3	HA4	HA5	HA6	HA7	HA8
Triple Sugar Iron agar	Slant	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	Bottom	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline	Alkaline
	H_2S production	–	–	–	–	–	–	–	–
	Gas production	–	–	–	–	–	–	–	–

Table (3-3) Morphological and biochemical tests of agarolytic bacterial isolates of (*Bacillus*)

Tests	HA9	HA10	HA11	HA12
Colony color	White	White	White	White
Cell Shape	bacilli	bacilli	bacilli	bacilli
Gram stain	+	+	+	+
Catalase production	+	+	+	+
Oxidase production	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Citrate utilization	+	–	+	–
Motility	+	+	+	+

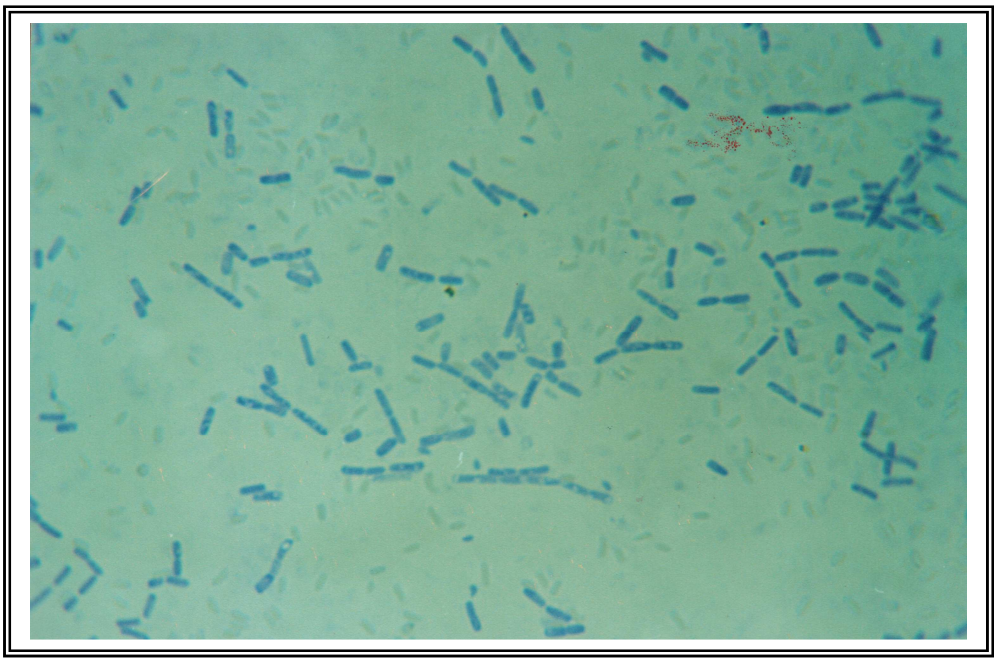

Figure (3-2) Gram's stain film of *Bacillus* (HA9) showed the morphological shape of cells 100x

3.3 Selection of Efficient Isolates

All the isolates that have been isolated and have the ability to degrad agar that seen or identified by the clearing zone around the growth of bacterial isolates as in figure (3-3). Efficient isolates were identified from these (12 isolates) was (HA1) from *pseudomonas* and (HA9) from *Bacillus* depending on the clearing zone that appearance more clearly than the other isolates as in figure (3-4) for *Pseudomonas* isolate (HA1), and that may be attributed to the physiological and genetic properties of these isolates. These efficient isolates were used for further study.

The gel of agar may be degrade by microorganisms in three ways. (1) Disruption of the double helical structure without breakdown of the polymer. This dose not generally produce any visible changes in gel and such limited activity can only be detected by failure to form the brown color that the normal gel forms with iodine. This color is thougt to depend on the integrity of the double helical structure (Ng Ying Kin and Yaphe, 1972). (2) Cleavage of the alpha-linkages of the agar molecule, giving rise to oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactose at the reducing end. (3) Cleavage of the beta-linkages, giving rise to the neo-agarobiose series of oligosaccharides with D-galactose at the reducing end. The enzymes involved in (2) and (3) are called alpha- and beta-agarases, respectively; most agarolytic bacteria so far described are consider to produce only beta-agarase (Vattuone *et al*., 1975; Van der Meulen and Harder, 1975).

