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Determination of the Optimum Conditions for Prodigiosin Production by the Locally Isolated *Serratia marcescens*

A Thesis

Submitted to the College of Science Al-Nahrain University as a Partial
Fulfillment of the Requirements for the Degree of Master of Science in
Biotechnology.

By

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Summary

A total of 57 samples were collected from different environments (soil, water, and sewage samples) from different locations in Baghdad governorate. The total isolates obtained from these samples were 30 isolates, 15 of them were identified as *Serratia spp* according to cultural and morphological characteristics. Biochemical tests were carried out on these 15 isolates. Results showed that 5 of these isolates were identified as *Serratia marcescens*.

The ability of these isolates in prodigiosin production was examined. Results showed that all these isolates are prodigiosin producers, among them *S.marcescens* S11 was the efficient one in prodigiosin production, the prodigiosin activity in culture medium of this isolate was 200 U/cell.

Optimum conditions for prodigiosin production by *S. marcescens* S11 were studied. Results showed that the optimum conditions for prodigiosin production were achieved when the production medium supplemented with olive oil as a carbon source, and casein hydrolysate as a nitrogen source in a concentration of 1.5% for broth, KH2PO4 as a phosphate source, initial medium pH 8, and incubation at 28° C. Under these conditions, prodigiosin activity in culture medium was increased to 3000 U/cell.

S. marcescens S11 was subjected to mutagenesis to increase its ability in prodigiosin production. Random mutagenesis was achieved using physical mutagen by UV irradiation, and chemical mutagen using Mitomycin C. Results showed that subjection of *S. marcescens* S11 to UV irradiation and Mitomycin C caused to obtain several mutants characterized with its high ability in prodigiosin production.

Prodigiosin activity culture medium of the most efficient over-producer mutant (S11H7) raised after physical mutagenesis was 350 U/cell, while the prodigiosin activity in culture filtrate of the most efficient over-producer mutant (S11H54) raised after chemical mutagenesis was 400U/cell in comparison with 200 U/cell for the wild-type.

Supervisors certification

We, certify that this thesis" Determination of the Optimum Conditions for Prodigiosin Production by the Locally Isolated *Serratia marcescens"* was prepared by "Dr. Hameed Majeed and Dr. Abdul Kareem Jasim" under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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

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Chapter Two: Materials and Methods

Chapter Three: Results and Discussion

Conclusions and Recommendations

Chapter One

Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

Microbes are single cell organisms that are the oldest form of life on earth. Microbes are makers or destroyers. They can promote health or cause disease. Microbes inhabit almost every niche of the world, from 20 miles beneath the earth's surface to 20 miles overhead. Numerous microorganisms synthesize small-molecular-weight compounds that have no demonstrable function in the cells. Some microbes produce pigments as secondary metabolism of cellular growth and multiplication, many of these pigments are neglected for long time and recent studies shows many of these pigments can be life saving (Srijith, 2006).

Prodigiosin, a secondary metabolite (red pigment) produced by *Serratia marcescens, S.rubidaea, Vibriopsychreorythrous, Alteromonas rubra, Rugamonas rubra* and Gram positive actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus* (Rowan and Fisher, 1997*)*. Prodigiosin is of great interest due to its antifungal, antibacterial, antiprotozoal, antimalarial, immunosuppressive, and anticancer activities (D'Alessio *et al.*, 2000; Montaner *et al.*, 2000).

Serratia spp are gram negative bacteria, classified in the large family of *Enterobacteriaceae*. *Serratia* spp can be distinguished from other genera by its production of three special enzymes DNAase, lipase and gelatinase (Giri *et al*., 2004). *Serratia* appears to be ubiquitous genus in nature, and ten species are recognized, *Serratia* spp occur in water and soil, on plant, in insects and in man and animal (Singlton *et al*., 2001).

Serratia marcescens has a historical background that may be described as literally colorful because of their red pigment (Sekiguchi *et al*., 2004).

Because of the limited studies and importance of both *Serratia marcescens* and prodigiosin in genetic engineering, biological control and medicine, this study work was aimed to:

- Isolation and identification of *S.marcescens* from different environmental sources.
- Screening the ability of the local isolates in prodigiosin production and determine the efficient one in prodigiosin production.
- Determination the optimum conditions for prodigiosin production by the selected isolate.
- Enhance prodigiosin production by mutagenesis using physical mutagenesis by UV irradiation, and chemical mutagenesis by Mitomycin C.

1.2 Literature Review

1.2.1 *Serratia* genus

The genus *Serratia* comprises gram-negative rods, 0.5–0.8µm in diameter and 0.9–2.0 µm in length and is part of the family *Enterobacteriaceae* and at now the genus *Serratia* consists of 12 recognized species: *S. entomophila, S. ficaria, S.fonticola, S. grimesii, S. liquefaciens, S. marcescens, S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea,* and *S. ureilytica* (Houdt *et al.*, 2007).

Generally motile, by means of peritrichous flagella and are facultative anaerobic. Colonies are white, pink, red in color. Almost all strain grow at temperature between 10 and 36ºC, at pH 5–9. *Serratia* can be distinguished from other genera by its production of three special enzymes DNAase, Lipase and Gelatinase (Giri and Anandkumar, 2004).

The most common sites for *Serratia* infection include the urinary tract, respiratory tract, bloodstream, gastrointestinal tract and central nervous system (CNS). In adults, CNS infection mostly occurs following neurosurgery. *Serratia* is a virulent organism, when it enters the bloodstream, endotoxins are released and can cause fever, septic shock, thrombocytopenia and Disseminated intravascular Coagulation (DIC). The mortality from *Serratia* bacteraemia is high (Law, 2001).

