vv vv シ1

Abbreviations

Symbols	Abbreviations
BCA	Biological Control agent
IR	infra red
ISR	Induced systemic resistance
MIC	Minimum inhibitory concentration
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
O.D.	Optical density
P.aeruginosa	*Pseudomonas aeruginosa*
PCA	Phenazin -1-carboxylic acid
PDA	Potato dextrose agar
Phz R	Phenazin regulator
R_F	factor of Retardation
rpm	Round per minute
SDS	Sodium dodecyl sulphate
TLC	Thin layer chromatography
W.T	Wild Type

Acknowledgments

*First of all Praise to **Allah** the lord of the unieverse, peace be upon **Mohammed** the messenger of **Allah** and upon his **Relatives**. First and foremost my thanks must go to my supervisors **Dr. Majed H. Al-Gelawi** and **Dr. Abdul Kareem Al-Kazaz** for their guides and great support throughout my studies. I specially appreciate their warm and gentle approach to my supervision, as well as their immense patience in all their dealings with me during my time at the laboratory. The knowledge I gained from them ,in both academic and non-academic matters, have been invaluable and will definitely be beneficial to my future career.*

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Simple words can not express my deep appreciation to my dear sister Dr. Sinaa for supporting me with materials and tools, and for her guidance and advice during the whole work.

Rasha…..

Supervisor Certification

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

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Date:

Signature:
Supervisor: Dr.Abdul Kareem Al- Kazaz
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In review of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:
Name: Dr. Nabeel Al-Ani
Title: Chairman of Biotechnology Department
Date:

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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Address: Dean of the College of Science

Date:

Conclusions:

1. The trait of antifungal compound production in *P.aeruginosa* OM13 is located on the chromosome.
2. Transformation between DNA of *P.aeruginosa* OM13 and *E.coli* MM294 does not succeed.
3. There may be a correlation between changing the colony color of *P.aeruginosa* OM13 and the antifungal compound production.
4. MNNG is an effective mutagenic agent in improving the antifungal compound production in *P.aeruginosa* OM13.
5. The partially purified antifungal compound is one of phenazine derivatives (pyocyanin)

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Recommendations:

1. Further studies are required to isolate other microbial genera capable of producing antifungal compounds.
2. Test the ability of this isolate and mutant isolate to inhibit the growth of other pathogenic fungi for humans.
3. Using other genetic methods such as transformation by electroporation in an attempt to improve the production of the antifungal compound by *P.aeruginosa* OM13.
4. It's of a great importance and interest to make a complete characterization and identification of our antifungal compound by using (^{1}HNMR, ^{13}CNMR).

Summary

The ability of *P.aeruginosa* OM13 in antagonizing or inhibiting the growth of some phytopathogenic fungi was tested by measuring the inhibition zone for the growth of the test fungi (*Alternaria alternate, Rhizoctonia solani*) caused by the growth of the bacterial isolate (*P.aeruginosa* OM13) and by the action of the culture filtrate of this isolate on test fungi. It was found that this isolate inhibited the growth of the test fungi and the inhibition zone was 5mm, 7mm for *A. alternate* and *R. solani* respectively.

The plasmid profile for the *P.aeruginosa* OM13 was studied. The results showed that this isolate has small plasmid DNA bands.

In order to determine the role of plasmid in antifungal compound production, firstly, the antibiotic sensitivity disc test was made to find a genetic marker for the curing and transformation experiment. Then the curing experiment was performed by using sodium dodecyl sulfate (SDS) and showed that a number of colonies (4%) had lost their ability to inhibit fungal growth. It was also found that the cured cell became pale (lost its color).

Transformation experiment between *P.aeruginosa* OM13 DNA and *E.coli* MM294 strain did not succeeded. These results confirm that the antifungal compound production trait in *P.aeruginosa* OM13 may be chromosomal born.

To improve the antifungal compound production by *P.aeruginosa* OM13, it was treated with mutagenic agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG). Fifty colonies were selected randomly and the antifungal activity of its filtrate was measured by using agar well method and measuring the optical density (O.D) at 520nm. The results reported

that the antifungal agent production of 5 isolate was highly increased, in which RA5 gave the highest inhibition zone and $O.D_{520nm}$ values when compared with others.

An attempt to characterize the partially purified antifungal compound produced by *P.aeruginosa* OM13 by using infrared (IR), UV absorbance and thin layer chromatography (TLC) were done and the results showed that the compound appeared as a single dark spot on a TLC plate under UV-light that had R_f value 0.32. From IR, UV analysis it was confirmed that the compound was aromatic and contained aliphatic group.

From the results of characterization tests, and depending on the R_f value, color, IR, UV analysis, it can be said that the active antifungal compound might be either pyocyanine which is a derivative of the antibiotic phenazine or might be a new derivative of phenazine.

الخلاصة

تمت دراسة قابلية العزلة *P.aeruginosa* OM13 على تثبيط نمو فطريات الاختبار (*A.alternata, R..Solani*) و ذلك بالاعتماد على قطر منطقة التثبيط للنمو الفطري بفعل النمو البكتيري و بفعل راشح مزرعة البكتيريا. ووجد ان القطر التثبيطي كان 5mm , 7mm لفطريات الاختبار *A.alternata*، *R .solani* بالتتابع.

تمت دراسة النمط البلازميدي لهذه العزلة و تبين انها تحتوي على حزم بلازميدية صغيرة.

درست حساسية هذه البكتريا لعدد من المضادات الحيوية و ذلك لايجاد دلائل وراثية يمكن استخدامها في تجارب التحييد و التحول في محاولة لدراسة دور بلازميدات هذه البكتريا في انتاج المضادات الفطرية و اظهرت نتائج التحييد باستخدام مادة الصوديوم دوديسيل سلفيت (SDS) الحصول على مستعمرات (4%) فقدت قابليتها على انتاج المضاد الفطري و لقد لوحظ ايضا انها اصبحت عديمة اللون اي فقدت قابليتها على انتاج صبغة ال (Pyocyanin).

اجريت تجارب التحول و ذلك باستخدام الدنا للعزلة *P.aeruginosa* OM13 و السلالة *E.coli*MM294 و اظهرت النتائج عدم الحصول على اي نوع من المتحولات . تشير هذه النتائج الى ان الجينات المسؤولة على انتاج المضادات الفطرية في بكتريا *P.aeruginosa* OM13 قد تكون محمولة على الكروموسوم.

لتحسين انتاج المضاد الفطري لعزلة *P.aeruginosa* OM13 تم تعريضها الى عامل مطفرهو النتروزكوانيدين (MNNG) تم اختبار قابلية الراشح البكتيري ل ٥٠ مستعمرة انتخبت بصورة عشوائية على انتاج المضاد الفطري و ذلك باستخدام agar well method و قياس الكثافة الضوئية للراشح ($O.D_{520nm}$). , تبين ان انتاجية خمس عزلات قد ازدادت بكمية كبيرة و تم الحصول على عزلة RA5 التي اعطت اكبرقطر تثبيطي واكبر كثافة ضوئية مقارنة مع طافرات اخرى.

جرت محاولة لتشخيص المركب المضاد للفطريات و المنقى جزئيا واظهرت النتائج بان ذلك المركب يظهر بشكل بقعة واحدة داكنة على صفائح الTLC تحت ضوء الاشعة الفوق

البنفسجيةال UV-light و التي لها قيمة R_f هي ٠,٣٢ . ومن خلال نتائج الIR,UV تم التاكيد بان المركب هو من المركبات الاروماتية (الحلقية) و يحتوي على مجموعة اليفاتية.

من خلال نتائج الاختبارات التشخيصية و اعتمادا على قيمة الR_f و اللون وتحليل ال (IR,UV) نستطيع القول بان المركب الفعال المضاد للفطريات محتمل ان يكون pyocyanine احد مشتقات المضاد الحيوي الphenazine او محتمل ان يكون مشتق جديد لل phenazine .

To My Family

Rasha

بِسمِ ٱللَّهِ ٱلرَّحْمَٰنْ ٱلرَّحِيمِ

﴿وَيَسْئَلُونَكَ عَنِ ٱلرُّوحِ قُلِ ٱلرُّوحُ مِنْ أَمْرِ رَبِّي وَ مَآ أُوتِيتُم مِنَ ٱلْعِلْمِ إِلاَّ قَلِيلاً﴾

صدق الله العظيم

(سورة الاسراء أية ٨٥)

chapter one

Introduction and Literature review

chapter two
Materials and Methods

chapter three

Results and Discussion

References

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

Genetic Improvement of Antifungal Compound Production by *Pseudomonas aeruginosa* OM13

A thesis
Submitted to the College of Science of Al-Nahrain University in partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Rasha Sadik Amin
B. Sc. In Biotechnology
(Al-Nahrain University 2004)

February 2007 Muharam 1428

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تحسين إنتاجية العزلة البكتيرية *Pseudomonas aeruginosa* OM13 من المركب المضاد للفطريات

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

من قبل

رشا صادق أمين
بكالوريوس تقانه احيائية
جامعة النهرين
(٢٠٠٤)

محرم ١٤٢٨ **شباط ٢٠٠٧**

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Literature review

1.2-Biocontrol of plant disease:

Bacterial secondary metabolites play critical roles in many aspects of bacterium–host interaction. Secondary metabolite that function as virulence factors play central role in disease suppression by altering host tissue (Rahme *et al* .,1995;Kimura *et al*.,2001).

Other secondary metabolites produced by beneficial bacteria can function to prevent infection by pathogens by altering the environment and improving the bacterium ability to compete with pathogens by inhibiting the activity of pathogens or by triggering host defenses (Bleomberg and Lugtenberg., 2001; Raaijmakers *et al*., 2002).

Certain beneficial strains of fluorescent *Pseudomonas* bacterial species colonize the roots of plants and provide biological control of soil borne plant pathogens through the production in situ of small organic molecules with antibiotic activity. Members of the *P.fluorescence, P. putida, P. aureofaciens,* and *P. aeruginosa* have demonstrated *in vitro* antagonism toward several soil microfungi, but with great variability among the strains (Shanahan *et al*., 1992) .Table (1-1) shows some secondary metabolites from fluorescent pseudomonads.

Table (1-1) Secondary metabolites from fluorescent pseudomonads (Leisinger and Margraff .,1979)

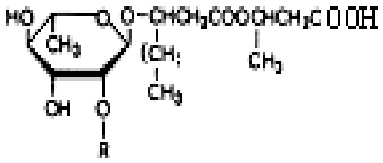
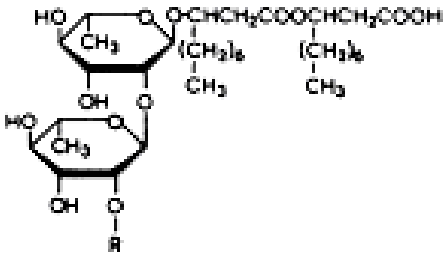
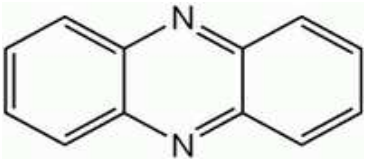
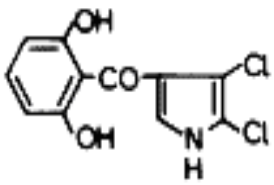
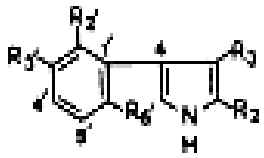
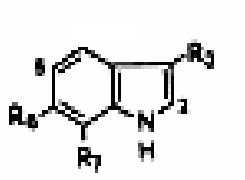
Compound (trival name)	structure
LIPIDS	
Rhamnolipids	
	R
Pyolipic acid	–H
Compound B	–COCH–CH$(CH_2)_4CH_3$
	
	R
Jarvia rhmnolipid	-H
Compound A	–COCH–CH$(CH_2)_4CH_3$
PHENAZINE	
PYRROLES	
pyoluteorin	

Table (1-1) continued

Compound (trival name)	structure
Phenylpyrroles	

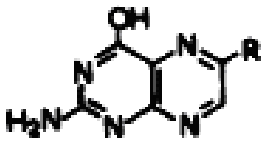
	R_2	R_2´	R_3	R_3´	R_6
Pyrrolnitrin	−H	−Cl	$-NO_2$	−Cl	−H
2-chloropyrolnitrin	−Cl	−Cl	$-NO_2$	−Cl	−H
Aminopyrrolnitrin	−H	−Cl	$-NH_2$	−Cl	−H
Oxypyrrolnitrin	−H	−Cl	$-NO_2$	−Cl	−OH
Monodechloropyrrolnitrin	−H	−Cl	$-NO_2$	−H	−H
Amino-3-chlorophenylpyrrol-2-carboxylic acid	−COOH	−H	$-NH_2$	−Cl	−H

INDOLES



	R_2	R_6	R_7
3-chloroindole	-Cl	−H	−H
Indol-3-carboxaldehyde	−CHO	−H	−H
6-Bromoindol-3-carboxaldehyde	−CHO	−Br	−H
7-chloroindolacetic acid	$-CH_3COOH$	−H	−Cl

PTERINES

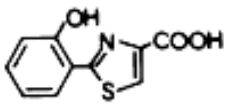


	R
Pterine	–H
6-Aminopterine	$–NH_2$
6-Hydroxymethylpterine	$– CH_2OH$
Monapetrine	$–CHOHCHOHCH_2OH$

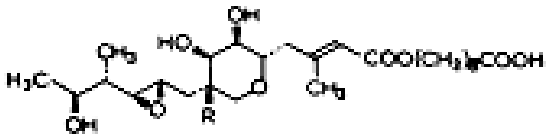
MISCELLANEOUS COMPOUNDS

Cyanhydric acid HCN

Aeruginoic acid



Pseudomonic acid



	R
Pseudomonic acid A	H
Pseudomonic acid B	OH

1.3-The genus *Pseudomonas*:

Straight or slightly curved rods, but not helical (0.5 –1.0 x 1.5-5.0). Many species accumulate poly β-hydroxybutyrate as carbon reserve materials, which appear as danophilic inclusion. They do not produce prostheace and are not surrounded by sheaths, no resting stages are known. Cells stains gram-negative. Motility occur by one or several polar flagella; they are rarely non motile.

In some species lateral flagella of shorter wavelength may also be formed. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. In some cases nitrate can be used as an alternative electron acceptor, allowing growth to occur an aerobically. Xanthomonadins are not produced. Most if not all species fail to grow under acidic conditions (pH 4.5). Most species do not require organic growth factors. Oxidase positive, catalase positive and chemoorganotrophic; some species are facultative chemolithotrophs, able to use H_2 or CO_2as energy sources. Widely distributed in nature. Some species are pathogenic for humans, animals or plants (Holt *et al.*, 1994).

Some *Pseudomonas* species produce characteristics pigment; it was found that a *Pseudomonas aeruginosa* produce yellow –green diffusible pigment (pyocyanine) and *P.fluorescens* produce a fluorescine pigment. *Pseudomonas* species are widely distributed in soil and aquatic ecosystem, occurring as free-living bacteria or in association with plants or animals and some species are plant and animal pathogen (Atlas *et al.*, 1995).

1.4- *Pseudomonas* as biocontrol agent:

Soil-borne, non-pathogenic bacteria with the ability to antagonize fungal phytopathogens and thus prevent plant disease represent realistic alternative to chemical fungicides. It was found that many soil bacteria have biocontrol abilities, these bacteria known by several generic names, including biological control agents (BCAs), plant growth promoting rhizobacteria (BGPR) and biopesticiedes. Because of their catabolic versatility, their excellent root-colonizing abilities, and their capacity to produce a wide range of antifungal metabolites, the soil-borne fluorescent *Pseudomonas* have received attention .In addition some *Pseudomonas* BCAS have been shown to elicit a disease-resistance response in crop species a phenomenon known as induced systemic resistance (ISR) (Van loon *et al* ., 1998;Pieterse and Van loon ., 1999).

This dual activity of *Pseudomonas* BCAs (i.e. direct antagonism at phytopathogens and induction of disease resistance in the host plant) further highlights their potential as plant protection products (PPPs) Suppressive soils contain rhizobacteria that are able to control plant disease that are caused by fungi or bacteria, the mechanism responsible for this biocontrol activity include competition for nutrients, niche exclusion, induced systemic resistance (ISR), and the production of antifungal metabolites (AFMs) (Walsh *et al.*, 2001)..

1.5-*Pseudomonas* as a source of antibiotics production:

Numerous soil –inhibiting pseudomonads colonize the surface of seeds and roots and inhibit the infection by soil borne plant pathogens. Many of these bacteria produce antibiotics and other compounds (siderophores, volatile substance) that are thought to account in part for their antimicrobial properties (Georgakopoulos *et al.,* 1994).

Siderophores have been widely isolated in soils (Akers, 1983) and considerable work has been done on their role in microbial interaction (Kloepper and Schroth 1981).

In contrast the role of antibiotics in affecting the population of the microorganism in the rhizosphere is not clear, however Mazzola *et al.,* (1992) reported that the production of phenazine antibiotic by *Pseudomonas fluorescence* 2-79 and *Pseudomonas aureofaciens* 30-84 contribute to their ecological competence in soil in the rhizosphere of wheat by enhancing their ability to compete with indigenous microflora.

Although phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol have been isolated from the rhizosphere of wheat (Thomashow *et al*.,1990; Hass *et al*., 1991), antibiotics have in general, only rarely been isolated from the soil, presumably because of sporadic production, rapid inactivation, lack of sensitive method (Williams and Vickers., 1986)or adsorption by soil colloids (Stotzky ,1986).

Thomashow and Pierson, (1991); Thomashow *et al* .,(2000) related the inhibition of wheat root infection from *Gaeumennomyces graminis*, var. *Tritici* by *Pseudomonas aureofaciens* 30-84 to phenazine antibiotic production.