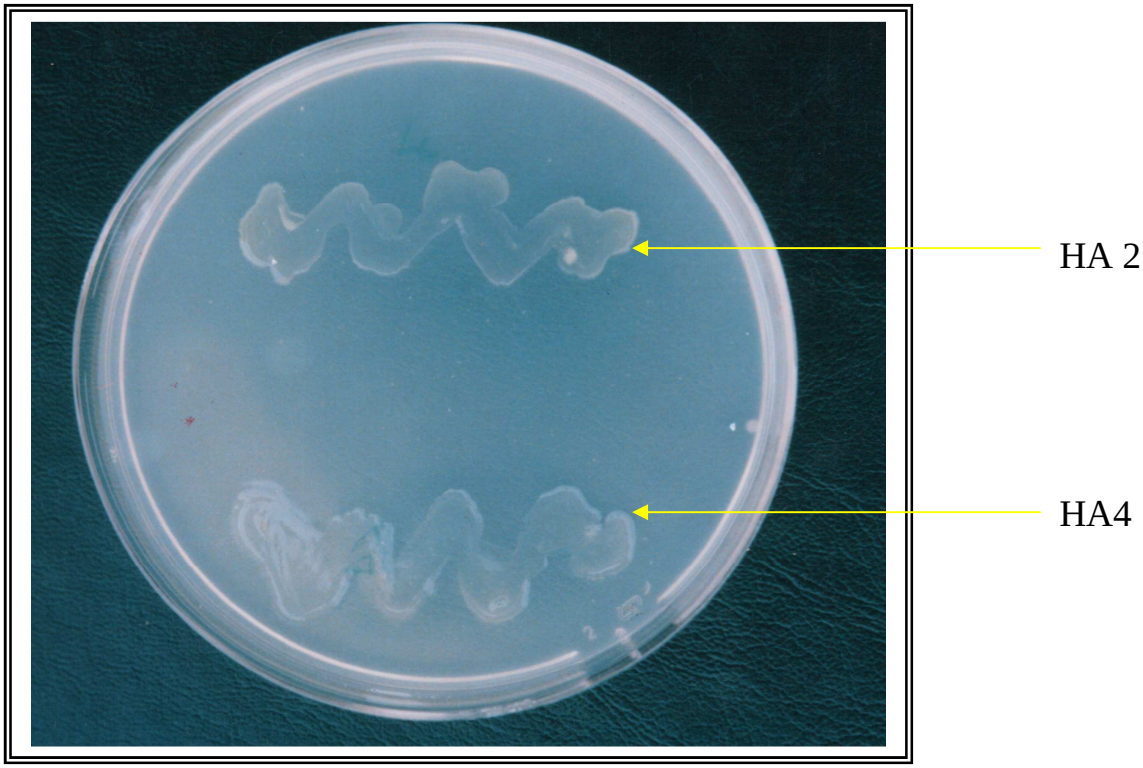

Figure (3-3) growth of bacterial isolates (HA2, HA4) on agar plate (defined media), identified by the clearing zone around the growth of bacteria.