Serratia genus responsible for 1.4% of nosocomial septicemia and can causes infection in several sites, wounds and the eye, where it may cause conjunctivitis, keratitis, endophthalmitis and tear duct infections. It's also a rare cause of pneumonia and meningitis (Khanafari *et al*., 2006).

1.2.2 *Serratia marcescens*

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Serratia*
Species: *S. marcescens*

Serratia marcescens comprises short rod with occasional longer rods, it form round, convex colonies with smooth, shiny surface on nutrient agar and blood agar plates. *S.marcescens* is motile by peritrichous flagella and are facultative anaerobic, chemoorganotrophic bacteria with both a respiratory and a fermentative type of metabolism. *Serratia* spp occur in air, water, bathtubs, soil, sewage, foodstuffs (starchy foods) on plant, in insects, in animals like (rabbits, horses, deer and water buffalo) and man, normally found in the human intestines (Singlton *et al*., 2001).

In 1819, Bartolomeo Bizio, a pharmacist from Padua, Italy, discovered and named *S.marcescens* when he identified the bacterium as the cause of a miraculous bloody discoloration in a cornmeal mush called polenta. Bizio named *Serratia* in honor of the Italian physicist named Serrati, who invented the steamboat and Bizio chose *marcescens* from the Latin word for decaying because the bloody coloration quickly disappeared.

Serratia marcescens exists in two cell forms and displays two kinds of motility depending on the type of growth surface encountered. In liquid medium, the bacteria are generally short rods with few flagella that exhibit swimming motility.

However, upon growth on solid surface (0.7-0.85% agar), they reportedly differentiate into elongated, hyper-flagellated cells that use a swarming motility. These morphological changes appear to be necessary to allow colony expansion, for swarming is a type of active surface motility that enables bacteria to move rapidly across a semi-solid surface in a coordinated manner (O'Rear *et al.*, 1992).

Serratia marcescens produces extracellular enzymes such as: nuclease, protease, haemolysin, lipase and chitinases. These factors are predicated to play a role in bacterial environmental adaptive capacity, in either pathogenic potential (Hejazi and Falkiner, 1997), and able to produce two types of pigments:

- Prodigiosin: a nondiffusible, water–insoluble pigment bound to the cell envelope, prodigiosin–producing colonies are totally red or show either a red center, a red margin or red sectors.
- Pyrimine: a water–soluble, diffusible pink pigment. Ferrous iron is required for the production of pyrimine. When the pyrimine is produced, the agar medium turns pink while the colonies are white to pinkish (Grimont and Grimont, 1984).

Cultures can produce two kind of odors, a fishy to urinary odor attributed to Trimethylamine (mixed with some NH_3), or a musty, potato–like odor resembling that of 2–methoxy–3–isopropyl–pyrazine. The musty odor is produced by *S.odorifera*, *S.ficaria* and a few strains of *S.rubidaea*. All other strains and species produce the fishy–urinary odor (Grimont and Grimont, 1984).

Several species can grow readily at 4–5ºC (*S. liquefaciens*, *S. odorifera*, *S. plymuthica* and *S. ficaria*) or at 40ºC (*S. marcescens* and several strains of *S. odorifera*, *S. rubidaea*) however, the temperature of 37ºC is not favorable for the isolation of *S. plymuthica* (Forbes *et al*., 2002).

Most *S. marcescens* are resistant to several antibiotics because of the presence of R-Factor, which a type of plasmid that carry one or more genes that encode resistance, all are consider intrinsically resistance to ampicillin and first – generation cephalosporins (such as cefalexin) (Carbonell *et al*., 2000).

1.2.3 Ecology of *Serratia marcescens*

The widespread occurrence of *S. marcescens* indicates a strong adaptive and survival potential and the ability to utilize wide range of nutrients. It is able to survive and grow under extreme conditions, including antiseptics and double-distilled water (Ajithkumar *et al.*, 2003; Church *et al.*, 2004; Horkajada *et al.*, 2006).

The bacterial cells readily colonize on surfaces and are enriched at the air-water interface, most probably because of the pronounced cell surface hydrophobicity of *S. marcescens*. In addition, *S. marcescens* is able to utilize surface-bound nutrients, such as long chain fatty acids (Ulrich, 1993) and it can be isolated from different plants such as: Eucalyptus, Pistachio, Bitter, Cherry, Acacia, Coconuts, Sorghum, Grass, and this bacteria was also isolated from vegetables such as: Mushrooms, tomatoes, leeks, green onions, lettuce, broccoli, artichokes, radish spinach, carrots, and figs (Burger and Bennett, 1985) and from different sources of foods like; fish, chicken and their products, milk and their products (Braun *et al*., 2001).

It can also grow in any moist location where phosphorous containing materials or fatty substances accumulate. Sources of these substances include soap residues in bathing areas and soap and food residues in pet water dishes. *Serratia* can also grow in tap water in locations such as toilets in guest bathrooms where the water is left standing long enough for the chlorine residual disinfectant to dissipate. *Serratia* will not survive in chlorinated drinking water (Ajithkumar *et al.*, 2003; Horkajada *et al.*, 2006).

Serratia marcescens is a well-recognized hospital acquired pathogen, outbreaks in environmental sources associated with cross-infection which included contaminated disinfectants, sinks, adhesive, tape, scalp vein needles, intravenous solutions, saline bottles, mechanical respirators, intravenous catheters, and ultrasonic nebulizers (Giles *et al*., 2006).