1.6-Antifungal compound produced by bacterial isolates:

The use of antagonistic microorganisms to control plant pathogenic microfungi is receiving increasing attention as current legislation restricts the use of synthetic chemical pesticides. Several years of searching for bacterial strains capable of exhibiting biocontrol have resulted in numerous antagonistic isolate, in which the antagonistic activity has been attributed to release of antibiotics (Keel and Defago., 1997).

Various bacterial genera produce peptide antibiotics showing antagonistic activity against a wide range of different plant pathogenic fungi (Miller *et al*., 1998), hence, the inhibition of mycelia growth by viscosinamide (Nielsen *et al*., 1999), fengycin (Vanittanakom and Loeffler., 1986),and tolaasin (Hutchison and Johnstone ., 1993)has been attributed to perturbation of cell membrane structure (Vanittanakom and Loeffler ., 1986)or to the formation of ion channels in the cell wall (Endo *et al* ., 1997; Thran *et al* ., 1999). Furthermore the synthetic peptide antibiotic and pneumocandine , was shown to inhibit 1,3-β-D-glucan synthesis in the ascomycete *Aspergillus fumigatus* (Kurtz *et al* ., 1998) .

Tensin, a new antifungal cyclic lipopeptide isolated from *pseudomonas fluorescence* strain 96.578, has antagonist activity against the plant pathogenic fungus *Rhizoctonia Solani* (Nielsen *et al*., 2000).

In particular, fluorescent pseudomonads show potential protective application against the fungal plant pathogen *Pythium ultimum*, which cause damage to pea, sugar beet, tobacco, cucumber and tomato crops. This fungus is a major cause of damping off a seedling disease that can greatly reduce crop yield. *Pseudomonas fluorescens* strain Q_2 –87, CHA0, and Fll3

have been shown to produce a natural antimicrobial metabolite, 2,4-diacetylphloroglucinol (phI) which inhibit *Pythium. ultimum* in plant (Bainton *et al* .,2004).

Pseudomonas chlororaphis PCL 1391 exhibits biocontrol activity against *Fusarium oxysporum, Radicis lycopersici* the causal agent of tomato foot and root through the production of phenazine –1-carboxamide (PCN) (Thomas *et al* .,2001).

Pseudomonas aureofaciens strain 30-84 is a biological control bacterium able to suppress take all disease of wheat caused by the fungal pathogen *Gaeumennomyces graminis,* var. *tritici* through the production of phenazine antibiotics (Whistler and Pierson ., 2003).

Pseudomonas aureofaciens PA147-2 produces an antibiotic-like compound that inhibits the growth of a plant fungal pathogen, *Aphanomyces euteiches* (Carruthers *et al*., 1994).

Pseudomonas aurantica isolated from soybean rhizosphere is able to produce a pigment characterized by a strong inhibiting capacity upon different pathogenic fungi such as *Phytium* spp., *Fusarium* spp., *Alternaria* spp., *Macrophomin*a spp., and *Rhizoctonia solani* (Altamirano *et al*., 1996) .

Pseudomonas aeruginosa is widely distributed opportunistic pathogen inhibit the growth of *Candida albicans,* other *Candida* spp., and *Aspergillus fumigatus* (Kerr *et al*., 1999). It secretes proteins which are toxic to a wide range of organism, it also secretes copious amount of phenazine pigment .The major phenazine produced by this organism is pyocyanin (1-hydroxy-5-methylphenazine). Interest in pyocyanin derived from its intense color, which make its presence so obvious from its antibiotic properties and from the correlation between its production and pathogenesis, furthermore, pyocyanin can accept a single electron yielding a relatively stable anion radical and readily undergo a redox cycle,

previously noted that pyocyanin induced the biosynthesis of manganese containing superoxide dismutase (MnSoD) and it caused enhance production of O^{-}_{2} and it appeared that the antibiotic action of pyocyanin might actually be an expression of the toxicity of the O^{-}_{2} and H_2O_2 produced in increased amount in its presence (Hassan and Fridovich., 1980).

1.7-Phenazine:

Certain strains of root-colonizing fluorescent *Pseudomonas* bacteria have gained attention in recent years because they produce broad-spectrum metabolites active against fungal pathogens that cause soil borne root diseases. One such class of compounds, the phenazines, includes over 50 members with the same core structure but with different chemical modifications that largely determine their physical properties and biological activity. In *P. aeruginosa*, the most widely-studied phenazine-producing pseudomonad, phenazines contribute not only to biological control activity but also to the activity of the bacteria as opportunistic pathogens of animals, insects, nematodes, and plants (Mavrodi *et al* .,2006).

Many phenazine compounds are found in nature and are produced by bacteria such as *Pseudomonas* spp., *Streptomyces* spp., *Nocardia, Sorangium, Burkholderia, Brevibacterium* and *Pantoea agglomerans*. These phenazine natural products have been implicated in the virulence and competitive fitness of producing organisms. Such as phenazine-1-carboxylic acid, produced by a number of Pseudomonads, increases survival in soil environments and has been shown to be essential for the biological control activity of certain strains. Phenazine chemical formula ($C_{12}H_8N_2$ or $C_6H_4N_2C_6H_4$) , it also called azophenylene , dibenzo-p-diazine , dibenzopyrazine, and acridizine, is the parent substance of many

dyestuffs, such as the eurhodines, toluylene red, indulines and safranines. It is a dibenzoparadiazine having the formula given in figure (1-1) (Turner and Messenger, 1986; McDonald et *al*., 2001).

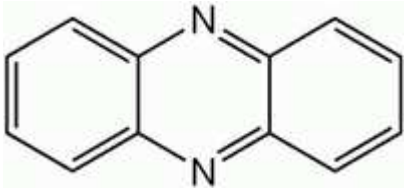


Figure (1-1) The general structure of phenazine
(Boland and Kuykendall, 1998)

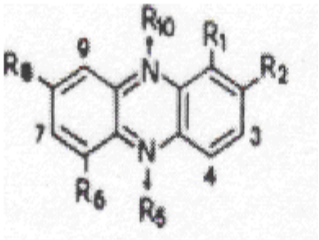
1.7.1-Mode of action:

The broad-spectrum activity exhibited by phenazine compounds against fungi and other bacteria, it is thought that they diffuse across the membrane and ,once inside the cell, accept a single electron ,disrupting respiration by interfering with the normal process of electron transport .This result in the overproduction of O^{-}_{2} and H_2O_2 ,which overwhelm cellular superoxide dismutase and ultimately cause cell death .The cellular superoxide dismutase of *P.aeruginosa*, a bacterium which produce the phenazine compound pyocyanin, are more active than those of phenazine nonproducing bacteria such as *Escherichia coli*, and they provide protection against phenazine (Hassett *et al* .,1995;Shannon *et al*.,2001).

1.7.2- Phenazine types:

There are currently over 50 known phenazine compounds with the same basic structure differing only in the derivatization of the heterocyclic core .These modifications largely determine the physical properties of the phenazines and influence their biological activity against plant and animal pathogens (Shannon *et al*., 2001).Table (1-2)show some kinds of phenazines differing in R –group and biosynthesis of phenazine by *Pseudomonas* strains is illustrated in figure (1-2) .

Table (1-2) Phenazine types (Leisinger and Margraff., 1979)

structure	Kind	R					
		R_1	R_2	R_5	R_6	R_8	R_{10}
	Pyocyanin	OH	H	CH_3	H	H	—
	Hemipyocyanin	OH	H	—	H	H	—
	Idoinin	OH	H	O	OH	H	O
	Tubermycine B	COOH	H	—	H	H	—
	Chlororaphine	$CONH_2$	H	H	H	H	H
	Oxychlororaphine	$CONH_2$	H	—	H	H	—
	Aeruginisin A	H	NH_2	—	COOH	H	CH_4
	Aeruginosin B	H	NH_2	—	COOH	SO_2H	CH_4

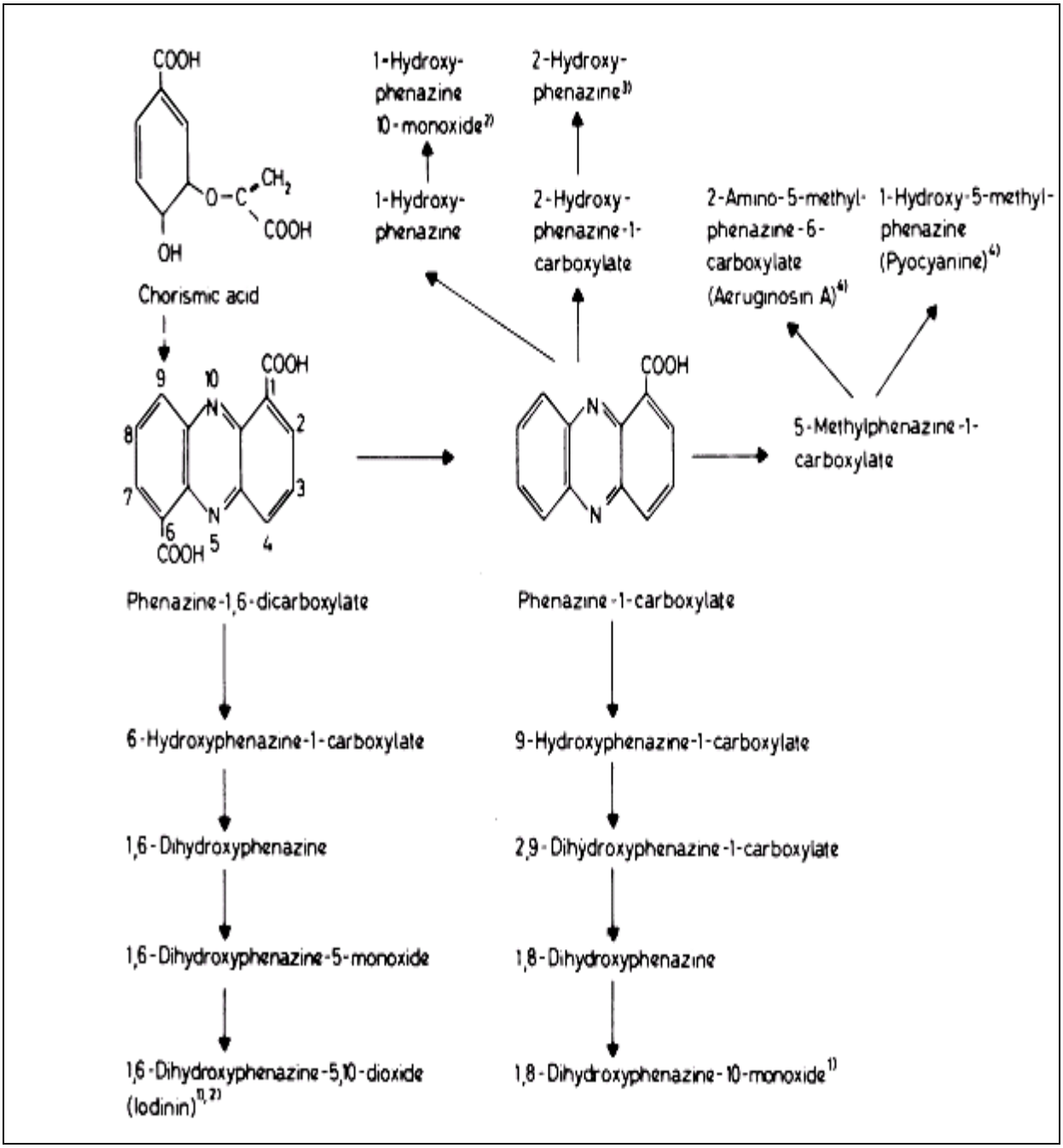


Figure (1-2): hypothetical scheme for phenazine biosynthesis in *Pseudomonas* species 1) *P.phenazinium* 2) *P.iodina* 3) *P .aureofaciens*

4) *P. aeruginosa* (Leisinger and Margraff .,1979).

1.8-Partial purification and characterization of antifungal metabolite:

chromatographic methods namely thin layer paper, gas liquid and high performance liquid chromatography ,are used widely for separation and identification of metabolites produced by microorganism .However such techniques may be suitable for small quantity rather than production (Warren *et al* .,1990) .

separation of pyocyanin ,the primary extract could be applied to a column of a lumina oxide (Watson *et al* .,1986).Although thin-layer chromatography (TLC) also well recognized as an ideal technique for the screening of drugs in toxicological analysis ,because of its low cost and convenience and the selectivity of the detection reagent ,objective criteria for the evaluation of the separation ability of TLC have often been lacking . Choice of system mostly depend on its ability to separate a product with low contaminant, such ability called discriminating power. However, system also selected in accordance to information gained during utilization of the unknown metabolites (Ulmer *et al* .,1990) .

Thin layer chromatography (TLC) used for the separation of the phenazine antibiotic, coupled with inherent fluorescens properties under ultraviolet illumination has provided to be the basis for extremely sensitive analytical methods (Betina ,1983).

A thin layer chromatography was carried out with the raw extract on silica-gel with chloroform as mobile phase. The relative mobility of each band was determined considering the relative flow of each solute (R_f) using an ultraviolet –light (λ 254-350) transilluminator, the elution of separate solutes was carried out by scraping each spot and dilution in 100% acetone. After removing silica gel by centrifugation, each fraction was

concentrated by acetone evaporation and dry residues were stored at -20ºC (Rovera *et al*., 2001).

Once a pure compound is obtained, its chemical structure will be determined by using a combination of gas chromatography mass–spectrometry, FT-infrared (FTIR), and nuclear magnetic resonance (NMR). Identification of the compound could lead to the chemical synthesis of this compound and analogues for use in the suppression of the destructive activities of pathogens in the field as well as in grain storage (Jayaswal *et al*., 1990).

1.9-Genetic of *Pseudomonas*:

Genetic studies on fluorescent *Pseudomonas* spp. the only microorganism for which the genes responsible for the assembly of the heterocyclic phenazine nucleus have been cloned and sequenced (Bloemberg and Lugtenberg ., 2001).

The phenazine biosynthetic loci from *P. fluorescens* 2-79, *P. aureofaciens* 30-84 *P. aeruginosa* PAO1and *P.chlororaphis* each contains a seven gene core operon regulate in a cell density dependent manner (Pierson *et al* .,1995; Mavrordi *et al* .,1998; ; Mavrordi *et al* ., 2006) .

In *P. fluorescens* 2-79 ,*P. aureofaciens* 30-84 , and *P.chlororaphis* pcL 1391,the phzI/R genes are found directly upstream from the phenazine core .But phenazine production in *P. aeruginosa* is controlled by two sets of regulatory genes rhlI/R and lasI/R located elsewhere in the genome .The seven gene core operon designated as phzABCDEFG and the core gene product phzC,phzD,phzE are homologous with phzF, phzA, phzB in strain 30-84 ,are similar to enzymes of shikimic acid metabolism (Shannon *et al* .,2001).

PhzA is thought to catalase one of the final steps in the formation of phenazine-1-caboxylic acid, the end product of phenazine biosynthesis in *p. fluorescens* 2-79 (Ahuja *et al*., 2004).

Pierson *et al*.,(1995)suggested that the phzC gene of ,*P. aureofaciens* 30-84 ,and in particular the last 28 amino acid of phzC protein are essential for the production of 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine(2-OH-PHZ).PhzG is similar to pyridoxamine-5-phosphate oxidase and probably is a source of cofactor for the PCA synthesizing enzymes (Mavrodi *et al* .,1998).

Functional studies in phenazine-nonproducing strains of fluorescent pseudomonads indicated that each of the biosynthetic operons from *P. aeruginosa* is sufficient for production of a single compound, phenazine-1-carboxylic acid (PCA). Subsequent conversion of PCA to pyocyanin is mediated in *P. aeruginosa* by two novel phenazine-modifying genes, phzM and phzS, that encode putative phenazine-specific methyltransferase and flavin-containing monooxygenase enzymes, respectively. Expression of phzS alone in *E. coli* or in pyocyanin-nonproducing *P. fluorescens* resulted in conversion of PCA to 1-hydroxyphenazine. *P. aeruginosa* with insertionally inactivated phzM or phzS genes developed pyocyanin-deficient phenotypes. A third phenazine-modifying gene, phzH, which has a homologue in *P. chlororaphis*, also was identified and shown to control synthesis of phenazine-1-carboxamide from PCA in *P. aeruginosa* PAO1(Mavrordi *et al* ., 2006).

1.10-Regulation of secondary metabolite production in root-colonizing *Pseudomonas* spp. :

Certain strains of fluorescent Pseudomonads are important biological components of agricultural soils that are suppressive to diseases caused by pathogenic fungi on crop plants. The biocontrol abilities of such strains depend essentially on aggressive root colonization, induction of systemic resistance in the plant, and the production of diffusible or volatile antifungal antibiotics. Evidence that these compounds are produced in situ is based on their chemical extraction from the rhizosphere and on the expression of antibiotic biosynthetic genes in the producer strains colonizing plant roots. Well-characterized antibiotics with biocontrol properties include phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide. In vitro, optimal production of these compounds occurs at high cell densities and during conditions of restricted growth, involving (i) a number of transcriptional regulators, which are mostly pathway-specific, and (ii) the GacS/GacA two-component system, which globally exerts a positive effect on the production of extracellular metabolites at a posttranscriptional level. Small untranslated RNAs have important roles in the GacS/GacA signal transduction pathway (Hass and Keel., 2003) .

Biosynthesis of these compounds and of the exoenzymes strictly depend on the GacS/GacA two-component system, which operates a switch from primary to secondary metabolism in various gram negative bacteria and which can also be involved in pathogenicity to plants and animals, in ecological fitness and in stress tolerance (Heeb and Hass ., 2001).