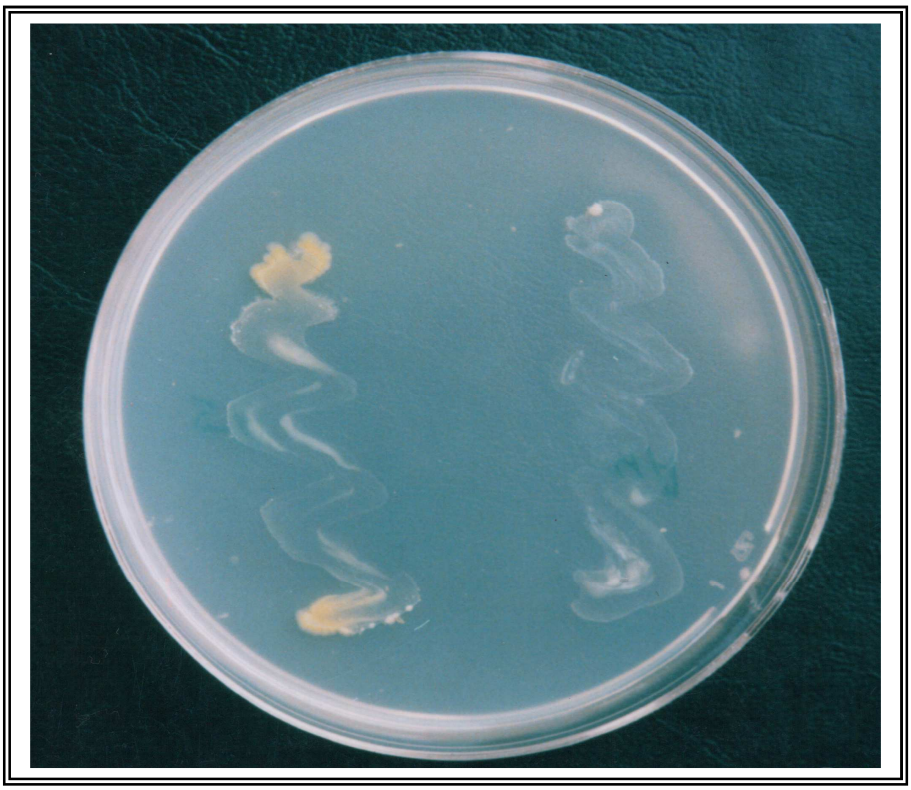

Figure (3-4) The clearing zone of *Pseudomonas* isolate (HA1) on agar plate (defined media), the most efficient isolate.

3.4 Plasmid Isolation

In order to determine the plasmid profile of the efficient isolates, the alkaline lysis and salting out method were used. The results showed that later method gave the best results.

The results in figure (3-5) were indicated that the isolates of *Pseudomonas* containing two (or more) small plasmid DNA bands and all these plasmid bands approximately in the same size compared with each other and with pBR322 plasmid (4.363Kb) as in figure (3-6). However bacterial isolates, tested in this study may be containing another plasmids does not detected (may be because of its large size).

Many studies were reported that *Pseudomonas* spp. containing agar degradative plasmids ranging in size from 10-300 Kb (Devereux and Sizemore, 1982; Carney and Leary, 1989). Also many studies were indicated that a different *Pseudomonas* isolates containing plasmids with the same size but have different set of genes or the plasmids are the same plasmids but they were transfer between the soil population of bacteria by conjugation or transformation (Devereux and Sizemore, 1982; Trevors, 1998).

For the *Bacillus* the same procedure were applied to extract and characterize agar degradation plasmids as illustrated in figure (3-7).

Other studies on the plasmid designated pSD15 isolated from the genus *Microscillia* which was isolated from Coastal California marine sediment.

This organism harbored a single 101-Kb circular DNA plasmid .The complete nucleotide sequence analysis indicated a number of genes putatively encoding a variety enzymes involved in polysaccharide utilization. The most striking feature was the occurrence of five putative agarase genes. They are: *ms*109, *ms*115, *ms*116, *ms*130, and *ms*132 (Zhong *et al.*, 2001).