1.2.4 Quorum sensing in *Serratia*

Diverse environmental and cellular cues are involved and a number of different regulatory systems have evolved to permit rapid bacterial adaptation to fluctuating environmental conditions. These include "quorum sensing", a mechanism which enables bacteria to sense their cell population density and use this information to coordinately regulate gene expression. Quorum sensing allows bacterial populations to efficiently adapt to changes in the surrounding environment (Henke and Blassler, 2004; Houdt *et al*., 2007). Like many other bacterial species, *Serratia* strains produce diffusible, low molecular –mass signal molecules which accumulate in their surroundings as the population increases. Two classes of quorum-sensing systems have been described in *Serratia*, first, the N-acylhomoserine lactones (AHL)-dependent LuxIR type and second, the autoinducer-2 (AI-2)/LuxS type (Winzer *et al*., 2003; Wei and Lai, 2006).

AHL-dependent quorum sensing regulates diverse phenotypes in *S. marcescens* including the production of prodigiosin, carbapenem antibiotic, extracellular and cell associated enzymes and biofilm maturation (Harris *et al*., 2004; Rice *et al*., 2005). AHLs also regulate swarming motility in *Serratia* by controlling the production of biosurfactants. For both prodigiosin and carbapenem, environmental factors play important roles in modulating the quorum–sensing response.

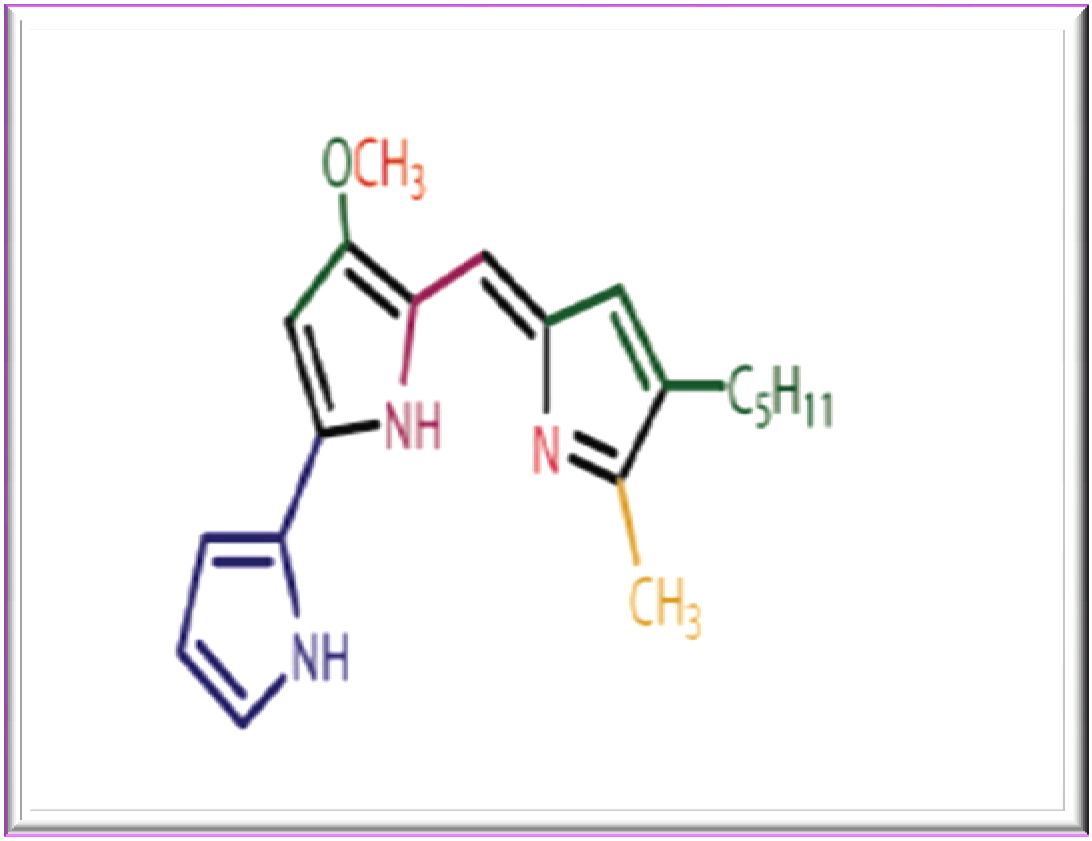
For example, phosphate availability regulates biosynthesis of prodigiosin and carbapenem in *Serratia spp*. ATCC 39006 *via* both quorum sensing – dependent and-independent pathways(Wei and Lai, 2006).

1.2.5 Prodigiosin

Prodigiosin is a red pigment insoluble in water, but soluble in chemical solvent like: alcohol, chloroform. Prodigiosin is a linear tripyrrole, a bifurcated pathway has been proposed for the biosynthesis of prodigiosin culminating in the enzymic condensation of the terminal products of the two pathways, the stable bipyrrole moiety 4-methoxy-2, 2-bipyrrole-5-carbaldehyde (MBC) and the volatile monopyrrole moiety 2-methyl-3-n-amyl-pyrrole(MAP) (Harris *et al*., 2004).

The prodigiosin group of natural products is a family of tripyrrole red antibiotic(2 – methyl – 3 – pentyl – 8 – methoxy prodigiosin) (Figure1-1).The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form prodigiosin. Prodigiosin have been shown to be associated in extracellular vesicles, cell associated or present in intracellular granules (Kobayashi and Ichikawa, 1991).