GacS/GacA control of secondary metabolites was shown to occur in strain CHA0 at the posttranscriptional level involving the RNA binding protein RsmA as a key regulatory element (Blumer *et al* .,1999).

RsmA is presumed to interact with specific ribosome – binding sites present in target genes and there by prevent translation such translation repression can be alleviated by the action of the small regulatory RNAs RsmY and RsmZ, whose expression is controlled by the GacS/GacA system in response to signal molecules produced by the strain CHA0 at the end of exponential growth (Heeb *et al* .,2002;Valverde *et al* .,2003).

RsmY and RsmZ bind multiple copies of the RsmA protein (Valverde *et al* .,2004)and , by a titration effect may thus render the ribosome-binding site of the target genes accessible for the translation machinery(Reimmann *et al* .,2005).

In *P. aeruginosa*, RsmA posttranscriptionally control the production of secondary metabolites directly as well as indirectly by modulating the quorum-sensing circuitry (Pessi and Hass., 2001 ;Pessi *et al.*, 2001).

Multiple regulators control phenazine production by *p. aureofaciens* 30-84 the quorum-sensing genes phzI and phzR (Pierson *et al*., 1994; Wood and Pierson., 1996) are directly responsible for the activation of the phenazine biosynthetic operon (phzXYFABCD)(Pierson *et al* .,1995).

In response to the cell density –dependent accumulation of N-hexanoyl homoserine lactone (HHL) produced by phzI, HHL association with phzR is believed to cause it to fold in to a structure enable it to bind to a sequence (phz box) upstream of the biosynthetic operon activating phenazine gene expression (Wood *et al* ., 1997).

In 1994 Pierson., *et al*, identified phzR (phenazine regulator) as a functional gene required for phenazine production. The predicted amino acid sequence of phzR is a homology with other bacterial positive transcriptional activator include LasR of *P.aeruginosa*, LuxR of *vibrio fischerii* and Tra R of *Agrobacterium tumefaciens*.

A second quorum-sensing system, CsaI/CsaR, also contribute to the accumulation of total N-acely-homoserine lactone (AHL) signal by strain 30-84 but only influences phenazine production when present in multiple copies or when grown in specific medium (Zhang and Pierson., 2001).

Chapter three

Results and Discussion

3.1-The antifungal activity of *P. aeruginosa* OM13:

It has been found in previous studies that the *P. aeruginosa* OM13 isolate, has the ability to inhibit fungal growth (Al -Ahmed, 2004).

In order to examine the antagonistic properties of the bacterial isolate against phytopathogenic fungi, two methods were used, in the first, a loopful of 24-hrs old culture of the bacterial isolate (*P.aeruginosa* OM13) was inoculated at 2 cm juxtaposed to the fungal as mentioned in (2.7.4) and the results shows that the *P.aeruginosa* OM13 isolate inhibited the growth of the test fungi (*A .alternata, R .solani)*and the inhibition zone was 5mm for *A.alternata* and 7mm for the fungi *R .solani* figure (3-1).

ii **i**

Figure (3-1): i)Effect of *P.aeruginosa* OM13 on fungal growth on PDA for 5 days at 28ºC ii)Control (A*lternaria alternata , Rhizoctonia solani)*

In the second method the antifungal activity of the bacterial isolate was examined by testing the effect of culture filtrate of the bacterium on the growth of the fungal pathogens, which was assessed by agar-well method (Gupta *et al* .,2001) as mentioned in (2.7.4) and the results of this method indicated that this isolate inhibited the growth of the fungi *A .alternata ,R .solani* and the inhibition zone was 5mm, 7mm respectively figure (3-2). The ratio between the growth of the fungus with bacterial isolate and bacterial filtrate to the growth of the fungus alone (control) was determined as illustrated in the table (3-1).

Table (3-1) Ratio*of fungus growth with bacterium isolate and cultural filtrate

Fungi	Ratio of the fungus growth (cm) by using	
	Bacterium isolate	Bacterium filtrate
A.alternata	0.5	0.54
R. solani	0.46	0.51

* The ratio between the growth of the fungus with bacterial isolate and bacterial filtrate to the growth of the fungus alone (control)

ii **i**

Figure (3-2): i) effect the filtrate of *P.aeruginosa* OM13 on the growth of the test fungi(*R. solani, A.alternata)* on PDA for 5 days at 28°C ii)control (*Rhizoctonia solani* , *Alternaria alternate*

3.2-Plasmid isolation:

In order to determine the plasmid profile of *P.aeruginosa* OM13, many methods were used, Alkaline method and boiling method (Maniatis *et al* ., 1982), salting out method (kieser , 1995) and plasmid isolation miniprep. kit (Us biological) has also been used. The last two methods gave acceptable results.

The results for both methods are shown in figure (3-3) which indicated that *P. aeruginosa* OM13 contained small plasmid DNA bands and these bands are approximately in the same size compared with pBR322 plasmid (4.363 Kb).

Many studies reported that the small plasmid DNA bands in *P.aeruginosa* are responsible for antibiotic resistance in this bacteria. Padilla and Vasquez (1993) reported that the plasmids of *P. aeruginosa* were responsible for the resistance of this bacteria to ampicillin and chloramphenicol. They also found that this bacteria harbored pAz-10 and pC-20 plasmids which are responsible for azlocillin and chloramphenicol resistance, respectively, this was demonstrated indirectly by their ability to confer resistance to these antibacterial when transformed into susceptible *Escherichia coli* hosts, and in (2004) Shahid and Malik showed that resistance of *P.aeruginosa* to amikacin was plasmid mediated.

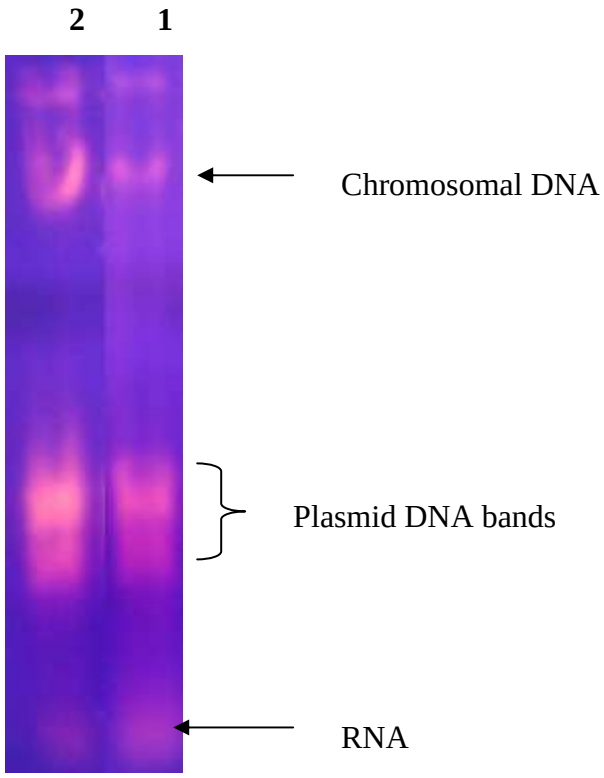


Figure (3-3): Gel electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.7%) in TBE buffer at (5V/cm). for 2 hrs

1. ***E.coli* HB101 harboring pBR322 plasmid**
2. ***Pseudomonas aeruginosa* OM13**

3.3-Antibiotic sensitivity test:

In order to find a genetic marker that can be useful in curing and transformation experiments, the standard disk diffusion method was used to determine the sensitivity of *P.aeruginosa* OM13 to antibiotics. Results in table (3-1) showed that *P.aeruginosa* OM13 was resistant to aztreonam, erythromycin, ampenicillin, carbencillin, and chloramphenicol, sensitive to tetracycline, streptomycin, norfloxacin, vancomycin, and gentamycin.

Table (3-2) Antibiotic sensitivity of *P.aeruginosa* OM13

Antibiotic / Strain	AZM	AMP	Nor	Cm	PY	TE	VA	GM	S	E
P.aeruginosa OM13	R	R	S	R	R	S	S	S	S	R

R	Resistance	PY	Carbenicillin
S	Sensitive	TE	Tetracycline
AZM	Aztreonam	VA	Vancomycin
AMP	Ampicillin	GM	Gentamycin
Nor	Norfloxacin	S	Streptomycin
Cm	Chloramphenicol	E	Erythromycin

Resistance of *P.aeruginosa* OM13 to chloramphenicol may be due to destruction of the antibiotic by an enzyme (chloramphenicol acetyltransferase) that is under plasmid control, and resistance to erythromycin may be results of an alteration (methylation) of the RNA receptor (this is under control of transmissible plasmid) (Jawetz *et al.*, 1998).

Resistance of *P.aeruginosa* OM13 to β- lactam antibiotics (ampicillin, carbencillin, aztroenam) may be attributed to degradation of the antibiotics by β-lactamase enzyme which is normally plasmid encoded, lack of penicillin binding protein (PBP) for a specific antibiotics or finally the microorganism

change their permeability to the drug. (Scott *et al*., 1999; Barlow and Hall, 2003).

In general it is well documented that very few of the conventional antibiotics are active against *P.aeruginosa*, because many studies demonstrated that a cluster of genes, which are either plasmid or chromosomally encoded, are responsible for antibiotics resistance in *P. aeruginosa* (Holloway *et al*., 1979; Padilla and Vasquez., 1993).

P.aeruginosa OM13 was capable to form viscous gels and this may be due to its ability to produce alginate. Alginate also plays a role in the resistance of *P.aeruginosa* to a wide range of antimicrobial agent which completely blocked diffusion of the antibiotics due to its ability to form viscous gels as well as to its ionic charge and that when the alginate is degraded, this aminoglycoside blocked activity is effectively reduced (Match and Schiller., 1998).

3.4- The role of plasmid in antifungal compound production:

In order to study the role of plasmid of *P. aeruginosa* OM13 in antifungal compound production curing and transformation experiments were done.

3.4.1- Curing

An attempt was made to cure *P. aeruginosa* OM 13 plasmids by using SDS. The results in table (3-2) indicated that the highest concentration of SDS that still allows the bacterial growth was 5%. From this treatment, appropriate dilution were made and spread on nutrient agar plates. Then 100 colonies were selected and tested on a selective medium containing antibiotics to which the W.T is resistant (ampicillin, chloramphenicol, aztroenam, erythromycin) in order to determine the cured colonies (which can not grow

on these antibiotic containing media), and then the ability of obtained cured colonies for fungal growth inhibition was tested.

Table (3-3) Effect of SDS on the growth of *P. aeruginosa* OM 13

SDS Concentration	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
Bacterial Growth	+++	+++	++	+	±	–	–	–	–	–

(+++): Very good growth (±): Slight growth
(++): Good growth (–): No growth
(+):moderate growth

After repeatedly plated of the selected colonies, results indicated that a number of colonies (4%) failed to grow on media containing chloramphenicol and, also failed to inhibit the growth of test fungi (*A .alternata, R .solani)*as shown in figures (3-4a), (3-4b),(3-4c),(3-4d). It was shown also that these colonies differ in color from green (color of the W.T) to pale and this difference was illustrated in figure (3-5).

ii **i**

Figure (3.4a): Growth of the fungus *A.alternata* on PDA for 5 days at 28 ºC with i) W.T ii) cured cell

ii i

Figure (3.4.b): Growth of the fungus *R.solani* on PDA for 5 days at 28 ºC with i)W.T ii)cured cell

ii iii i

Figure (3.4.c): Growth of the fungus *A.alternata* on PDA for 5 days at 28 ºC with the filtrate of i)*P.aeruginosa* (W.T) ii)cured cell iii) *A.alternata* (control)

i **ii**

iii

Figure (3.4.d): Growth of the fungus *R.solani* on PDA for 5 days at 28 ºC with the filtrate of i)*P.aeruginosa* (W.T) ii)cured cell iii) *R.solani* (control)

ii **i**

Figure (3-5): The difference in color between the filtrate of wild type (i) and cured cell (ii).

In order to determine the inability of the cured colonies to produce antifungal compounds because of plasmid elimination or not, plasmid from the cured cells were isolated and compared with that from the W.T. The results showed that there is no change in plasmid patterns between the cured cells and the wild type(figure (3-6)). This indicated that the small plasmid DNA bands in *P. aeruginosa* OM13 are not responsible for the antifungal compound production. It can be concluded that chloramphenicol resistance and antifungal compound production trait in *P.aeruginosa* OM13 may be located on the chromosome and this conclusion agreed with Georgakopouls *et al* .,(1994) results about the location of the genes responsible for antifungal compound production. Since the curing agent (SDS) effect on the bacterial cell wall (Trevors, 1986), the inability of cured cell to produce antifungal compound may be due to the physiological change in the permeability of the cellular membrane and this conclusion was supported by changing in the color of cured cells which refers to change in the bacterial cell wall had been occurred. May also be that the genes which were responsible for the antifungal compound production located on plasmid did not appeared in the gel (possibly due to its large size)

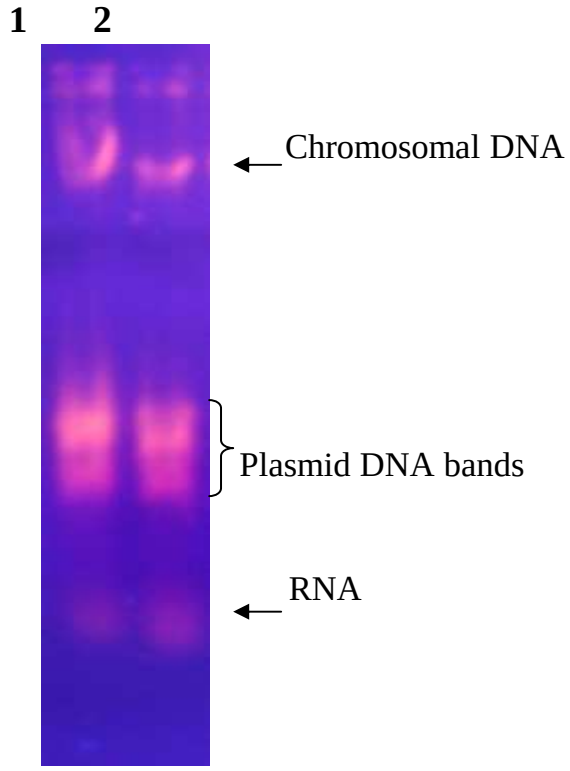


Figure (3-6): Gel electrophoresis of plasmids content of *P.aeruginosa* OM13 before and after treatment with SDS on agarose gel (0.7%) in TBE buffer at 5V/cm for 2 hrs.

1. **Plasmid content of the *P.aeruginosa* OM13**
2. **Plasmid content of the *P.aeruginosa* OM13 (cured)**

3.4.2-Transformation

The ability of antifungal compound production trait to transfer from one bacterium to another by transformation is another method to determine whether antifungal production genes are located on plasmid or not. For this reason transformation experiments were performed between DNA of *P.aeruginosa* OM13 (Cm^r, produce antifungal compound) as a donor and *E.coli* MM294 (Cm^s, does not produce antifungal compound) as a recipient.

Transformation mixtures were spread on a nutrient agar medium containing chloramphenicol in order to select transformant colonies. Results showed that all attempts to isolate transformed colonies which have the ability to resist chloramphenicol and inhibit fungal growth were not succeed.

Failure to select transformants *(E.coli* able to inhibit fungal growth) may be due to one of the following reasons:

- -Transformation between different strains of different species is not always possible.
- The DNA were taken up by *E.coli* but have been degraded by host nucleases enzymes.
- The DNA were taken up by *E.coli* but were unable to replicate or express their genetic marker (chloramphenicol) in the new host (*E.coli* MM294)

3.5- Mutagenesis of *P.aeruginosa*:

In an attempt to enhance antifungal compound production by *P. aeruginosa* OM13 the survival curve of *p.aeruginosa* OM 13 after treatment with MNNG at different time is shown in figure (3-6).

It was found that this bacterium was sensitive to MNNG. The survival curve showed increase of the lethal percentage exponentially with increasing the time of exposure to MNNG, and the highest lethal percentage or the less survival percentage was (0.1%) when the bacterium was treated with MNNG (30µg/ml) for 60 min. The treatment (15min) led to a survival percentage of 5.8% (94.2% killing). Therefore, it was selected for mutants isolation.

It is known that MNNG is an effective mutagenic compound. It can generate mispairing lesion by adding alkyl group (e.g methyl group) to various position on nucleic acids and hence missreplication of DNA, or missrepair of damaged DNA. Some of these lesions are potentially lethal as they can interfere with the unwinding of the DNA during replication and transcription (Freifelder, 1987). It also can induce mutation by an error prone DNA repair pathway (Stonesifer *et al*., 1985). Many studies dealing with the effect of MNNG on different bacteria (*Clostridium butyricum, Micrococcus* spp.) showed that these bacteria were sensitive to MNNG (Carrasco and Soto, 1987; Al-Bakri and Umran, 1994).