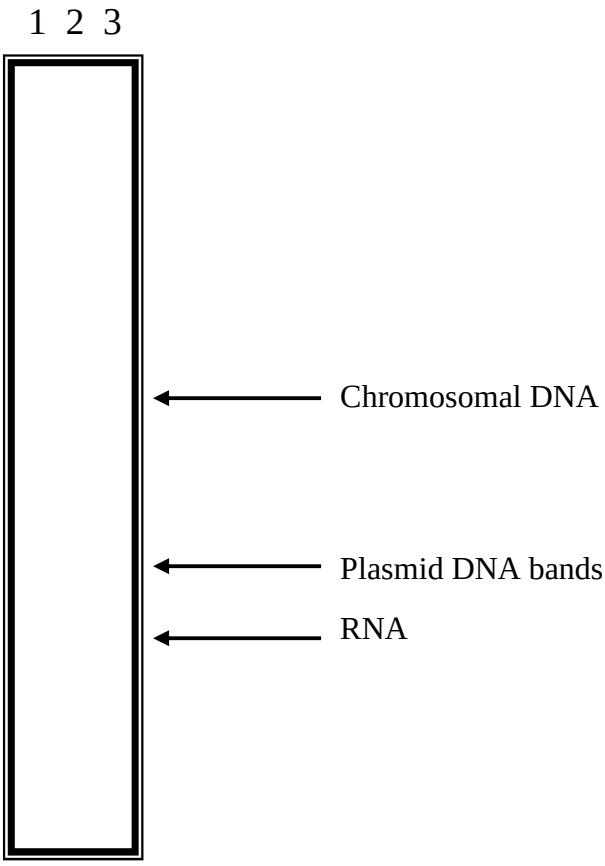

Figure (3-5) Gel electrophoresis of isolated plasmid from the bacterial isolate of (1, 2 and 3) *Pseudomonas* HA1 migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2½ hrs.

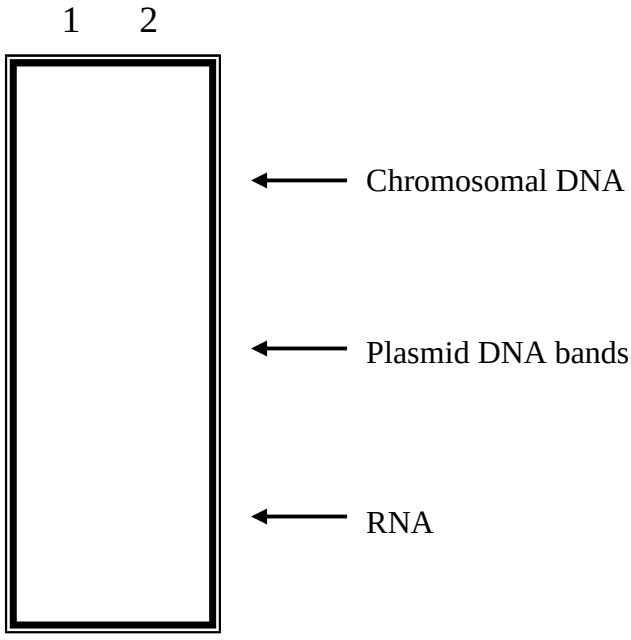

Figure (3-6) Gel electrophoresis of isolated plasmid from the bacterial isolate of *E. coli* HB 101 (1), and *Pseudomonas* HA1 (2) migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2½ hrs.