The molecular formula of the compound is ($C_{20}H_{25}N_3O$), prodigiosin possesses an apparent pk_a of 7.6 in aqueous dioxane, and 8.25 in aqueous ethanol and that the pigment is yellow in alkali and pink in acidic media. Interestingly, the proton affinity of the prodigiosin has now been shown to be governed by the presence of two geometrical isomers(α and β forms) that have very different apparent pk_a value, i.e. pk_α = 8.23, pk_β = 5.4 and have small molecular weight of 323.4 Dalton (Manderville, 2001).



Figure(1-1): The structure of prodigiosin (Srijith, 2006)

1.2.5.1 Prodigiosin production

Prodigiosin is typical secondary metabolite only appearing in the later stages (stationary phase) of bacterial growth (Williams *et al.*, 1971; Khanafari *et al.*,2006). The production of prodigiosin has been shown to be influenced by numerous environmental factors including: inorganic phosphate availability, media composition, incubation period, age of bacterial colony, temperature, pH, oxygen contents (Slater *et al.*, 2003) and this pigment are kept to red color in dark for long time because prodigiosin is sensitive to light (Allen, 1967; Hearn *et al.*, 1968).

Its produced by *Serratia marcescens, S.rubidaea, Pseudomonas magneslorubra, Vibrio psychreorythrous, Vibrio gazogenes, Alteromonas rubra*, *Rugamonas rubra* , and various marine bacteria, including *Hahella chejeunsis* KCTC 2396, and *Pseudoalteromonas denitrificans,* and Gram positive Actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus* (Rowan and Fisher, 1997*).*

Prodigiosin has no defined role in the physiology of producing strains. A recent study has suggested prodigiosin may be an important factor for the trypanolytic activity of a *S.marcescens* strain. Other suggested roles for prodigiosin have included a role in metabolic overflow from primary metabolism (Srijith *et al.*, 2006). Prodigiosin has potential clinical interest because it is reported to have anti-fungal, anti-bacterial, anti-protozoal/anti-malarial, immunosuppressive and anti-cancer activities (D'Alessio *et al.*, 2000; Montaner *et al.*, 2000).

Previous attempts to reconstitute prodigiosin production in *Escherichia coli* have been unsuccessful but in 1984, Dauenhauer isolated an *S.marcescens* genomic clone capable of condensing the two prodigiosin precursors, monopyrrole moiety 2-methyl-3-n-amyl-pyrrole (MAP) and bipyrrole moiety 4-methoxy-2,2-bipyrrole-5-carbaldehyde (MBC), to form

prodigiosin, so can able to demonstrate expression of prodigiosin in a heterologous host. The *pig*. gene cluster from *Serratia* was expressed in *Erwinia carotovora* subsp., though it was not expressed in several other members of the Enterobacteriaceae, including *E.coli* (Thomson *et al.*, 2000).

Many factors affects the biosynthesis of the pigment e.g. Fe(III), and sodium dodecyl sulphate have been shown to enhance the synthesis of prodigiosin (Geron *et al*., 1988), while many factors have been shown to inhibit prodigiosin synthesis e.g. temperature, glucose, ATP, ribose, inorganic phosphate, NaCl, KCI, polymyxin B, and streptomycin (Geron and Rokem, 1988).

1.2.5.2 Prodigiosin biosynthesis

The biosynthesis of prodigiosin in *Serratia sp.* is controlled by a complex regulatory network of both N-acyl-L-homoserine lactone quorum sensing dependent and independent pathways. The *Serratia* 39006 *pig*. cluster consists of 15 biosynthesis genes *pig*.(A-O) transcribed as a single polycistronic mRNA (Harris *et al*., 2004; Fineran *et al*., 2005).

The biosynthesis of prodigiosin can be done by two steps:

First: Also called the cytoplasmic step to produce; MAP, MBC.

- **MAP**(monopyrrole moiety 2-methyl-3-n-amyl-pyrrole): Three *pig*. gene can be share from total *pig*. genes to produce MAP: (*PiB*, *PigD*, *PigE*), the first steps in the MAP pathway are thought to be performed by enzymes of fatty acid biosynthesis to give 2–octenal. Alternatively 2–octenal may be derived by autoxidation of unsaturated fatty acids. *Pig*D also decarboxylates pyruvate but that the two–carbon fragment then adds to C-3of 2-octenal giving 3-acetyloctanal. Then transamination on the aldehyde group of 3-acetyloctanal and the resulting aminoketone by

PigB, would cyclize spontaneously to give the cyclic imine H_2MAP. Finally *PigB* is responsible for a 2-electron oxidation of H_2MAP to MAP (Harris *et al.*, 2004; Williamson *et al.*, 2005) as in figure (1-2).

- **MBC**(bipyrrolemmoietym4-methoxy-2,2-bipyrrole-5-carbaldehyde): The precursors to produce pyrrole were shown to be acetate, serine and proline. The first step to create this pyrrole by activate the L-proline by ATP and then transfer the L-proline group to sulfate group in *Pig G* to form prolyl-*Pig G*, then oxidation by *PigA* forming Prolyl-2-carboxyl-*Pig* G, then transfer of the pyrrole-2-carboxyl unit from *Pig* G to the active-site cystine of *PigJ*, following then transfer of a malonyl group from malony group from malonyl CoA to the phosphopantetheinyl side-chain of *PigH* and decarboxylative attack of the malonyl unit on the pyrrole-2-carboxyl thioester giving a pyrrolyl-β-Ketothioester attached to *PigH*, then condensation of serine from *PigH* with Pyrrole-2-carboxy thioester forming HBM (4-hydroxy-2,2bipyrrole5-methanol). The oxidation of the alcohol group by *PigM* forming HBC (4-hyroxy-2,2-bipyrrole-5-carbaldehyde).