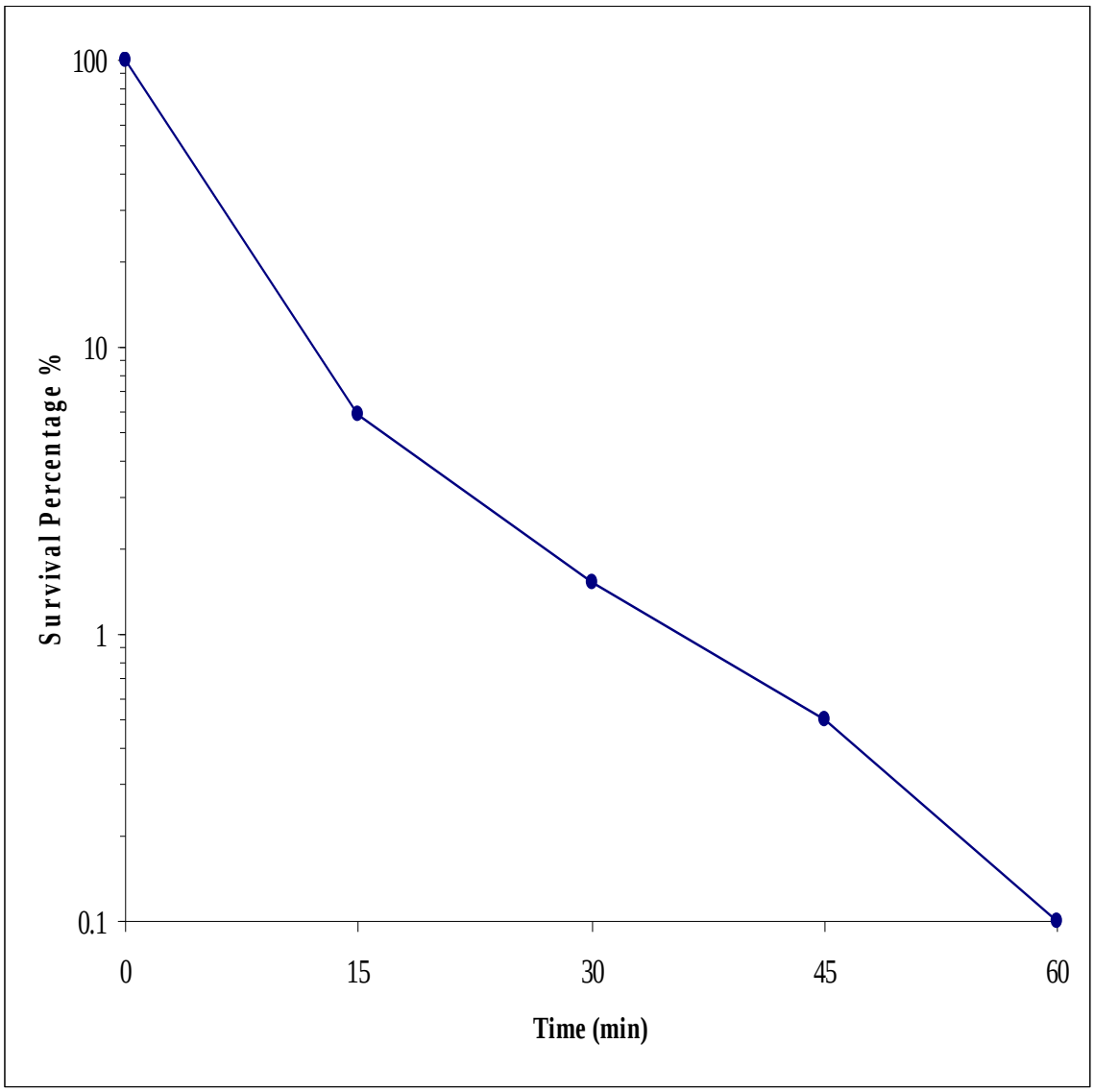


Figure (3-7) effect of MNNG (30 µg/ml) on a suspension of *P.aeruginosa* OM13 in phosphate buffer (pH= 7).

3.6-Bioassay the antifungal activity of the Mutants:

In order to know the effect of mutagenesis by MNNG to improve the production of antifungal compound by *P.aeruginosa* OM13, mutagenized culture from the selected treatments (bacteria treated with 30µg/ml of MNNG for 15 min) were replica plated on King A agar plate in an attempt to detect any difference in color which is used as an indicator for antifungal compound production.

Results showed that there is no difference in color of obtained colonies compared with W.T. For this reason, an alternative method for the detection of antifungal compound production was employed for screening of antifungal compound produced by *P.aeruginosa* OM13 mutants, in which (50) colonies named (RA1-RA50) were grown in peptone broth incubated at 37˚C for 18 hrs then cells was pelleted by centrifugation, then the supernatant was taken and the antifungal compound production was determined by measuring the optical density at 520 nm. In addition, agar well method was also used in which the filtrate of the mutants were cultured with the fungus *A. alternata* and the inhibition zone was measured after 5 days of incubation and compared with the inhibition zone caused by W.T.

Results in table (3-3) indicated that colonies obtained after treatment with MNNG varied in production of antifungal compound, in which(34%) of the mutants showed an increase in O.D. while (26%) showed decrease, at the same time other mutants(40%) remained the same as the wild type.

Results showed also the relationship between the O.D. and the inhibition zone, in which (5) colonies showed increasing more than four times. It was also seen that (RA5) gave a highest O.D measurement (nine times) and the highest inhibition zone.

Table (3-4) $O.D_{520nm}$ values and inhibition zone caused by *P.aeruginosa*OM13 mutants after treatment with MNNG (30µg/ml for 15min)

Bacterial isolates	**$O.D_{520nm}$**	**Inhibition zone (mm)**
OM13	0.1	5
RA*1	0.45	10
RA2	0.56	10
RA3	0.77	10
RA4	0.8	10
RA5	0.9	12
RA6	0.29	9
RA7	0.28	9
RA8	0.28	9
RA9	0.26	8
RA10	0.23	8
RA11	0.23	8
RA12	0.21	7
RA13	0.21	8
RA14	0.2	8
RA15	0.2	8
RA16	0.2	7
RA17	0.2	8
RA18	0.15	5
RA19	0.16	6
RA20	0.12	5
RA21	0.16	6
RA22	0.12	5
RA23	0.18	7

RA24	0.16	6
RA25	0.15	6
RA26	0.14	5
RA27	0.17	6
RA28	0.1	5
RA29	0.17	6
RA30	0.16	6
RA31	0.14	6
RA32	0.18	8
RA33	0.13	6
RA34	0.16	7
RA35	0.17	٧
RA36	0.16	8
RA37	0.18	8
RA38	0.06	4
RA39	0.018	2
RA40	0.076	5
RA41	0.012	2
RA42	0.03	3
RA43	0.05	3
RA44	0.07	4
RA45	0.01	2
RA46	0.03	3
RA47	0.01	2
RA48	0.07	4
RA49	0.07	5
RA50	0.08	5

OM13 : W.T , *: *P. aeruginosa* after treated with MNNG (30µg/ml)

Results indicated that MNNG caused random mutation in the genes responsible for antifungal compound production. The mutation may have occurred in the regulatory genes rhl I/R las I/R that located elsewhere in the genome which is controlling the phenazine production in *P.aeruginosa* due to decreasing in the antifungal compound production (Shannon *et al*., 2001).

Mavrodi *et al*., (2006) reported that *P.aeruginosa* have biosynthetic operons which are sufficient for production of single compound phenazine-1-carboxylic acid (PCA). And subsequent conversion of PCA to pyocyanin is mediated by two novel phenazine modifying genes, phzM and phz S, that encode putative phenazine –specific methyl transferase and flavin containing monooxygenase enzyme respectively. So the mutation may have occurred in this operon and led to decrease in the production of antifungal compound by these mutants.

The increase in the antifungal compound production from other mutants may be attributed to the mutation occurring in the (Gac S /Gac A) which controlls the secondary metabolite production at the posttranscriptional level involving the RNA binding proteins RsmA as a key regulatory element. RsmA is presumed to interact with specific ribosome binding sites present in the target genes and thereby prevent translation. Such translation repression can be elevated by the action of small regulatory RNA, Rsm Y and RsmZ whose expression is controlled by the Gac S /Gac A system in response to signal molecules produced by *Pseudomonas* at the end of exponential growth phase (Reimmann *et al* .,2005).

MNNG was used successfully to mutate and to get different mutants from different bacteria. In 1974 Carson and Jensen used MNNG in recognition of phenazine pigment (pyocyanin) mutants in *P.aeruginosa.* Al-Gelawi (1999) used MNNG to isolate salt sensitive mutants from *Micrococcus* spp. strain G1.

3.7- Characterization of the antifungal compound:

The following tests were performed in order to characterize the partially purified antifungal compound, and the results of these tests were as follows:-

- **TLC analysis of antifungal compound:**

When the partially purified antifungal compound was spotted on silica-gel TLC plate (10 x10 cm) and examined under UV transilluminator for characterization and purification, the result showed that the active antifungal compound has a single dark fluorescent spot under UV light and has an R_f value of (0.32) as illustrated in figure(3-8).

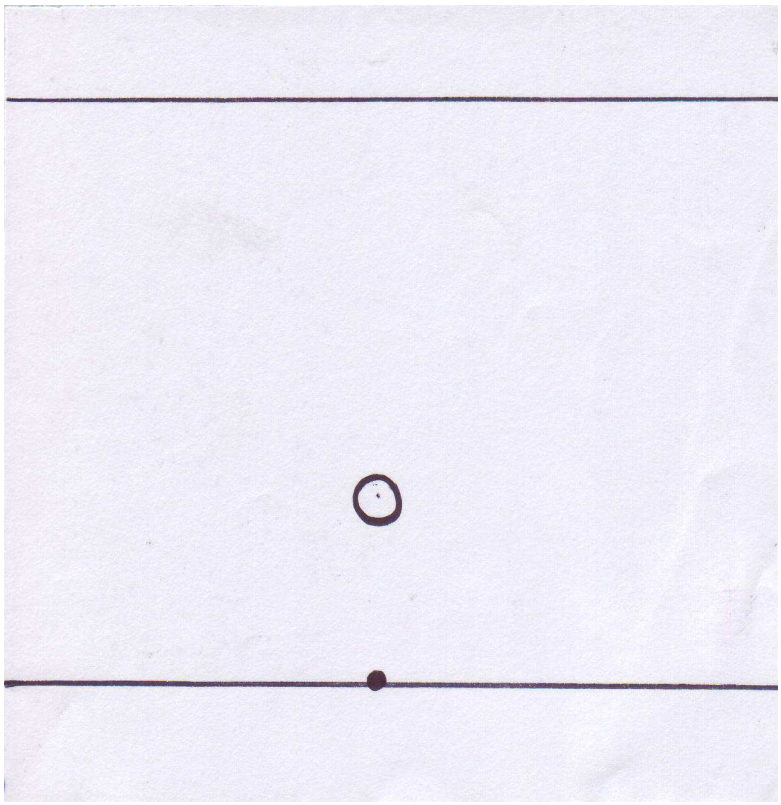


Figure (3-8) TLC analysis for the partially purified antifungal compound

- **UV absorption:**

The UV absorption spectrum figure (3-9) for the partially purified antifungal compound shows two peak at λ=319nm and 356 nm. These bands may be attributed to a π-π* and n-π* transition respectively.

A similar result was reported by authors working with pyocyanine produced by *P.aeruginosa* (Vukomanovic *et al*., 1997).

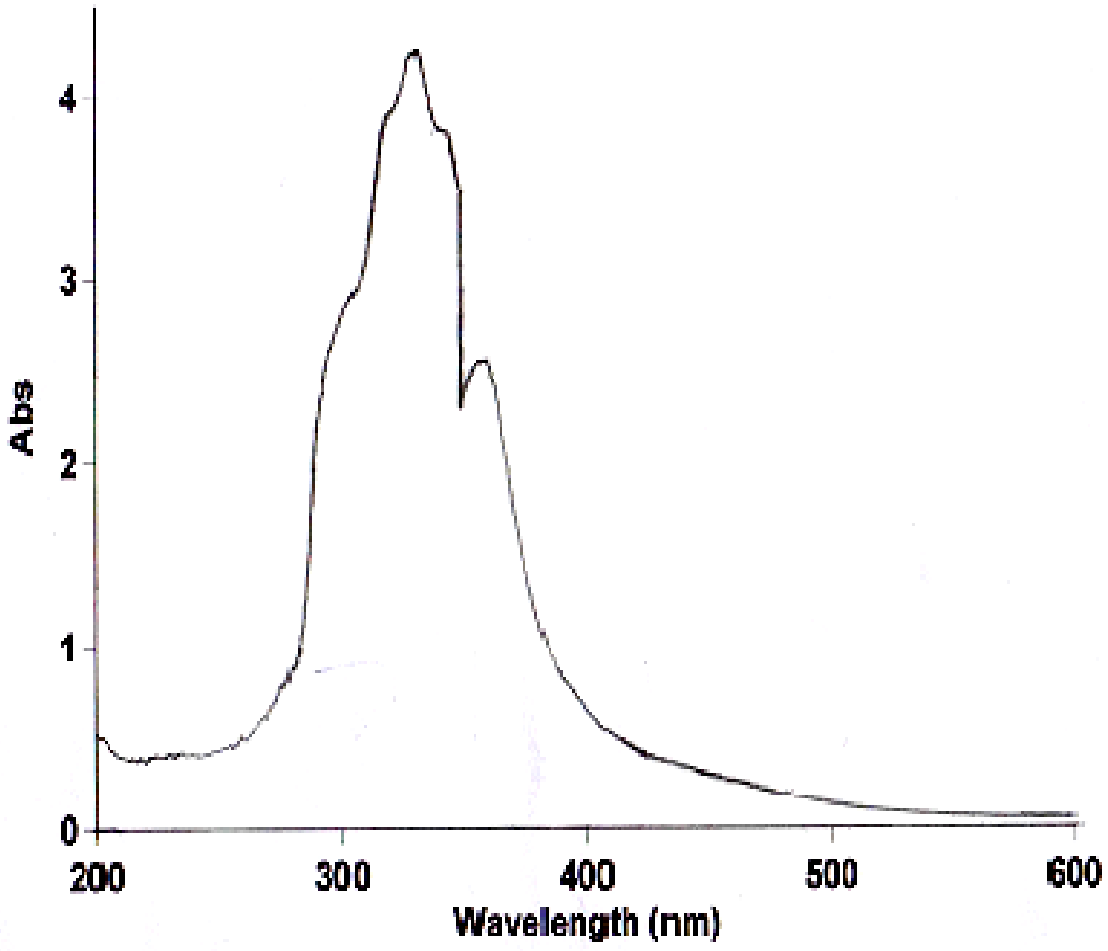


Figure (3-9) UV spectrum of the partially purified antifungal compound

- **IR analysis:-**

The partially purified compound was subjected to the IR analysis, figure (3-11), in order to know the functional chemical groups that are found in the compound that may lead us to propose a possible chemical structure of the compound. Depending on Socrates, (1980) for infra-red spectrum characterization of organic compounds and on specialist chemist at the Chemistry Department at Al-Mustansirya university. The IR results of the partially purified compound (the main important absorption bands) shows the presence of different bands corresponding to the following functional groups present in the molecular structure: stretching bands at 2950 cm^{-1} for ($C\text{-}H_{aliph.}$) due to methyl group, 3090 cm^{-1} for ($C\text{-}H_{arom}$), 1600 cm^{-1} for ($C{=}C_{arom.}$), 3200 cm^{-1} for (O-H),1650 $cm^{-1}$for (C=N), 1450 cm^{-1} for ($C\text{-}N_{cyclic}$), 1200 cm^{-1} for ($C\text{-}N_{arom}$), 1100 cm^{-1} for (C-O). This result was also supported by IR analysis of pyocyanin from Integrated Spectral Data Base System for Organic Compounds (SDBS) [intrenet 1].

As shown from the IR results and by depending on specialist chemists we can propose the possible chemical structure of the partially purified antifungal compound fig (3-10) as follows:-

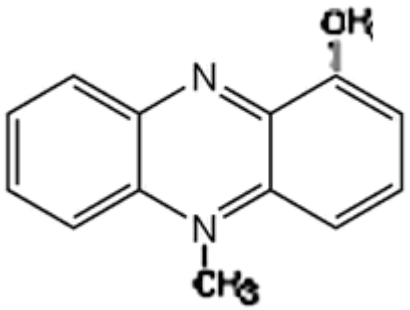


Figure (3-10) The proposed chemical structure of the partially purified antifungal compound from the IR results.

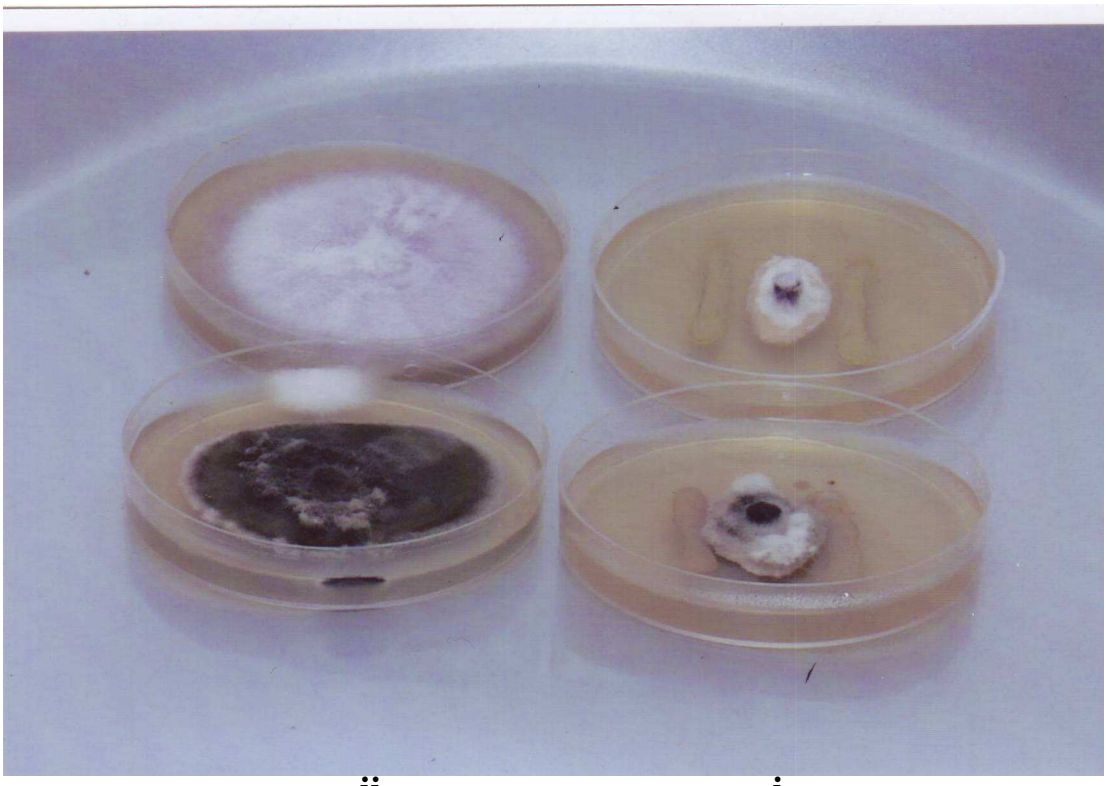
Chapter three

Results and Discussion

3.1-The antifungal activity of *P. aeruginosa* OM13:

It has been found in previous studies that the *P. aeruginosa* OM13 isolate, has the ability to inhibit fungal growth (Al -Ahmed, 2004).