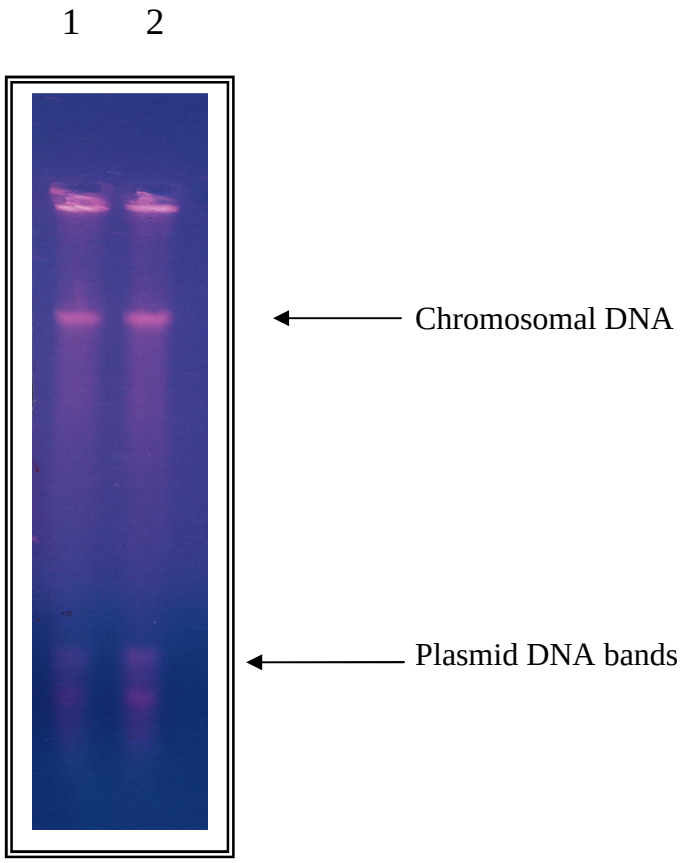

Figure (3-7) Gel electrophoresis of isolated plasmid from the bacterial isolate of (1 and 2) *Bacillus* HA9 migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2½ hrs.

3.5 The Role of Plasmids in Utilization of Agar

Pseudomonas (HA1) and *Bacillus* (HA9) were candidate for study the role of their plasmids in agar degradation, because these isolates showed a growth density and growth yield more than other isolates, along five days of incubation on agar plate (defined media), utilizing agar as a sole source of carbon and energy, for that curing and transformation experiments were performed on these two types of bacterium.

3.5.1 Curing of Agar Degradation Plasmids

A varity of chemical agents, such as acridine dyes, ethidium bromide and sodium dodecyl sulfate are able to free or cure bacterial cells of plasmid DNA molecules. Plasmid molecules, which exist as autonomously replicating circular DNA duplexes, are eliminated by these agents either because of interference with their replication (acridines and ethidium bromide) or by alteration of their membrane attachment sites (SDS) (Novick, 1969).

Many attempts were made in order to cure *Pseudomonas* and *Bacillus* agar degradation plasmids using SDS. The results indicated that the highest concentration of SDS that allows the bacterial growth, were 4% for *Pseudomonas* (HA1) and 0.4% for *Bacillu*s (HA9) as illustrated in table (3-4) and (3-5), from these concentrations of curing agents, appropriate dilutions were made and spread on nutrient agar plates, then colonies were tested on (defined media) in order to determine the cured colonies, which can not utilize agar as a sole source of carbon and energy.

Results showed that a number of colonies (9%) failed to grow on agar plate (defined media) for *pseudomonas* (HA1), while *Bacillus* (HA9) didn’t loss their ability to degrade agar.

In order to determine the failure of the colonies to grow on the agar plate (defined media), were because of plasmid elimination or not, plasmid isolation from the cured cells were made and compared with that from the wild isolate.

The results indicated that there is no plasmids on the gels for the cured colonies as illustrated in (Fig 3-8), which means that the plasmid is responsible for agar degradation property.

Many studies indicated that SDS were used successfully as a curing agent (Trevors, 1998), and Aislablie and co-workers, (1990) have successfully cured of hydrocarbon degradative plasmid using SDS.

Table (3-4) Effect of SDS on the growth of *Pseudomonas* HA1

SDS Concentration %	**Bacterial growth**
1%	+++
2%	++
3%	+
4%	±
5%	–

– = no growth
± = slightly growth
\+ = moderate growth
++ = good growth
+++ = very good growth

Table (3-5) Effect of SDS on the growth of *Bacillus* HA9

SDS Concentration %	**Bacterial growth**
0.1%	+++
0.2%	++
0.3%	+
0.4%	±
0.5%	–

– = no growth
± = slightly growth
\+ = moderate growth
++ = good growth
+++ = very good growth