The final step of the MBC pathway, methylation of the hydroxy group of HBC, involves the two enzymes *PigF* and *PigN* (Dairi *et al.*,2006; Williamson *et al.*, 2005) as in figure(1-2).

Second: the final biosynthetic step is the condensation of the terminal products of the two parallel pathways, MAP and MBC to form prodigiosin. This presented by *PigC* as the condensing enzyme (Ding and Williams , 1983; Williamson *et al*., 2006).

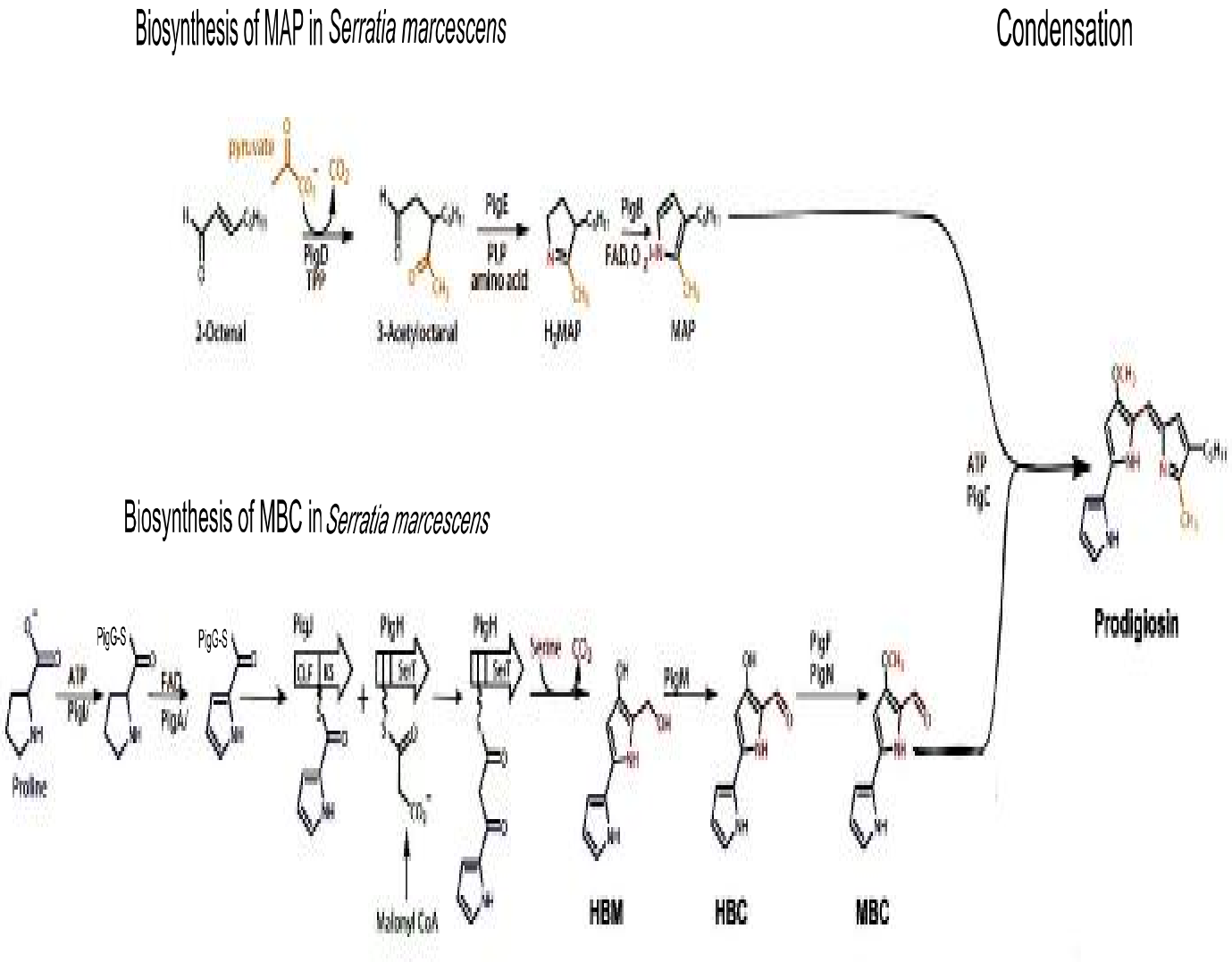


Figure (1–2) Bifurcated pathway for prodigiosin biosynthesis (Williamson *et al.*, 2006).

1.2.6 Prodigiosin gene expression

Assays of bacterial gene expression make attractive teaching tools for several reasons**. First**, bacteria modulate their gene expression quickly in response to environmental cues such as cell density, growth temperature and growth medium. Bacterial messenger RNA molecules are typically degraded with half-lives measured in minutes versus the hours of stability for eukaryotic transcripts. **Second,** many bacteria express pigments under certain conditions. Because most pigments absorb light at some defined wavelength, pigment expression may be easily monitored spectrophotometrically. **Third,** and perhaps most importantly, bacteria are easy to propagate in the teaching laboratory (Khanafari and Assadi, 2006).

Prodigiosin is expressed as a secondary metabolite in the general method of gene expression called quorum sensing. Growth in liquid culture at low cell density allows low–level expression of a membrane permeable positive regulator of gene expression. The intracellular concentration of the regulator remains low at low cell density due to its diffusion across the cell membrane after synthesis. However, as cell density increases in a closed system, the intracellular concentration of regulator increases to a threshold needed for activation of prodigiosin expression. Thus, high levels of prodigiosin are expressed in liquid culture only at high cell density. A similar phenomenon operates with colonies grown from single cells on agar plates (Haddix and Werner, 2000).