In order to examine the antagonistic properties of the bacterial isolate against phytopathogenic fungi, two methods were used, in the first, a loopful of 24-hrs old culture of the bacterial isolate (*P.aeruginosa* OM13) was inoculated at 2 cm juxtaposed to the fungal as mentioned in (2.7.4) and the results shows that the *P.aeruginosa* OM13 isolate inhibited the growth of the test fungi (*A .alternata*, *R .solani)*and the inhibition zone was 5mm for *A.alternata* and 7mm for the fungi *R .solani* figure (3-1).



ii **i**

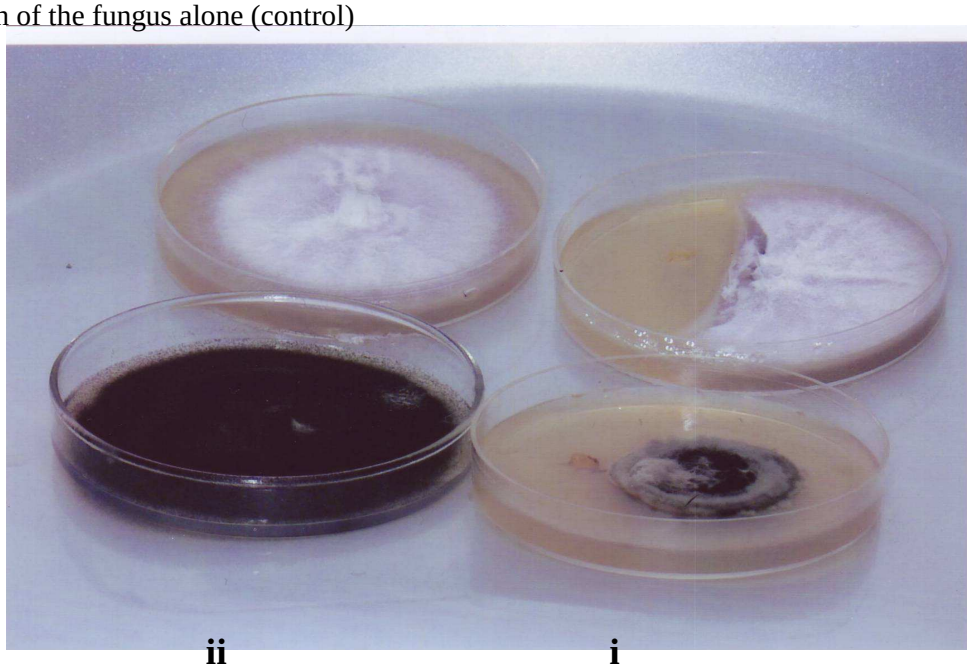
Figure (3-1): i)Effect of *P.aeruginosa* OM13 on fungal growth on PDA for 5 days at 28ºC ii)Control (A*lternaria alternata , Rhizoctonia solani)*

In the second method the antifungal activity of the bacterial isolate was examined by testing the effect of culture filtrate of the bacterium on the growth of the fungal pathogens, which was assessed by agar-well method (Gupta *et al* .,2001) as mentioned in (2.7.4) and the results of this method indicated that this isolate inhibited the growth of the fungi *A .alternata ,R .solani* and the inhibition zone was 5mm, 7mm respectively figure (3-2). The ratio between the growth of the fungus with bacterial isolate and bacterial filtrate to the growth of the fungus alone (control) was determined as illustrated in the table (3-1).

Table (3-1) Ratio*of fungus growth with bacterium isolate and cultural filtrate

Fungi	Ratio of the fungus growth (cm) by using	
	Bacterium isolate	Bacterium filtrate
A.alternata	0.5	0.54
R. solani	0.46	0.51

* The ratio between the growth of the fungus with bacterial isolate and bacterial filtrate to the growth of the fungus alone (control)



ii **i**

Figure (3-2): i) effect the filtrate of *P.aeruginosa* OM13 on the growth of the test fungi(*R. solani, A.alternata)* on PDA for 5 days at 28°C ii)control (*Rhizoctonia solani* , *Alternaria alternate*

3.2-Plasmid isolation:

In order to determine the plasmid profile of *P.aeruginosa* OM13, many methods were used, Alkaline method and boiling method (Maniatis *et al* ., 1982), salting out method (kieser , 1995) and plasmid isolation miniprep. kit (Us biological) has also been used. The last two methods gave acceptable results.

The results for both methods are shown in figure (3-3) which indicated that *P. aeruginosa* OM13 contained small plasmid DNA bands and these bands are approximately in the same size compared with pBR322 plasmid (4.363 Kb).

Many studies reported that the small plasmid DNA bands in *P.aeruginosa* are responsible for antibiotic resistance in this bacteria. Padilla and Vasquez (1993) reported that the plasmids of *P. aeruginosa* were responsible for the resistance of this bacteria to ampicillin and chloramphenicol. They also found that this bacteria harbored pAz-10 and pC-20 plasmids which are responsible for azlocillin and chloramphenicol resistance, respectively, this was demonstrated indirectly by their ability to confer resistance to these antibacterial when transformed into susceptible *Escherichia coli* hosts, and in (2004) Shahid and Malik showed that resistance of *P.aeruginosa* to amikacin was plasmid mediated.

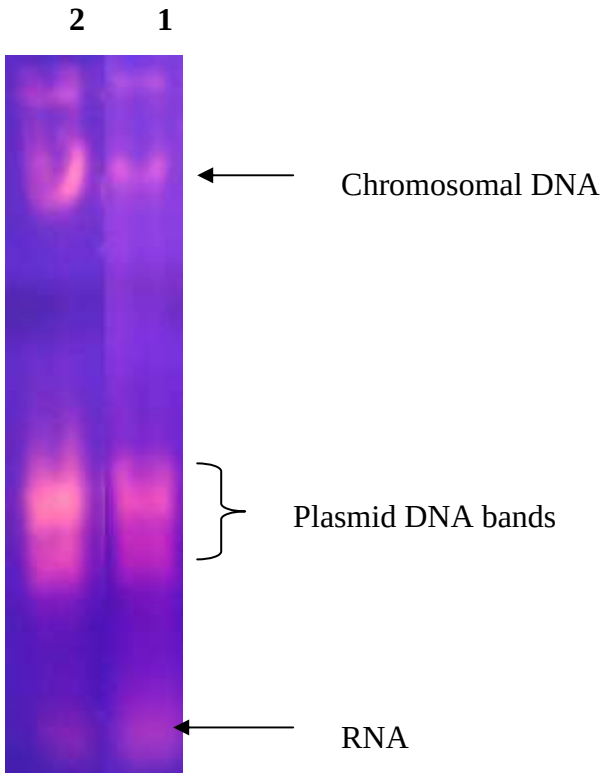


Figure (3-3): Gel electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.7%) in TBE buffer at (5V/cm). for 2 hrs

1. ***E.coli* HB101 harboring pBR322 plasmid**
2. ***Pseudomonas aeruginosa* OM13**

3.3-Antibiotic sensitivity test:

In order to find a genetic marker that can be useful in curing and transformation experiments, the standard disk diffusion method was used to determine the sensitivity of *P.aeruginosa* OM13 to antibiotics. Results in table (3-1) showed that *P.aeruginosa* OM13 was resistant to aztreonam, erythromycin, ampenicillin, carbencillin, and chloramphenicol, sensitive to tetracycline, streptomycin, norfloxacin, vancomycin, and gentamycin.

Table (3-2) Antibiotic sensitivity of *P.aeruginosa* OM13

Antibiotic / Strain	AZM	AMP	Nor	Cm	PY	TE	VA	GM	S	E
P.aeruginosa OM13	R	R	S	R	R	S	S	S	S	R

R	Resistance	PY	Carbenicillin
S	Sensitive	TE	Tetracycline
AZM	Aztreonam	VA	Vancomycin
AMP	Ampicillin	GM	Gentamycin
Nor	Norfloxacin	S	Streptomycin
Cm	Chloramphenicol	E	Erythromycin

Resistance of *P.aeruginosa* OM13 to chloramphenicol may be due to destruction of the antibiotic by an enzyme (chloramphenicol acetyltransferase) that is under plasmid control, and resistance to erythromycin may be results of an alteration (methylation) of the RNA receptor (this is under control of transmissible plasmid) (Jawetz *et al.*, 1998).

Resistance of *P.aeruginosa* OM13 to β- lactam antibiotics (ampicillin, carbencillin, aztroenam) may be attributed to degradation of the antibiotics by β-lactamase enzyme which is normally plasmid encoded, lack of penicillin binding protein (PBP) for a specific antibiotics or finally the microorganism

change their permeability to the drug. (Scott *et al*., 1999; Barlow and Hall, 2003).

In general it is well documented that very few of the conventional antibiotics are active against *P.aeruginosa*, because many studies demonstrated that a cluster of genes, which are either plasmid or chromosomally encoded, are responsible for antibiotics resistance in *P. aeruginosa* (Holloway *et al*., 1979; Padilla and Vasquez., 1993).

P.aeruginosa OM13 was capable to form viscous gels and this may be due to its ability to produce alginate. Alginate also plays a role in the resistance of *P.aeruginosa* to a wide range of antimicrobial agent which completely blocked diffusion of the antibiotics due to its ability to form viscous gels as well as to its ionic charge and that when the alginate is degraded, this aminoglycoside blocked activity is effectively reduced (Match and Schiller., 1998).

3.4- The role of plasmid in antifungal compound production:

In order to study the role of plasmid of *P. aeruginosa* OM13 in antifungal compound production curing and transformation experiments were done.

3.4.1- Curing

An attempt was made to cure *P. aeruginosa* OM 13 plasmids by using SDS. The results in table (3-2) indicated that the highest concentration of SDS that still allows the bacterial growth was 5%. From this treatment, appropriate dilution were made and spread on nutrient agar plates. Then 100 colonies were selected and tested on a selective medium containing antibiotics to which the W.T is resistant (ampicillin, chloramphenicol, aztroenam, erythromycin) in order to determine the cured colonies (which can not grow

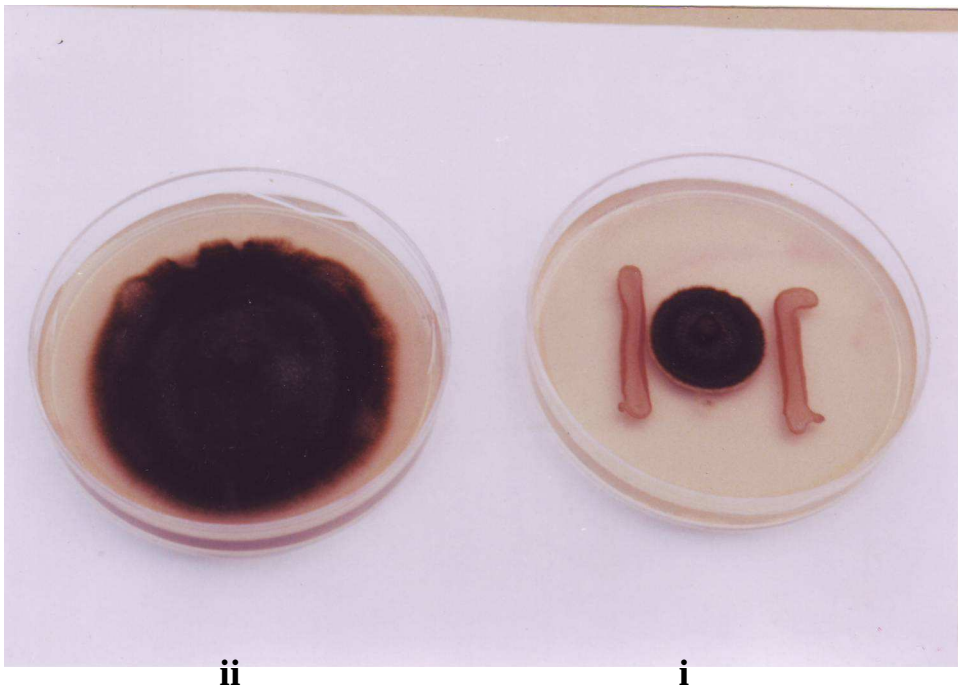
on these antibiotic containing media), and then the ability of obtained cured colonies for fungal growth inhibition was tested.

Table (3-3) Effect of SDS on the growth of *P. aeruginosa* OM 13

SDS Concentration	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
Bacterial Growth	+++	+++	++	+	±	–	–	–	–	–

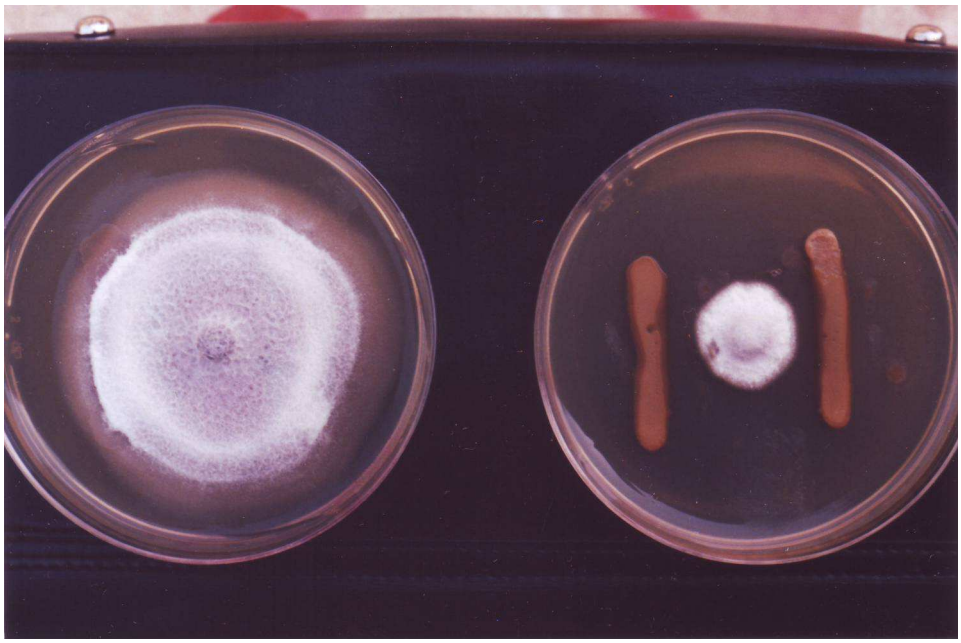
(+++): Very good growth (±): Slight growth
(++): Good growth (–): No growth
(+):moderate growth

After repeatedly plated of the selected colonies, results indicated that a number of colonies (4%) failed to grow on media containing chloramphenicol and, also failed to inhibit the growth of test fungi (*A .alternata, R .solani)*as shown in figures (3-4a), (3-4b),(3-4c),(3-4d). It was shown also that these colonies differ in color from green (color of the W.T) to pale and this difference was illustrated in figure (3-5).