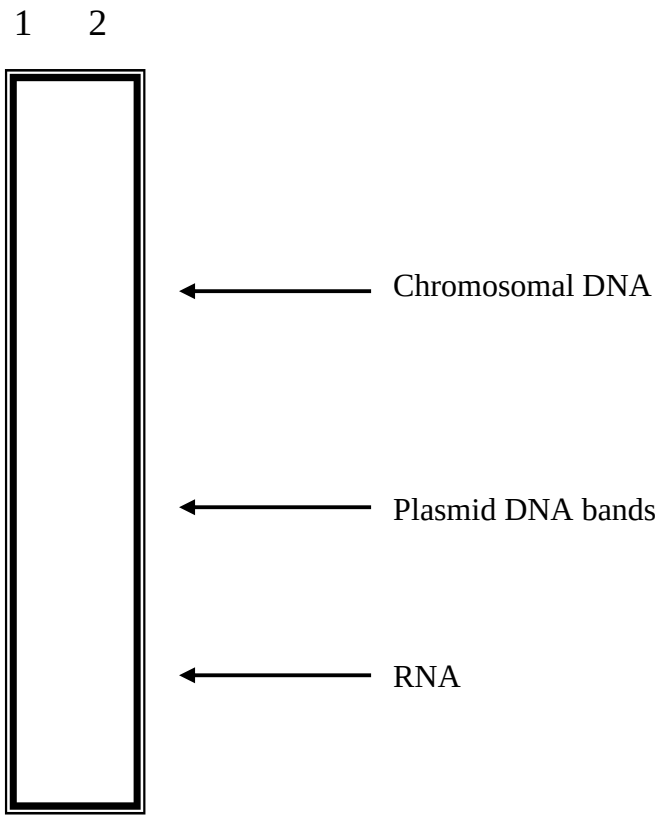

Figure (3-8) Gel electrophoresis of isolated plasmid from the bacterial isolate of *Pseudomonas* HA1 before and after treatment with SDS migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2½ hrs.

1- The wild isolate (HA1), 2-isolate after curing with SDS

3.5.2 Transformation:

The transformation of agar degradation plasmid, from one bacterium to another was another method to determine whether agar degradation genes are located on plasmid or not. For this reason transformation experiments using *E. coli* MM294 (Rif^r) as a recipient to the agar degradative plasmids DNA isolated from *Pseudomonas* (HA1), were performed as illustrated in chapter two.

Transformants colonies spreaded on agar plate (defined media) were isolated and screened for existence of plasmids.

Results showed that ($4.5x10^{-3}$) transformants for *Pseudomonas* plasmids transformation respectively, were grown on agar plate (defined media) i.e.: they utilize agar as a sole source of carbon and energy compared with the wild (controls).

Agarose gel electrophoresis results (Fig. 3-9) showed the plasmid bands extracted from transformants colonies as compared with controls (wild isolates).

Transformation results confirmed that genes responsible for utilizing agar as a sole source of carbon and energy, are located on plasmids which is in agreement with curing experiments results.

Schroeder and his co-workers (2003) have been isolated pDA1 plasmid from *Pseudoalteromonas gracilis* B9 which was responsible for the agarolytic activity. Transformation experiments using *E. coli* as recipient to pDA1 plasmid was done. *E. coli* transformants exhibited this property (i.e agarolytic activity) when grown on solid medium.

aagA gene located on pDA1 had 85% identity to the agarase (*dag A*) from *pseudoalteromonas atlantica* ATCC 19262 at the amino acid level. AagA was purified from extracellular medium of an *E. coli* transformant harboring pDA1 by using a combination of gel filteration and ion-exchange chromatography. *aagA* showed that the enzyme cleaves the beta-(1,4) linkages of agarase to yield predominately neoagarotetraose (Schroeder *et al*., 2003)

Then Genomic DNA was extracted from *P. gracilis* B9 and partially digested as described by Ausuble *et al*. (1989) with *Sau*3A. The *Sau*3A DNA restriction fragments were size-fractionated on 10-40% (w/v) sucrose gradient (Sambrook *et al*., 1989). DNA fragments of 10 Kb in size were pooled and ligated with T4 ligase into the *Bg/*11site of plasmid pEcoR251 at 15°C overnight (Ausubel *et al*., 1989). The recombinant plasmids were recovered by transformation into *E. coli* HB101 (Dagert and Ehrlich, 1979; Schroeder *et al*., 2003)