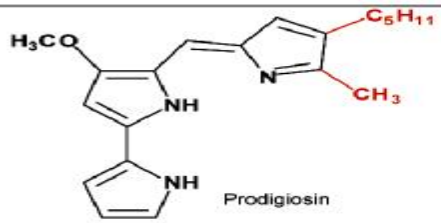
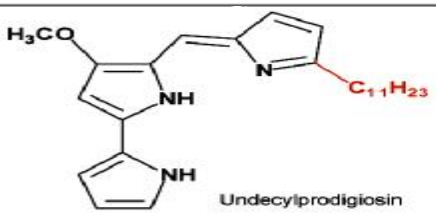
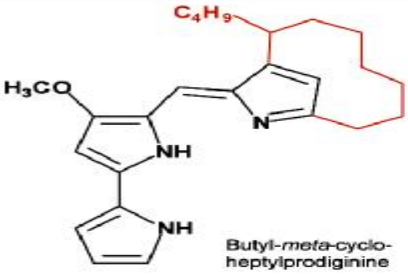
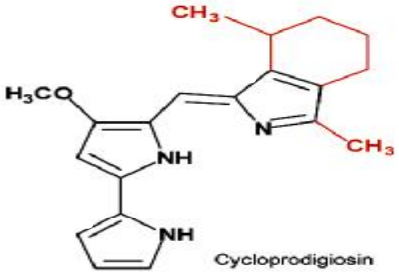
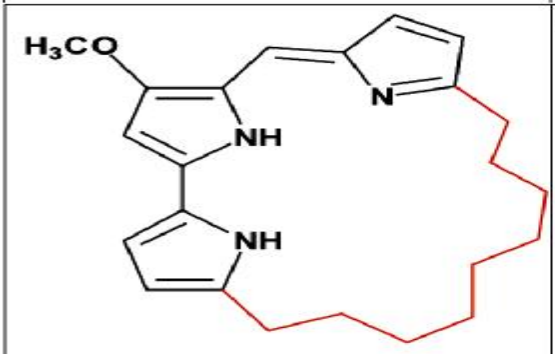
1.2.7 Naturally occurring prodigiosins and related compounds

The naturally occurring prodigiosins may be divided into **acyclic** and **cyclic** forms. All the naturally occurring prodigiosins contain the 4-methoxy-2, 2-bipurrole ring system; variation in structure is found in the C-pyrrole ring. Prodigiosin itself is acyclic and contains a 2-methyl-3pentyl-pyrrole as the C-ring.

The other acyclic analogue, Undecylprodigiosn, is often referred to as prodigiosin 25-C, as it was the third C_{25} pigment of the prodigiosin series found in nature. The more interesting analogues are the cyclic forms that include Cycloprodigiosin, Cyclonylprodigiosin, Butyl-metacycloprodigiosin (Manderville, 2001) prodigionine structures shown as in figure (1-3).

The macrocyclic nonylprodigiosin analogues is particularly interesting as its alkyl chain spans all three pyrrole rings. Related natural products include the Tambjamine class alkaloids that have been isolated from marine organisms which include Bryozoans(Blackman, 1994), Nudibranches and Ascidiants (Manderville, 2001).

Table (1-1): Prodiginine groups and sources for this compounds (Williamson *et al.*,2006).

Prodiginine	Group	Source
 Prodigiosin	1	*Serratia S. marcescens, Serratia plymuthica, Pseudomonas magnesiorubra, Vibrio psychroerythreus*
 Undecylprodigiosin	1	*Steptomyces longisporus ruber, Streptoverticillium rubrireticuli, Actinomadura madurae, Str. coelicolor* A3 (2), *Saccharopolyspora* sp. nov.,
 Butyl-*meta*-cyclo-heptylprodiginine	2	*Saccharopolyspora* sp. nov., *Str. coelicolor* A3 (2)
 Cycloprodigiosin	3	*Vibrio gazogenes, Alteromonas ruber, P. denitrificans*
 Cyclononylprodigiosin	4	*Actinomadura pelletieri, Actinomadura madurae*

1.2.8 Applications of prodigiosin in Biotechnology

Prodigiosin exhibits antibacterial because this pigment showed high inhibition activity against gram positive bacteria e.g. *Staphylococcus aureus*, *Streptococcus pyogenes*, while showed little inhibition activity against gram negative bacteria e.g. *Klebsiella pneumonia, Proteus vulgaris* (Nakashima *et al.*, 2005b).

This pigment, showed potent algicidal activity against various red tide phytoplankton's *H. akashiwo, H. circularisquama, C. polykrikoides, G. impudicum* and *A. tamarense*, in a concentration-dependent manner (Jeong *et al.*, 2005; Nakashima *et al*., 2006; Kim *et al*., 2007).

One such antagonistic bacterium, *Serratia marcescens* which produce prodigiosin, has been isolated from the phylloplane of tomato plants (Someya *et al*., 2000). This pigment effectively inhibits the *in vitro* growth of several phytopathogenic fungi and suppresses cyclamen gray mold caused by *Botrytis cinerea*, cyclamen soil-borne diseases caused by *Rhizoctonia solani AG-4* and *fusarium oxysporum,* rice sheath blight caused by *Rhizoctonia solani AG*-1 AI, and rice blast caused by *Pyricularia oryzae* in greenhouses (Iyozumi *et al.*, 1996; Someya *et al*., 2000, 2001). *S.marcescens* is particularly effective in inhibiting both hyphal growth and sclerotium germination of *R.solani* (Someya *et al.*, 2005; Someya *et al.*, 2007).