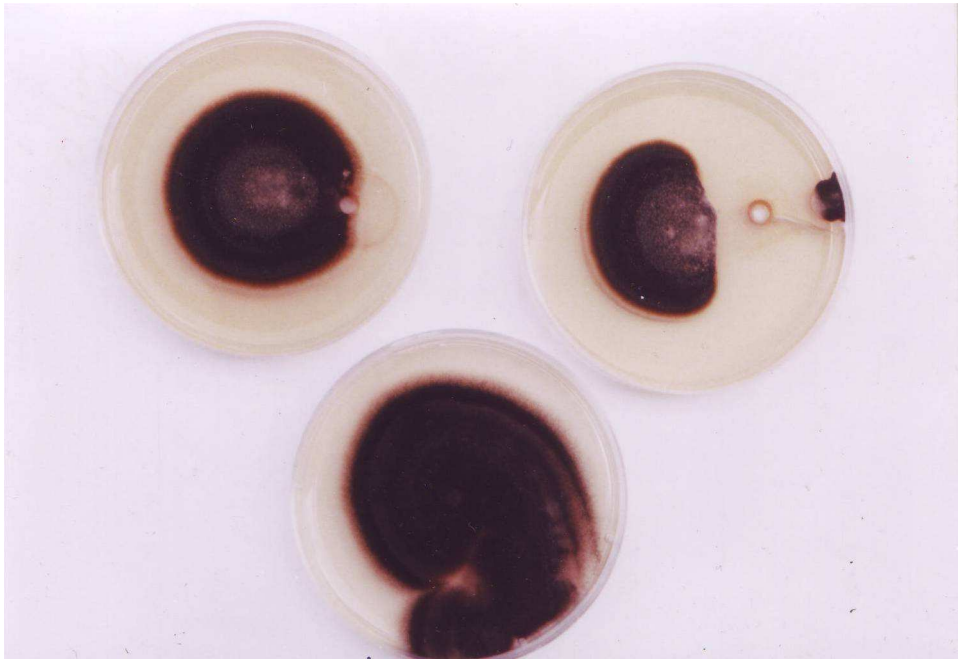
ii **i**

Figure (3.4a): Growth of the fungus *A.alternata* on PDA for 5 days at 28 ºC with i) W.T ii) cured cell



ii **i**

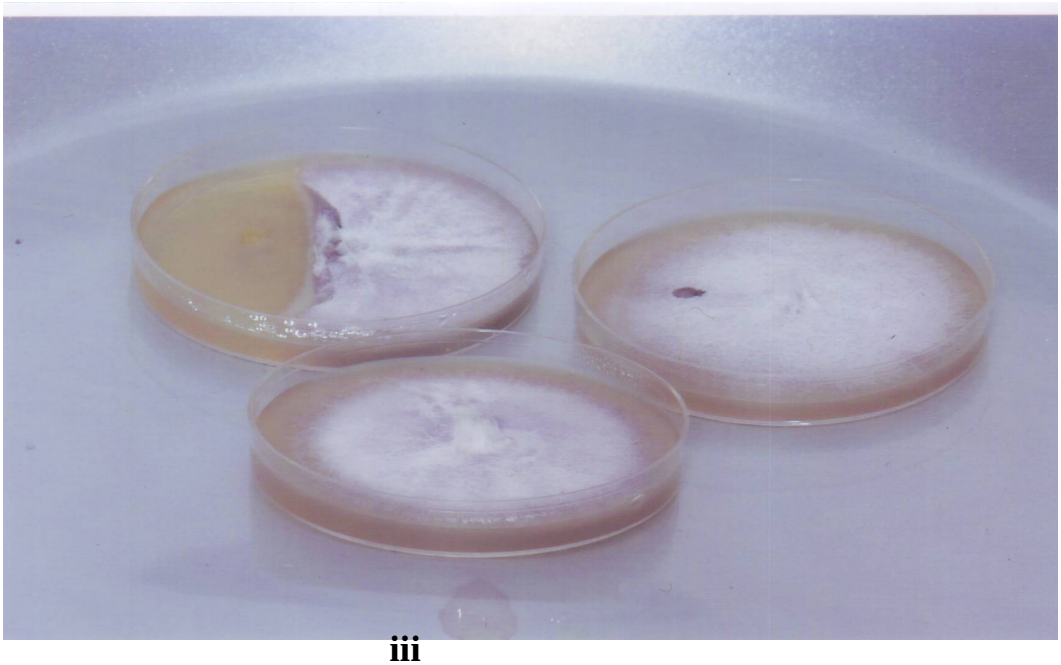
Figure (3.4.b): Growth of the fungus *R.solani* on PDA for 5 days at 28 ºC with i)W.T ii)cured cell



ii **iii** **i**

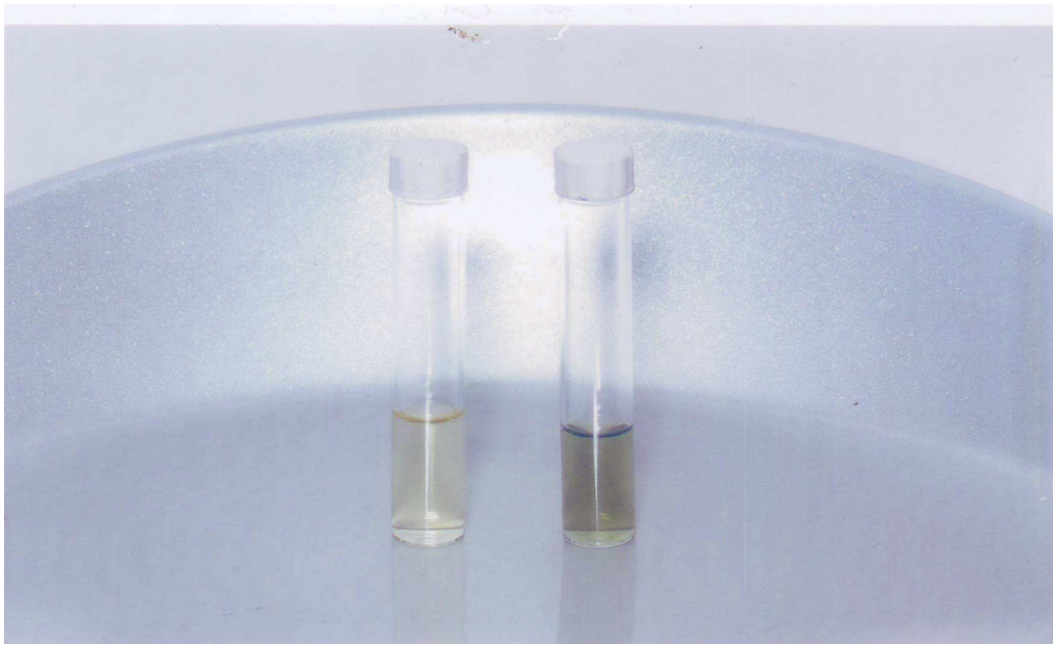
Figure (3.4.c): Growth of the fungus *A.alternata* on PDA for 5 days at 28 ºC with the filtrate of i)*P.aeruginosa* (W.T) ii)cured cell iii) *A.alternata* (control)

i ii



iii

Figure (3.4.d): Growth of the fungus *R.solani* on PDA for 5 days at 28 ºC with the filtrate of i)*P.aeruginosa* (W.T) ii)cured cell iii) *R.solani* (control)



ii i

Figure (3-5): The difference in color between the filtrate of wild type (i) and cured cell (ii).

In order to determine the inability of the cured colonies to produce antifungal compounds because of plasmid elimination or not, plasmid from the cured cells were isolated and compared with that from the W.T. The results showed that there is no change in plasmid patterns between the cured cells and the wild type(figure (3-6)). This indicated that the small plasmid DNA bands in *P. aeruginosa* OM13 are not responsible for the antifungal compound production. It can be concluded that chloramphenicol resistance and antifungal compound production trait in *P.aeruginosa* OM13 may be located on the chromosome and this conclusion agreed with Georgakopouls *et al* .,(1994) results about the location of the genes responsible for antifungal compound production. Since the curing agent (SDS) effect on the bacterial cell wall (Trevors, 1986), the inability of cured cell to produce antifungal compound may be due to the physiological change in the permeability of the cellular membrane and this conclusion was supported by changing in the color of cured cells which refers to change in the bacterial cell wall had been occurred. May also be that the genes which were responsible for the antifungal compound production located on plasmid did not appeared in the gel (possibly due to its large size)

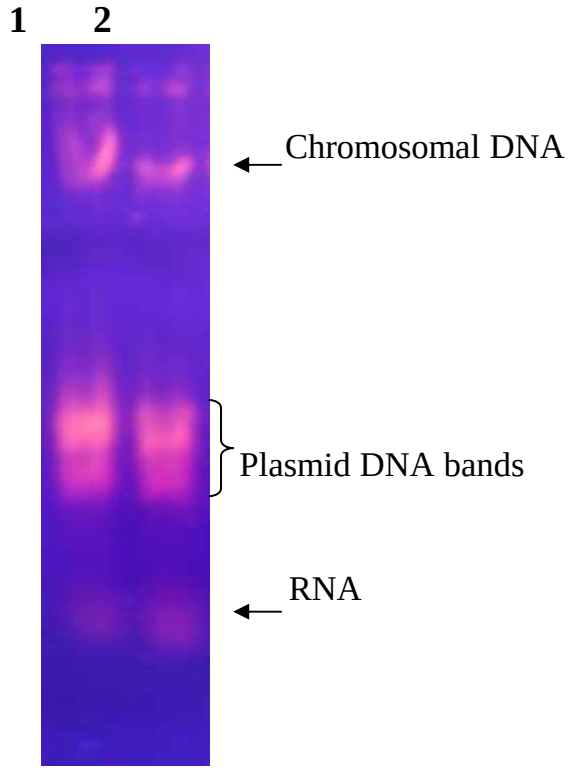


Figure (3-6): Gel electrophoresis of plasmids content of *P.aeruginosa* OM13 before and after treatment with SDS on agarose gel (0.7%) in TBE buffer at 5V/cm for 2 hrs.

1. **Plasmid content of the *P.aeruginosa* OM13**
2. **Plasmid content of the *P.aeruginosa* OM13 (cured)**

3.4.2-Transformation

The ability of antifungal compound production trait to transfer from one bacterium to another by transformation is another method to determine whether antifungal production genes are located on plasmid or not. For this reason transformation experiments were performed between DNA of *P.aeruginosa* OM13 (Cm^r, produce antifungal compound) as a donor and *E.coli* MM294 (Cm^s, does not produce antifungal compound) as a recipient.

Transformation mixtures were spread on a nutrient agar medium containing chloramphenicol in order to select transformant colonies. Results showed that all attempts to isolate transformed colonies which have the ability to resist chloramphenicol and inhibit fungal growth were not succeed.

Failure to select transformants *(E.coli* able to inhibit fungal growth) may be due to one of the following reasons:

- -Transformation between different strains of different species is not always possible.
- The DNA were taken up by *E.coli* but have been degraded by host nucleases enzymes.
- The DNA were taken up by *E.coli* but were unable to replicate or express their genetic marker (chloramphenicol) in the new host (*E.coli* MM294)

3.5- Mutagenesis of *P.aeruginosa*:

In an attempt to enhance antifungal compound production by *P. aeruginosa* OM13 the survival curve of *p.aeruginosa* OM 13 after treatment with MNNG at different time is shown in figure (3-6).

It was found that this bacterium was sensitive to MNNG. The survival curve showed increase of the lethal percentage exponentially with increasing the time of exposure to MNNG, and the highest lethal percentage or the less survival percentage was (0.1%) when the bacterium was treated with MNNG (30µg/ml) for 60 min. The treatment (15min) led to a survival percentage of 5.8% (94.2% killing). Therefore, it was selected for mutants isolation.

It is known that MNNG is an effective mutagenic compound. It can generate mispairing lesion by adding alkyl group (e.g methyl group) to various position on nucleic acids and hence missreplication of DNA, or missrepair of damaged DNA. Some of these lesions are potentially lethal as they can interfere with the unwinding of the DNA during replication and transcription (Freifelder, 1987). It also can induce mutation by an error prone DNA repair pathway (Stonesifer *et al*., 1985). Many studies dealing with the effect of MNNG on different bacteria (*Clostridium butyricum, Micrococcus* spp.) showed that these bacteria were sensitive to MNNG (Carrasco and Soto, 1987; Al-Bakri and Umran, 1994).

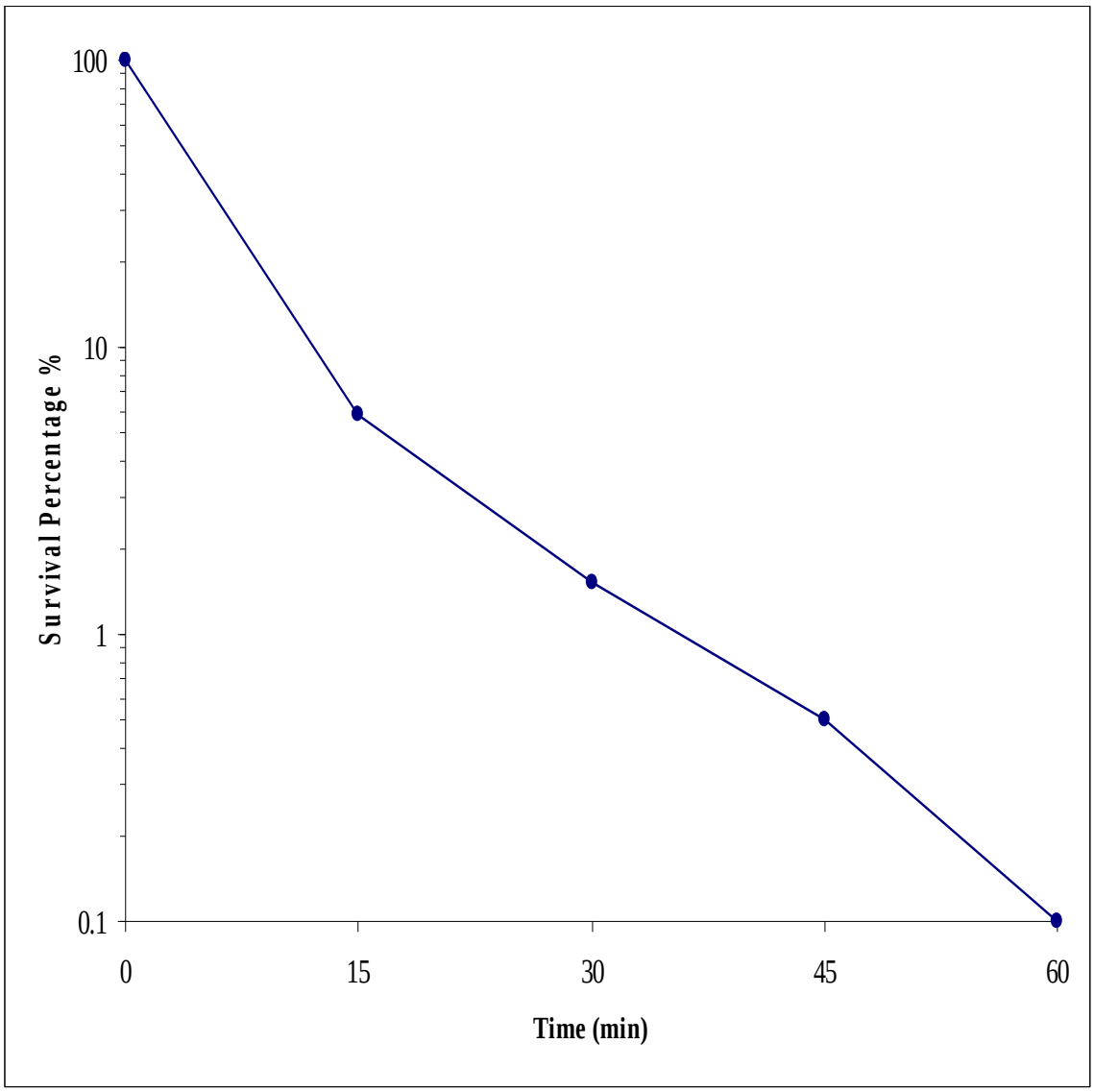


Figure (3-7) effect of MNNG (30 µg/ml) on a suspension of *P.aeruginosa* OM13 in phosphate buffer (pH= 7).

3.6-Bioassay the antifungal activity of the Mutants:

In order to know the effect of mutagenesis by MNNG to improve the production of antifungal compound by *P.aeruginosa* OM13, mutagenized culture from the selected treatments (bacteria treated with 30µg/ml of MNNG for 15 min) were replica plated on King A agar plate in an attempt to detect any difference in color which is used as an indicator for antifungal compound production.

Results showed that there is no difference in color of obtained colonies compared with W.T. For this reason, an alternative method for the detection of antifungal compound production was employed for screening of antifungal compound produced by *P.aeruginosa* OM13 mutants, in which (50) colonies named (RA1-RA50) were grown in peptone broth incubated at 37˚C for 18 hrs then cells was pelleted by centrifugation, then the supernatant was taken and the antifungal compound production was determined by measuring the optical density at 520 nm. In addition, agar well method was also used in which the filtrate of the mutants were cultured with the fungus *A. alternata* and the inhibition zone was measured after 5 days of incubation and compared with the inhibition zone caused by W.T.

Results in table (3-3) indicated that colonies obtained after treatment with MNNG varied in production of antifungal compound, in which(34%) of the mutants showed an increase in O.D. while (26%) showed decrease, at the same time other mutants(40%) remained the same as the wild type.

Results showed also the relationship between the O.D. and the inhibition zone, in which (5) colonies showed increasing more than four times. It was also seen that (RA5) gave a highest O.D measurement (nine times) and the highest inhibition zone.

Table (3-4) $O.D_{520nm}$ values and inhibition zone caused by *P.aeruginosa*OM13 mutants after treatment with MNNG (30µg/ml for 15min)

Bacterial isolates	**$O.D_{520nm}$**	**Inhibition zone (mm)**
OM13	0.1	5
RA*1	0.45	10
RA2	0.56	10
RA3	0.77	10
RA4	0.8	10
RA5	0.9	12
RA6	0.29	9
RA7	0.28	9
RA8	0.28	9
RA9	0.26	8
RA10	0.23	8
RA11	0.23	8
RA12	0.21	7
RA13	0.21	8
RA14	0.2	8
RA15	0.2	8
RA16	0.2	7
RA17	0.2	8
RA18	0.15	5
RA19	0.16	6
RA20	0.12	5
RA21	0.16	6
RA22	0.12	5
RA23	0.18	7

RA24	0.16	6
RA25	0.15	6
RA26	0.14	5
RA27	0.17	6
RA28	0.1	5
RA29	0.17	6
RA30	0.16	6
RA31	0.14	6
RA32	0.18	8
RA33	0.13	6
RA34	0.16	7
RA35	0.17	٧
RA36	0.16	8
RA37	0.18	8
RA38	0.06	4
RA39	0.018	2
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RA46	0.03	3
RA47	0.01	2
RA48	0.07	4
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RA50	0.08	5

OM13 : W.T , *: *P. aeruginosa* after treated with MNNG (30µg/ml)

Results indicated that MNNG caused random mutation in the genes responsible for antifungal compound production. The mutation may have occurred in the regulatory genes rhl I/R las I/R that located elsewhere in the genome which is controlling the phenazine production in *P.aeruginosa* due to decreasing in the antifungal compound production (Shannon *et al*., 2001).