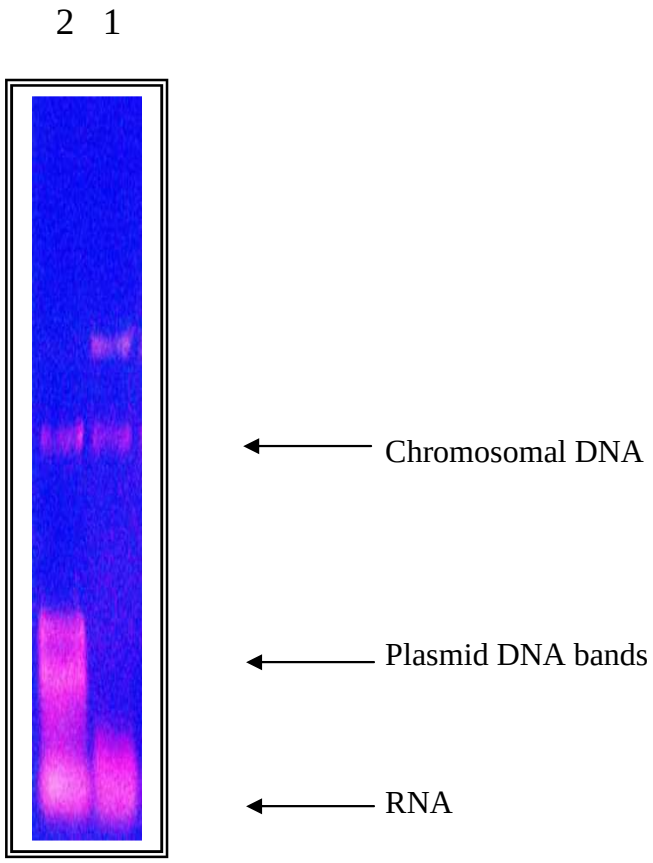

Figure (3-9) Gel electrophoresis of *E. coli* MM294 before (1) and after (2) transformation migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2½ hrs.

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الخلاصة

جُمِعت اربعون عينه (ماء و تربه) من مناطق مختلفه في العراق ومن سوريا، وأظهرت 25 عزله القابليه على النمو على وسط الاكار . أُخِذت هذه العينات وأُعيد زراعتها على وسط الاكار للتأكد من قابليتها على النمو والتحلل، فأظهرت النتائج أن (12) عزله فقط لها القابليه على النمو واظهار منطقه تحلل حول النمو البكتيري وتم تشخيص هذه العزلات وتبين ان (8) عزلات تعود لجنس *Pseudomonas* و(4) عزلات تعود لجنس *Bacillus* .

و أنتخبت العزلتانHA1 و HA9 و العائدتان لجنسي *Pseudomonas* و *Bacillus* على التوالي و أظهرتا القابليه على أستهلاك الاكاركأكفأ عزلتين لهما القابليه على النمو وأستهلاك الاكار كمصدر وحيد للكاربون، بالاعتماد على منطقه التحلل

أجريت الدراسه الوراثيه لصفة تحلل الاكار للعزلتين (HA1 وHA9) وذلك بدراسه المحتوى البلازميدي لكلا العزلتين واظهرت النتائج ان العزلتين تحويان على بلازميدات صغيره عند عزلها بأستخدام طريقه الترسيب بالاملاح.

أظهرت نتائج تجارب التحييد بأستخدام ال SDS ان العزله *Pseudomonas* (HA1) فقدت القابليه على النمو وأستهلاك الاكاركمصدر وحيد للكاربون بينما *Bacillus* (HA9) لم تفقد هذه الصفه.

وهذا يدل على ان صفة تحلل الاكار هي بلازميديه الموقع بالنسبه للعزله *Pseudomonas* (HA1) و كروموسوميه الموقع لعزله *Bacillus* (HA9).

نقلت صفه تحلل الاكار من العزله *Pseudomonas* (HA1) الى *E. coli* MM294 بأستخدام النقل بالتحويل وتمت بنجاح، وهذا يعزز نتائج التحيد السابقه.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

عزل وتشخيص البكتريا المحللة للاكار ودراسة الطبيعة الوراثية لهذه الصفة

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

هبة عبد القادر النجار

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٢

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اذار ٢٠٠٦

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