Chagas' disease is a protozoan infection caused by *Trypanosoma cruzi*. This disease is one of the most important public health problems in many South American countries, affecting 16–18 million people in Latin America.

Although there is intense research on drugs for the treatment of infection by *T. cruzi* , only one drug, benznidazole, has been recommended for the treatment of acute and congenital cases (Melo *et al*., 2000).

However, benznidazole has severe limitations related to its efficacy and toxicity as well as the development of parasite resistance. Studies with bacteria and mammalian cells have demonstrated the genotoxic potential of nifurtimox and benznidazole and rabbits treated with both drugs displayed a high incidence of malignant lymphomas. For this reason, the development of safer and more effective drugs, particularly against the chronic form of Chagas' disease, is an urgent priority (Isaka *et al*., 2002).

The search for new trypanocides is currently being done through the development of in vitro screening assays. Recent studies of antifungal agents with trypanocidal activities have shown that prodigiosin, an antimycotic drug, can kill these parasites(Azambuja *et al*., 2004). Prodigiosin had a potent trypanocidal activity against the trypomastigote forms of *T. cruzi* Y strain (IC_{50}=5 μM) compared with Nifurtimox which had an IC_{50} of 150 μM and higher trypanocidal activity compared with benznidazole (IC_{50}=19 μM).

Prodigiosin promote H+/Cl- symport and induce neutralization of the acidic compartment of cells which in turn results in the acidification of the cytoplasm and thus cell cycle arrest and eventually apoptosis (Yamamoto *et al*., 2000). It triggers apoptosis in cancer cell lines, with no marked toxicity in nonmalignant cell lines (Montaner and PerezTomas, 2001). Prodigiosin exhibits selective activity against breast, colon, hematopoietic cancer, and liver cancer cells (Soto–cerrato *et al*., 2004).

1.2.9 Factors affecting the productivity of the prodigiosin pigment

There are many conditions, which affect the productivity of prodigiosin pigment, which include pH, temperature, carbon source, nitrogen source, and phosphate source.

1.2.9.1 Effect of pH in prodigiosin productivity

Pigment productivity and continuing of metabolic pathway is highly affected by pH of the medium.

Optimum growth of all strains of *Serratia* has been observed at pH 7 and growth of all strains of *Serratia* is inhibited at a pH of < 4.5, while optimum pH for prodigiosin production is between 8.0-8.5 (Sole *et al.*, 1997).

1.2.9.2 Effect of temperature on prodigiosin productivity

Temperature is considered as one of the most important factors affecting pigment productivity and the growth of the microorganisms. Variable growth has been observed at 5˚C and 40˚C. Optimum growth of all strains of *Serratia* has been observed at temperatures from 20-37˚C. Growth of all strains of *Serratia* is inhibited at > 45˚C and prodigiosin was produced at temperature ranging from 12-37˚C, while the optimum temperature of prodigiosin production was at 30˚C (Holt *et al.*, 1994).

1.2.9.3 Effect of nutritional factors on prodigiosin production

1.2.9.3.1 Effect of carbon source

Microorganisms differ in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources e.g. (glucose, sucrose and fructose) is expensive from the economical case, so the industrial

fermentation try to use cheap carbon sources especially industrial and a variety of plant seed oils have also been used as carbon substances for prodigiosin production and displayed stimulatory effects on the production by *S.marcescens*.

The optimum carbon source is olive oil while prodigiosin production was inhibited by glucose due to catabolic repression (Giri *et al.*, 2004).

1.2.9.3.2 Effect of nitrogen source

Nitrogen Source is one of the most important part of the components of the culture medium for the producing microorganism such as inorganic nitrogen salts or organic sources like amino acids and proteins and these nitrogen sources can be used either as oxidized form ($NO\text{-}_3$, $NO\text{-}_2$) or as reduced form (NH_2, NH^+_4). Amino acids and ammonium salts considered as a good nitrogen sources, which can be used easily by the microorganisms.

It was observed that casein hydrolysate 1.5% is the best nitrogen source for the production of prodigiosin by *S.marcescens* while peptone is the best nitrogen source for bacteria cell growth (Kim *et al.*, 1999).

1.2.9.3.3 Effect of phosphate source

Mineral salts have an effect on the production of prodigiosin and there is several studies demonstrated that synthesis of prodigiosin by non-proliferating cells of *S.marcescens* is depended to presence of inorganic phosphate (Pi) concentrations. A high elevation of pigment formation was obtained at less than or equal to 0.3 mM and a broader but much lower elevation was obtained at 10 to 250 mM Pi (Witney *et al.*, 1977; Bennett and Bentley, 2000).

1.2.10 Extraction and Purification of prodigiosin

In order to make any study about the pigments to understand its characteristics and its role in any reaction, it is very important for the prodigiosin to be partially purified to get exact results during the study.

Pigments purification process means separation of the prodigiosin from another products and materials presents in the crude filtrate, prodigiosin is sometimes bound to proteins, thus, extracts may require acid treatment before isolation of the pigment. Higher homologs of prodigiosin have been detected by mass spectroscopy (Gerber, 1975).

The pigment was extracted from the bacterial cells by the method of Williams's *et al,*. (1965). To obtain the dry pigment, the petroleum ether extract was evaporated under vacuum at room temperature in the dark. Thin–layer chromatography (TLC) of the extracted pigments on silica gel, with chloroform: ethyl acetate: acetic acid (8:1:1 vol/vol). Sample of purified pigment obtained by preparative TLC were analyzed for infrared absorbance with a Perkin Elmer model spectrophotometer (Roberts *et al.*, 2007). Also this pigment can be purified by high performance liquid chromatography (HPLC) (Nakashima *et al*., 2005b), was performed as preliminary analysis for distinguishing closely related analogs of prodigiosin.