Mavrodi *et al*., (2006) reported that *P.aeruginosa* have biosynthetic operons which are sufficient for production of single compound phenazine-1-carboxylic acid (PCA). And subsequent conversion of PCA to pyocyanin is mediated by two novel phenazine modifying genes, phzM and phz S, that encode putative phenazine –specific methyl transferase and flavin containing monooxygenase enzyme respectively. So the mutation may have occurred in this operon and led to decrease in the production of antifungal compound by these mutants.

The increase in the antifungal compound production from other mutants may be attributed to the mutation occurring in the (Gac S /Gac A) which controlls the secondary metabolite production at the posttranscriptional level involving the RNA binding proteins RsmA as a key regulatory element. RsmA is presumed to interact with specific ribosome binding sites present in the target genes and thereby prevent translation. Such translation repression can be elevated by the action of small regulatory RNA, Rsm Y and RsmZ whose expression is controlled by the Gac S /Gac A system in response to signal molecules produced by *Pseudomonas* at the end of exponential growth phase (Reimmann *et al* .,2005).

MNNG was used successfully to mutate and to get different mutants from different bacteria. In 1974 Carson and Jensen used MNNG in recognition of phenazine pigment (pyocyanin) mutants in *P.aeruginosa.* Al-Gelawi (1999) used MNNG to isolate salt sensitive mutants from *Micrococcus* spp. strain G1.

3.7- Characterization of the antifungal compound:

The following tests were performed in order to characterize the partially purified antifungal compound, and the results of these tests were as follows:-

- **TLC analysis of antifungal compound:**

When the partially purified antifungal compound was spotted on silica-gel TLC plate (10 x10 cm) and examined under UV transilluminator for characterization and purification, the result showed that the active antifungal compound has a single dark fluorescent spot under UV light and has an R_f value of (0.32) as illustrated in figure(3-8).

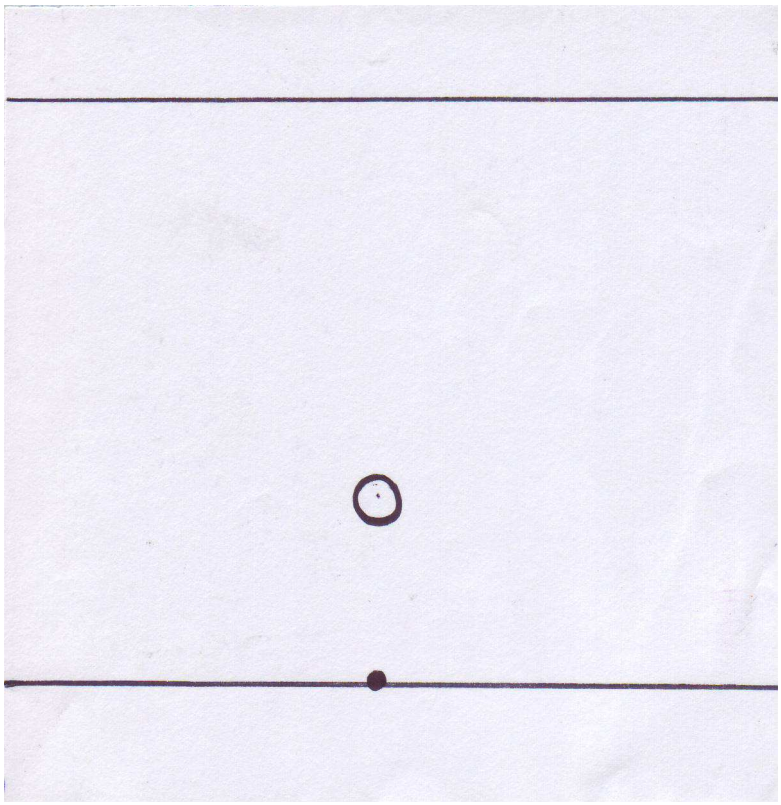

Figure (3-8) TLC analysis for the partially purified antifungal compound

- **UV absorption:**

The UV absorption spectrum figure (3-9) for the partially purified antifungal compound shows two peak at λ=319nm and 356 nm. These bands may be attributed to a π-π* and n-π* transition respectively.

A similar result was reported by authors working with pyocyanine produced by *P.aeruginosa* (Vukomanovic *et al*., 1997).

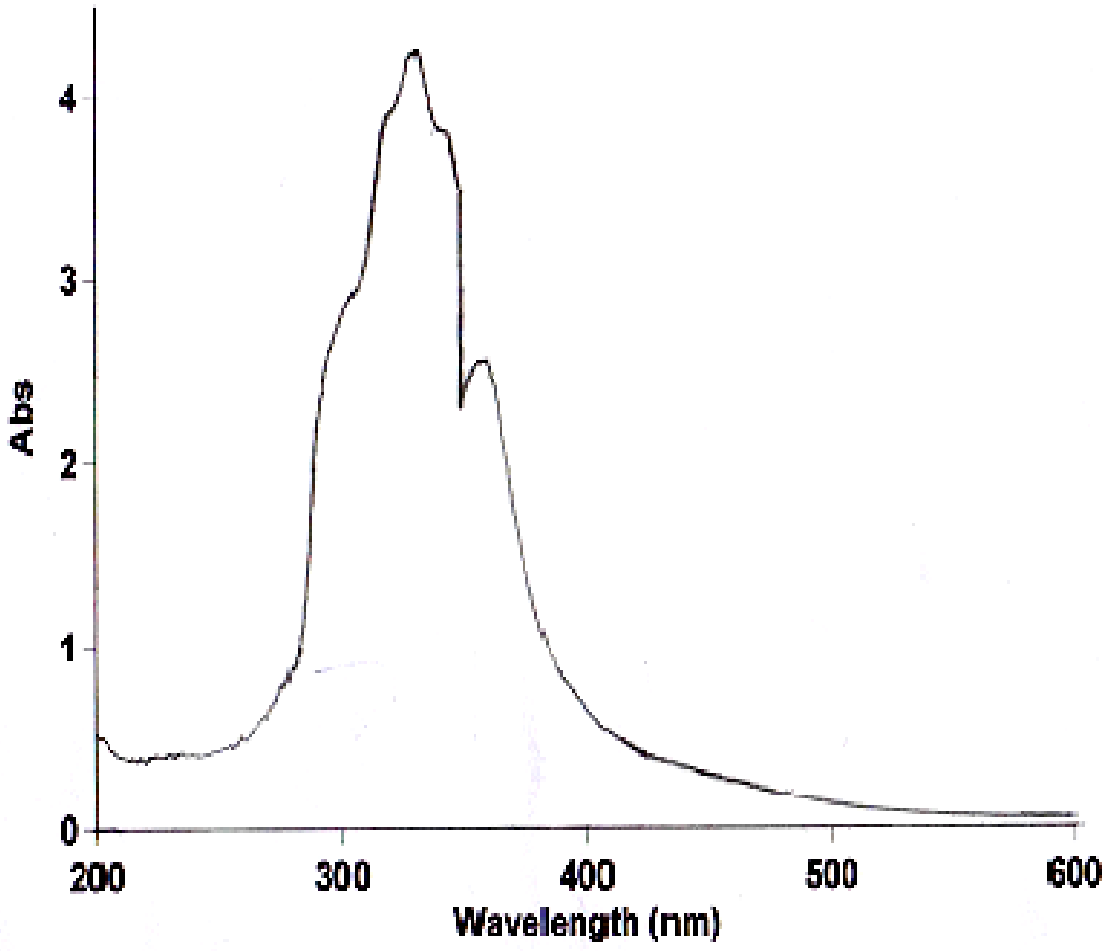

Figure (3-9) UV spectrum of the partially purified antifungal compound

- **IR analysis:-**

The partially purified compound was subjected to the IR analysis, figure (3-11), in order to know the functional chemical groups that are found in the compound that may lead us to propose a possible chemical structure of the compound. Depending on Socrates, (1980) for infra-red spectrum characterization of organic compounds and on specialist chemist at the Chemistry Department at Al-Mustansirya university. The IR results of the partially purified compound (the main important absorption bands) shows the presence of different bands corresponding to the following functional groups present in the molecular structure: stretching bands at 2950 cm^{-1} for ($C\text{-}H_{aliph.}$) due to methyl group, 3090 cm^{-1} for ($C\text{-}H_{arom}$), 1600 cm^{-1} for ($C{=}C_{arom.}$), 3200 cm^{-1} for (O-H),1650 $cm^{-1}$for (C=N), 1450 cm^{-1} for ($C\text{-}N_{cyclic}$), 1200 cm^{-1} for ($C\text{-}N_{arom}$), 1100 cm^{-1} for (C-O). This result was also supported by IR analysis of pyocyanin from Integrated Spectral Data Base System for Organic Compounds (SDBS) [intrenet 1].

As shown from the IR results and by depending on specialist chemists we can propose the possible chemical structure of the partially purified antifungal compound fig (3-10) as follows:-

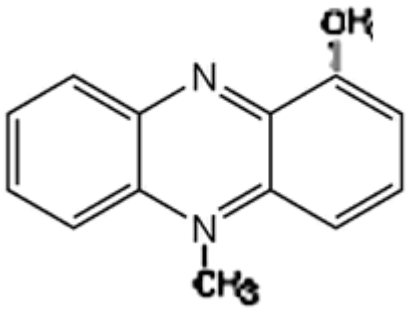

Figure (3-10) The proposed chemical structure of the partially purified antifungal compound from the IR results.

1.1-Introduction

Plant diseases, caused primarily by fungal and bacterial pathogens, cause severe losses to agricultural and horticultural crops every year. These losses result in reduced food supplies, poorer-quality agricultural products, economic hardships for growers and processes, and ultimately, higher price. Traditional chemical control methods are not always economical or effective for many diseases, and some chemical controls may have unwanted health, safety and environmental risks.

Biological control involves the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause. Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated with cultural and physical controls and limited chemical usage for an effective and integrated disease-management system. Biological control can be an important component in the development of more sustainable agricultural systems (Rovera *et al*., 2000).

Certain strains of root colonizing fluorescent *Pseudomonas* spp. particularly *Pseudomonas putida* and *Pseudomonas fluorescens* which are commonly isolated from the plant rhizosphere, have shown to protect plant from fungal infection. Two factors have been sited as essential for biocontrol: colonization of the rhizosphere and production of antibiotics (Jayaswal *et al*., 1990).

The fluorescent *Pseudomonas* produce a variety of biologically active natural products (Budzikiewicz, 1993), many of which have an ecological function in these gram-negative bacteria. Some of these natural products contribute to the suppression of plant-pathogenic fungi (Thomashow,

1996), where as other are important virulence factors of certain plant-pathogenic *Pseudomonas* species (Thompson *et al* ., 2003).

Disease suppression is mainly due to the antifungal metabolites phenazine, pyoluteorin, 2,4-diacetylphloroglucinol, pyrolnitrin and hydrogen cyanide (HCN) which are produced at the end of the exponential growth (Maurhofer *et al* ., 1998; Schnider-Keel *et al* ., 2000) .

Certain member of the genus *Pseudomonas* produce diverse low molecular weight secondary metabolites including nitrogen containing heterocyclic pigments known as phenazine compound (Whistler and Pierson .,2003) since phenazines are involved directly in successful disease control. Understanding the regulatory systems that contribute to phenazine production may result in more consistent disease control under various field conditions. Increase the production of such compound by using genetic method and understanding the environmental factors that regulate the biosynthesis of these antimicrobial compounds by disease-suppressive strains of *Pseudomonas* is an essential step towards improving the level and reliability of their biocontrol activity.

According to the above and because the very limited studies about the genetics of antifungal compound production by *Pseudomonas*, this study was conducted with the following aims : -

1. Determination the plasmid profile of *Pseudomonas aeruginosa* OM13.
2. Determination the role of plasmid of this bacterium in antifungal compound production by curing and transformation experiments.
3. Improving the ability of *Pseudomonas aeruginosa* OM13 to produce antifungal compound by mutagenesis.
4. Identification and characterization the antifungal compound by IR analysis and UV absorption spectrum.

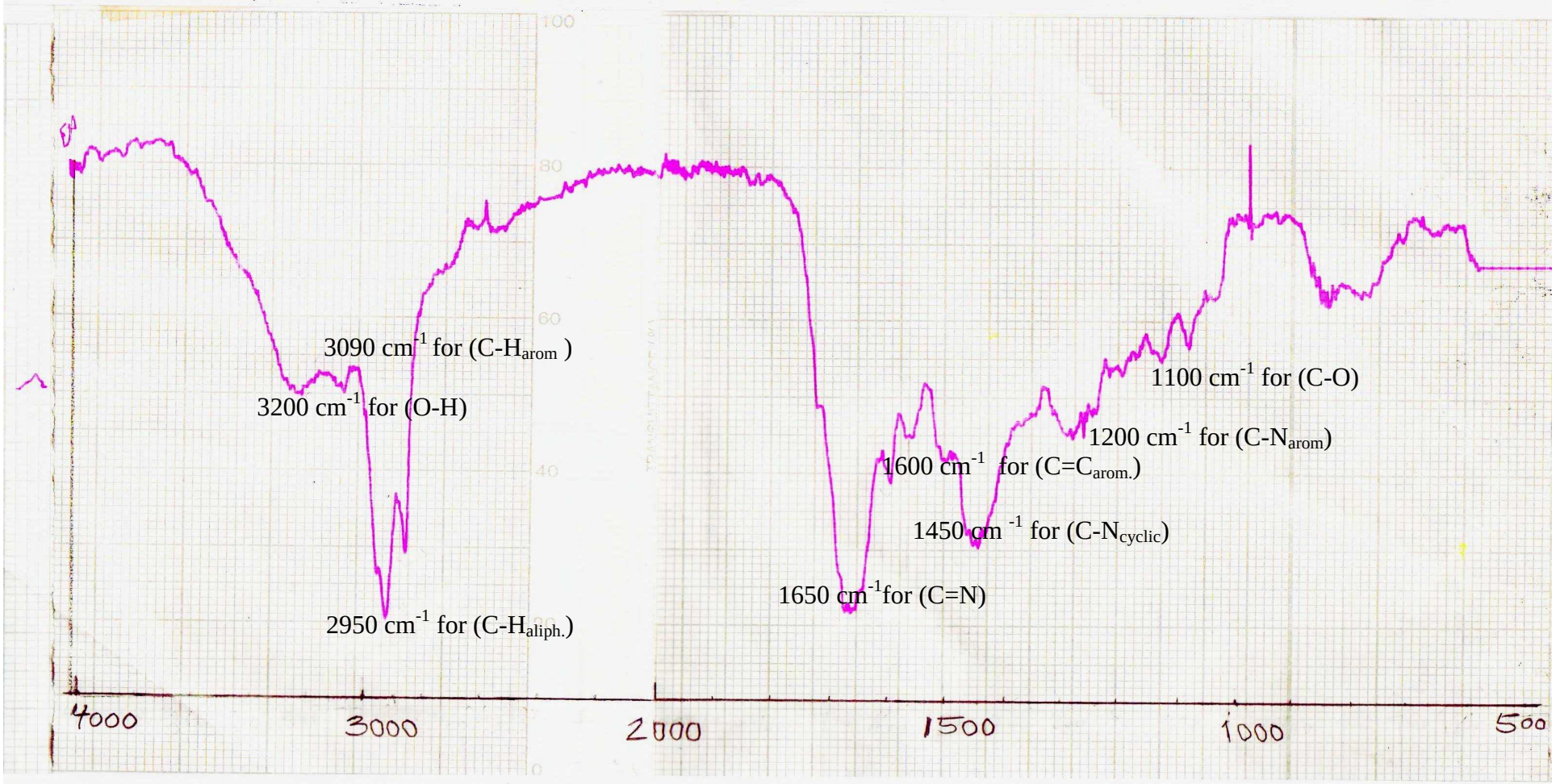

Figure (3-11) IR spectrum for the partially purified antifungal compound

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[MAIN]
Bandwidth=8
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Materials and methods

2.1- Chemicals:

The chemicals used in this study are the following: -

Materials	Company (Origin)
Glucose, Sucrose	Analar (U.K)
Glycerin, ethanol, NaOH	Merck (Germany)
Chloroform,Acetone,Urea,peptone	BDH (U.K)
NaCl, HCl, NaH_2PO_4, Na_2HPO_4	BDH (U.K)
Agar	Biolife (Italy)
Boric acid , Bromophenol blue	Riedel-Dettaen (Germany)
Ethidium bromide , Agarose	Sigma (USA)
Sodium dodecylsulfate (SDS), Tris(hydroxy methyl) amino methane base, Ethylenediamine-tetraacetic acid (EDTA), N-methyl-N-nitro-N-nitrosoguanidine (MNNG)	Fluka (Switzer-land)

2.2-Antibiotics:

The following antibiotic discs were used during this study:

Antibiotics	**Abbreviations**	**Concentration (µg/disc)**	**Source (origin)**
Ampicillin	Amp	10	Bioanalyse LTD
Tetracycline	TE	30	Bioanalyse LTD
Chloramphenicol	C	30	Bioanalyse LTD
Erythromycin	E	15	Bioanalyse LTD
Aztreonam	ATM	30	Bioanalyse LTD
Streptomycin	S	10	Bioanalyse LTD
Gentamycin	CN	10	Bioanalyse LTD
Vancomycin	V	30	Bioanalyse LTD
Norfloxacin	NOR	10	Bioanalyse LTD
Carbencillin	CRB	100	Bioanalyse LTD

2.3- Equipments and Apparatus:

The following equipments and apparatus were used during this study:

Equipment	Company (Origin)
Autoclave	Express (West-Germany)
Incubator	Termaks (U.K)
Distillator	GFL (Germany)
Oven	Gallen Kamp Sayo (U.K)
Millipore filter paper unit	Miliporecorp (USA)
pH-meter	Metler Toledo (U.K)
Centrifuge	Hermle Z_{200} A (Germany)
Shaker incubator	GFL (Germany)
Sensitive balance	Metler AE_{260} (Switzerland)
UV-transilluminator	Vilber Lourmat (France)
Silica gel thin layer chromatographic plate	Merek(Germany)
Cooling centrifuge	Harrier (U.K.)
Micropipette	Witey (Germany)
Water bath	GFL (Germany)
Minimal electrophoresis apparatus	Bio Rad (Italy)
Balance	Ohans (France)
Spectrophotometer	Aurora instruments Ltd.(England)
Eppendrof bench centrifuge	Netherler and Hinz (GMBH) (Germany)
Vortex	Buchi (Swissrain)

2.4-Microorganisms:

Microorganisms used in this study are listed bellow: -

Bacteria strain	Phenotype	Source
E.coli MM294	Rif ^{r}	Department of Biotechnology, Al-Nahrain University
*E.coli*HB101 harboring pBR322	Amp ^{r} ,Tc ^{r}	
P.aeruginosa OM13	Cm ^{r}	
Rhizoctonia solani	W.T	
Alternaria alternata	W.T	

2.5- Media:

2.5.1- Ready to use media:

These media were prepared according to the manufacturing companies and sterilized by autoclave at 121 C° for 15 min:

1-Nutrient broth (Biolife)

2-Nutrient agar (Fluka)

3-Trypticase soy broth (Biolife)

4- Trypticase soy agar (Biolife)

5-Potato dextrose agar (Oxoide)

2.5.2-Laboratory prepared media:

- Antifungal compound assay media (peptone broth):

It is composed of:

Peptone 1%

NaCl 1%

Glycerol 1%

Sterilized by autoclave at 121ºC for 15 min

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

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

pH was adjusted to 7.2 and sterilized by autoclaving at 121ºC for 15 min.

2.6- Buffers and Solutions

2.6.1- Plasmid extraction buffers and solutions:

-TE buffer (Maniatis *et al*., 1982):

EDTA 1mM

Tris- OH 10mM

pH was adjusted to 8.0 and sterilized by autoclave.

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

EDTA 25 mM

Tris- HCl 20 mM

NaCl 75 mM

pH was adjusted to 8.0 and sterilized by autoclave.

-NaCl solution (5M) in distilled water (Kieser, 1995).

-SDS solution (Kieser, 1995):

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

-Lysozyme solution (Kieser, 1995):

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

2.6.2-Plasmid isolation by miniprep kit solutions:

1. Solution No .1 (suspension buffer): Tris-HCl, EDTA pH:8
2. Solution No .2(lysis buffer): NaOH, SDS pH: 12
3. Solution No .3(potassium acetate): potassium acetate
4. Solution No .4(precipitation buffer): mineral salts
5. Solution No .5(binding buffer): guanidine and sorbet.
6. Solution No .6(elution buffer): Tris-HCl .

2.6.3- Electrophoresis buffer:

-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 1L	

-Ethidium bromide (Maniatis *et al*., 1982):

Prepared in concentration 10mg/ml in distilled water and store at 4 ºC.

-6X Gel loading buffer (**Maniatis *et al*., 1982):**

Bromophenol blue	0.25 % (w/v)
Sucrose in D.W	40 % (w/v)

2.6.4- Curing solution:

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

2.6.5- Transformation solutions (Sambrook and Russell., 2001):

a-$MgCl_2$ –$CaCl_2$ solution (80mM, 20mM)

b-$CaCl_2$ solution (0.1 M)

2.6.6- Antibiotics solutions (Sambrook and Russell ., 2001):

Ampicillin, chloramphenicol, aztreonam, erythromycin, were prepared as stock solution of 100mg/ml of antibiotic powders in distilled water, sterilized by filtration and stored at –20ºC.

2.6.7-Mutagenesis solutions

2.6.7.1-phosphate buffer (pH =7.0)

NaH_2PO_4	14.3g
Na_2HPO_4	1.14g
D.W	1L

2.6.7.2- N-methyl-N-nitro-N-nitrosoguanidine (MNNG) solution:

It was prepared as stock solution of MNNG (10mg/ml) in phosphate buffer (pH = 7.0).

2.7- Methods

2.7.1- Sterilization methods (Collins and Lyne, 1987):

a- Culture media (liquid and solid) and solutions were sterilized by autoclaving at 121ºC, 15 lb/in^2 for 15 minutes.

b- Glasswares (cylinders, petridishes, etc….) were sterilized in an electric oven at 180-200 ºC for 2 hours.

c- Thermolabile components or materials (such as sugars and amino acids) were sterilized by filtration through Millipore filter paper (0.2 μm).

2.7.2- Maintenance of bacterial strains:

Maintenance of bacterial strains were performed according to Maniatis *et al.*, (1982) and as follows:

1- Short – term storage:

Colonies of bacteria were maintained for periods of few weeks on the surface of nutrient agar media, the plates were tightly wrapped in parafilm and stored at 4ºC.

2- Medium – term storage:

Bacterial isolates were maintained by streaking on slant 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 37ºC for 24 hr, the slants with well growth were wrapped with parafilm and stored at 4ºC.

3- **Long – term storage:**

Bacteria can be stored for many years in nutrient broth medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria in a small screw-capped bottle with final volume 10 ml and stored at –20 ºC.

2.7.3- Maintenance of fungal isolates:

Fungal isolates were maintained for periods of few weeks on potato-dextrose agar (PDA) plates and maintained on slants of the same medium for few months at 4ºC.

2.7.4-Bioassay the antifungal activity of *P.aeruginosa* OM13 (Kobayashi *et al*., 2000 ; Gupta *et al*., 2001):

In order to examine the antagonistic properties of bacterial isolate against phytopathogenic fungi, a dual culture technique was carried out which is the simplest method to detect antifungal activity. In this technique an agar block (5 mm-diameters) of 5- day-old culture of fungal pathogen was placed in the center of plate containing potato dextrose agar (PDA).
A loopful of 24-hrs-old culture of the tested bacteria *Pseudomonas aeruginosa* OM13 was inoculated at 2 cm juxtaposed to the fungal, and some plates were left without inoculation of the bacteria which served as control. The plates were incubated at 28ºC ± 1 for 5 days and inhibition of fungal growth was measured.

The antifungal activity of bacterial filtrate (*P.aeruginosa* OM13) was also examined by agar-well method, Trypticase soy broth (TSB) inoculated with *P. aeruginosa* OM13 was incubated at 28±1 ºC for 5days.

Then centrifuged at 7000 rpm for 15 min. The supernatant was finally passed through a millipore filter (0.2 μm) to get cell-free culture

filtrate. One or two wells (5 mm diameter) were prepared with the help of arterial cork borer on (PDA) plates in one radius, 2 cm away from the center. An actively growing fungal mycelia disc (5mm) was placed in the center of the plate. The culture filtrate (crude extract, purified antifungal compound) (100 µl) was pipetted in each well. The plates were incubated at 28 ±1ºC and inhibition of the fungal growth was recorded after 5 days.

2.7.5- Antibiotic sensitivity test (Atlas *et al*., 1995):

The disc diffusion method was used to test the antibiotic sensitivity of the *P. aeruginosa* OM13, in order to find a genetic marker that could be useful in transformation experiment. A sterile cotton swab was dipped in to the inoculums (18 hr culture) and the entire surface at the nutrient agar plate was swabbed three times by rotating the plate approximately 60º between streaking to ensure even distribution. Then the discs of antibiotic were applied and the plates incubated at 37 ºC. The zone of inhibition was observed after incubation for 18 hrs. The antibiotic sensitivity of the bacterium was determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 1991).

2.7.6- Plasmid extraction:

Salting out method and plasmid isolation miniprep kit were used to isolate plasmid DNA as follows:

2.7.6.1-Salting out method (Kieser, 1995):

- Culture of bacteria grown in 20 ml of nutrient broth, was pelleted by centrifugation at 6000 rpm for 15 min.
- The pellet washed with 3 ml of SET buffer, and the cells were resusped with 1.6 ml of SET buffer, and then freshly prepared lysozyme (final concentration 1mg /ml) was added and incubated at 37ºC for 30 min.
- One ml of 10% SDS was added and mixed by inversion, then incubate at room temperature for 30 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 (6000 rpm at 4ºC) for 20 min.
- The aqueous phase 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 4ºC.
- The isopropanol was discarded and the precipitated DNA dissolved in 100 µl TE buffer and stored at –20 ºC.

2.7.6.2-Plasmid isolation kit:

The plasmid DNA was isolated by using a plasmid isolation miniprepe kit (US biological) as following:

- Two ml of bacterial suspension was pelletted by centrifugation (6000 rpm 4°C) for 1 min supernatant discarded.

- One hundred micro liter of solution NO.1 (2.6.2) was added and vortex vigorously (up to complete cell homogenization).
- One hundred micro liter of solution NO.2 (2.6.2) were added. The tube was inverted five times, incubated at room temperature (25°C for 3 min).
- One hundred micro liter of solution NO.3 (2.6.2) were added. Then mixed by inverting the tube five times, and incubated at room temperature for 3 min.
- Two hundred microliter of solution NO.4 (2.6.2) were added and centrifuged at 12000 rpm for 3 min.
- The aqueous phase (400µl) was transferred to a new 1.5 eppendroff tube, and (800µl) of solution No.5 (2.6.2) was added vortex 3-5 second and then incubated at room temperature for 3 min. Vortex 3-5 second and centrifuged it for 10 min in 6000 rpm.
- The supernatant was discarded.
- One ml of 50% ethanol was added. Vortexed for 5 –10 seconds, pelletted by centrifugation at 6000 rpm for 1 min.
- The supernatant was discarded. The last step was Repeated.
- The pellet was dried for 3 min in 45º C.
- The pellet was suspended in 30µl of solution No.6 (2.6.2) centrifuged for 1 min at 12000 rpm and then gently 25µl of supernatant was transferred to a new tube, which contain the plasmid DNA.

2.7.7- Agarose gel electrophoresis (Maniatis *et al.*, 1982) :

Agarose gels (0.7%) were run horizontally in Tris-borate-EDTA (TBE 1X). Sample 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 5 V/ 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 µg/ml for 30-45 min. DNA bands were visualized by U.V.Illumination at 302 nm on a U.V Transilluminator. Gels were destained in distilled water for 30-60 min. to get ride of background before photographs were taken.

2.7.8- Role of plasmid in antifungal compound production:

2.7.8.1-Curing of plasmid DNA

Curing experiments were performed on *P. aeruginosa* OM13 by using curing agent (SDS) and depending on Trevors (1986). Bacterium was grown in 5 ml of nutrient broth to mid log phase, then 0.05 ml inoculums of the culture were inoculated in a series of 5ml fresh nutrient broth tubes containing various concentration of SDS (0% , 1% , 2% , 3% , 4% , 5% , 6%, 7%, 8%, 9%, 10%) . All tubes were incubated at 37 ºC 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 bacterial isolate considered as the minimum inhibitory concentration (MIC).

Samples were taken from tube containing the highest concentration of SDS that still allowed bacterial growth, and diluted appropriately, then 0.1 ml from proper dilution were spread on nutrient agar plates and incubated overnight at 37 ºC to score the colonies that survived.

Survived colonies (100 colonies) were analyzed for the presence or absence of antibiotics resistance as a result of eliminating the plasmid. Those colonies were replica plated (using toothpick) on nutrient agar (master plates), and on nutrient agar containing antibiotic to which the original isolate is resistant.

If colonies were able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it means that the cells of this colony are cured cells that lost the resistance to this antibiotic. These colonies (cured) were tested for their ability to inhibit fungal growth.

2.7.8.2-Transformation (Sambrook and Russel ., 2001):

Attempts were done to transform *E.coli* MM294 (Rif^r)with DNA of *P.aeruginosa* OM13 as follow :-

- One hundred milliliter of nutrient broth were inoculated with a single colony of *E.coli* MM294. the culture was incubated for 3 hrs at 37ºC with vigorous agitation.
- The bacterial cells were transferred to sterile, disposable ice cold 50 ml falcon tube, cooled the culture to 0ºC by storing the tubes on ice for 10 minutes
- The cells were recovered by centrifugation at 6000 rpm for 15 min at 4ºC.
- The pellet was resuspended by gentle vortexing in 30 ml of ice cold [$MgCl_2$-$CaCl_2$ (80mM, 20 mM)].
- The cells were recovered by centrifugation at 6000 rpm for 10 min at 4ºC.
- The pellet was resuspended by gentle vortexing in 2 ml of ice-cold 0.1M $CaCl_2$ for each 50 ml of original culture.
- Two hundred micro liter of each suspension of competent cells were transferred to a sterile tube using a chilled micropipette tip, DNA was add to each tube (10µl). the tubes was Mixed by swirling gently, and stored on ice for 30 minutes.
- The tubes were transferred to a rack, placed in a preheated 42ºC circulating water bath, tubes stored in a rack for 90 seconds (the tubes should not be shaken).

- The tubes were transferred rapidly to an ice bath. the cells were allowed to chill for 1-2 minutes.
- Nutrient broth medium (800 µl) was added to each tube , the cultures incubated for 45 min. at 37 ºC to allow bacteria to recover and to express the marker encoded by the DNA .
- Then 0.1 ml samples of the transformation mixture were diluted properly and plated on to nutrient agar medium containing chloramphenicol.
- The plates were inverted and incubated at 37ºC. Transformed colonies (Cm^r) should appear and tested for fungal growth inhibition.
- Two controls were made. In the first, 10µl of DNA was added to 100µl of TE buffer, while in the second 10µl of TE buffer was added to 100µl of cell suspension of competent cells and spreaded on nutrient agar medium containing chloramphenicol and incubated as with the transformation mixture.

2.7.9- Mutagenesis:

Mutagenesis of *P.aeruginosa* OM13 were made in an attempt to improve the production of antifungal compound by this bacterium and as follow:

Cells were harvested from 10 ml of mid-exponential phase by centrifugation (6000 rpm, 10 min., 4ºC), washed and resuspended in 10 ml phosphate buffer.MNNG was added to the bacterial suspension to a final concentration of 30µg/ml. The mixture was incubated at 37 ºC with shaking. At various time intervals (0, 15, 30, 45, 60) min. two samples of each treatment were taken and as follow : 0.1 sample diluted properly and spread on nutrient agar for 24 hrs to determine the total viable count(survival percentage), and 0.2 ml sample added to20 ml nutrient broth

and incubated for 24 hr at 37 ºC for expression , then diluted properly and spread on nutrient agar .Plates were incubated for 24 hrs .according to the MNNG survival curve ,the treatment that led to a survival percentage of approximately 10% as compared with the control was suspected to have the higher mutation frequency .From this treatment a number of colonies (50) were selected and tested for their ability to inhibit fungal growth and compared with the original isolate.

2.7.10-Bioassay the antifungal activity of mutant cell:

2.7.10.1-Spectrophotometrical method:

The antifungal compound production by *P.aeruginosa* OM13 after being treated with mutagenic agent (MNNG) was determined as described by (Essar *et al*., (1990); Sarkisova *et al*.,(2005) as follows:

W.T and (50) mutants were incubated for 18 h at 37°C in peptone broth (1% peptone, 1% NaCl, 1% glycerol). Then cells were pelleted by centrifugation at 6000 rpm for 20min and the amount of antifungal compound was determined for the supernatant spectrophotometrically at 520 nm.

2.7.10.2-Inhibition zone method:

The antifungal activity of 50 mutants were determined by measuring the zone of inhibition as described in (2.7.4).

2.7.11-Isolation and purification of the antifungal compound (Kobayashi *et al*., 2000; Rovera *et al*., 2001):

Trypticase soy broth (TSB) medium was inoculated with a loopful of fresh (24-hour old) culture of *P. aeruginosa* OM13 and incubated at 28±1ºC for 5 days. It was then centrifuged at 7000 rpm for 15 min, and the supernatant was finally passed through a Millipore filter (0.2 µm porosity) to get cell free culture filtrate that contains the antifungal compound.

In order to extract the antifungal compound, the filtrate was treated with chloroform (20 ml for 20 ml filtrate) in separating funnels, the chloroform was added in two batches, and each batch contains 10 ml of chloroform. In each time of extraction the separating funnel was shake vigorously, after two phase were separated and clear , the organic phase (which contain the antifungal compound) was taken and chloroform was evaporated in an oven at 40ºC for 24 hrs . The precipitate was purified by using preparative TLC on a silica gel plate , in which thin layer chromatography was carried out with crude extract and acetone as a mobile phase and the relative mobility of each spot was determined considering relative flow (R_f).

The activity of purified antifungal compound was compared with that of the crude extract.

2.7.12-Characterization of the partially purified antifungal compound:

In an attempt to characterize the partially purified antifungal compound the following tests were performed:

- **Determination of the R_f value:**

Another type of characterization is to know the R_f value of the partially purified antifungal compound and these R_f values were measured at the same conditions that were used in this study [using silica gel thin layer chromatographic plate (TLC) and using chloroform as mobile phase].

- **Determination of UV absorbance:**

In order to identify the antifungal compound produced by *P. aeruginosa* OM13, the partially purified antifungal compound was subjected to UV absorption spectrum to show the absorption bands in the sample.

The UV absorbance was done at the Chemistry Department at the College of Science/ Al-Mustansirya University and this will give the absorbance of the functional chemical groups that are found in the compound in order to propose a possible chemical structure of that compound.

- **IR analysis:**

Depending on Socrates, (1980) for the identification of antifungal compound produced by *P. aeruginosa* OM13, the partially purified antifungal compound was subjected to the measurement of the infrared (IR) for the characterization of the compound.

The IR spectrum was done at the Chemistry Department at the College of Science/ Al-Mustansirya University and this spectrum will give the functional chemical groups that are found in the compound in order to propose a possible chemical structure of that compound.