1.2.11 Mutagenesis

Mutagenesis, the creation or the formation of a mutation can be used as a powerful genetic tool. By inducing mutation specific ways and then observing the phenotype of the organism, the function of the genes and even individual nucleotides can be determined. Mutagenesis was used to ability to overproduce a desired metabolite. So now the study of mutagenizing microorganisms is important, interesting and potentially profitable.

The mutation of a gene or genes under study can be achieved by first altering the DNA of the microorganism in some fashion and then screening or selecting for the desired phenotype (Maki, 2002).

Three general treatments can be used to mutagenize microorganism: physical radiation, chemical mutagens and transposons. Mutation by radiation involves exposing the microbe to high energy waves (UV light, laser or X-ray). This procedure damages the target DNA and sometimes, during repair, an improper base pair (or pairs) is incorporated in the DNA, causing a mutation. Chemical mutagens are also employed. These compounds are added to a growing culture of an organism for a given time period and interfere with the replication of the DNA (Paustian and Kurtz, 1994).

Some mutagens achieve this by serving as base analogs, others chemically modify the DNA, and yet another class can insert or intercalate between the base pairs of DNA causing DNA polymerase to make mistakes. In all cases, the mutagen causes incorrect copying of the DNA resulting in base substitutions (exchange of one base pair for another), insertions (additions of one base pairs), or deletions (removal of one or more base pairs) (Paustian and Kurtz, 1994).

The third method of mutagenesis involves the use of mobile genetic elements. The most commonly employed of these are a sub-class called transposons. Transposons are relatively short pieces of DNA that replicate by inserting into other pieces of DNA (plasmid, chromosomes, and viruses). They encode two sets of functions. One set is involved in regulating and performing the movement of the transposons from one place of "host" DNA to the next (transposition functions). The other set of functions encode genes that may provide an advantage for the host of example (Paustian and Kurtz, 1994).

1.2.11.1 Physico – chemical mutagens

Physical mutagens are different types of radiations having mutagenic properties such as UV light and ionizing radiation. The energy content of a radiations depends upon its wavelength i.e.: shorter the wavelength, the greater the energy value of radiation. While the ionizing radiation which is one of the physical mutagens, has the greater penetration power than non ionizing radiation. Ionizing radiation causes single strand breaks in DNA and produces deletion. Ultraviolet rays are the only non–ionizing rays with mutagenic properties (Settey and Sreekrishna, 2004).

1.2.11.1.1 Mutagenic Properties of Ultraviolet Radiation

UV light is the portion of the spectrum with wavelength of 100-400 nm, which is just shorter than visible light (Miller *et al.*, 1999).

UV radiation (UVR) is lethal and potentially mutagenic to all organisms greatly dependant on the source of radiation and the time exposure. UV can be classified into UV-A (320-400nm), UV-B (290-320nm), and UV-C(< 290). Photons of UVB and UVC wavelengths cause direct DNA damage by inducing the formation of DNA photoproducts such as cyclobutyl pyrimidine dimmers(CPD) and the pyrimidine (6-4) pyrimidinione. The accumulation of DNA photoproducts can be lethal to cells through the blockage of DNA replication and RNA transcription.

The UVA typically cause only indirect damage to cellular DNA through catalyzing the formation of chemical intermediates such as reactive oxygen species. Distinct differences between far-UVC and near-UV (UVB and UVA) damage have been observed in bacteria and bacteriophage, UVC has the most potential for directly damage DNA (Miller *et al.*, 1999; Kim and Sundin, 2001; Qiu *et al.*, 2004).

UV light is widely used as mutagen that generates a broad spectrum of lesions in DNA. The most important mutagenic effect of UV irradiation is believed to be stimulation of misrepair (Goodenough, 1984).

1.2.11.2 Chemical Mutagens

A chemical mutagen is a substrate that can alter a base that is already incorporated in DNA by change its hydrogen-bonding specifically (Freifelder, 1987). Among the most widely used mutagenic reagent with microorganisms are the alkalating agents, ethyl methane sulfonate (EMS) and Mitomycin C (MMC) also used as chemical mutagen which causing cross-link formation in cellular DNA by deletion (Szybalski and Iyer, 1964).

1.2.11.2.1 Mitomycin C

Mitomycin C was identified in 1956 as an antibiotic produced by S*treptomyces lavendulae* and subsequently established as an important antitumor agent. Mitomycin C functions as a pro drugs and requires enzymatic and chemical reduction to become a highly reactive alkylating agent(Tomasz *et al.*,1988; Henderson, 1993). It has been reported that the antibiotic mitomycin C has a specific effect on cellular DNA, but has little or no effect on either RNA and protein formation (Suzuki and Kilgore, 1966). The ability of Mitomycin C to inhibit bacterial cell growth involves the combined action of DNA alkylation and the formation of reactive oxygen species (Sheldon *et al.*, 1999). The primary action of Mitomycin C is believed to be associated with either the inhibition of DNA biosynthesis or the breakdown of the nuclear apparatus.

The molecular formula of the compound is ($C_{15}H_{18}N_4O_5$), Mitomycin C seem to form covalent bonds with DNA, Mitomycin C can cross-link the complementary strands of DNA *in vivo* and under appropriate conditions, *in vitro* as well. It was proposed that the bacteriocidal effect of Mitomycin C is the indirected consequence of the cross-links (Mercado and Tomasz, 1972) which cause the deletion mutation type. Mitomycin C structure shown in figure (1-4).

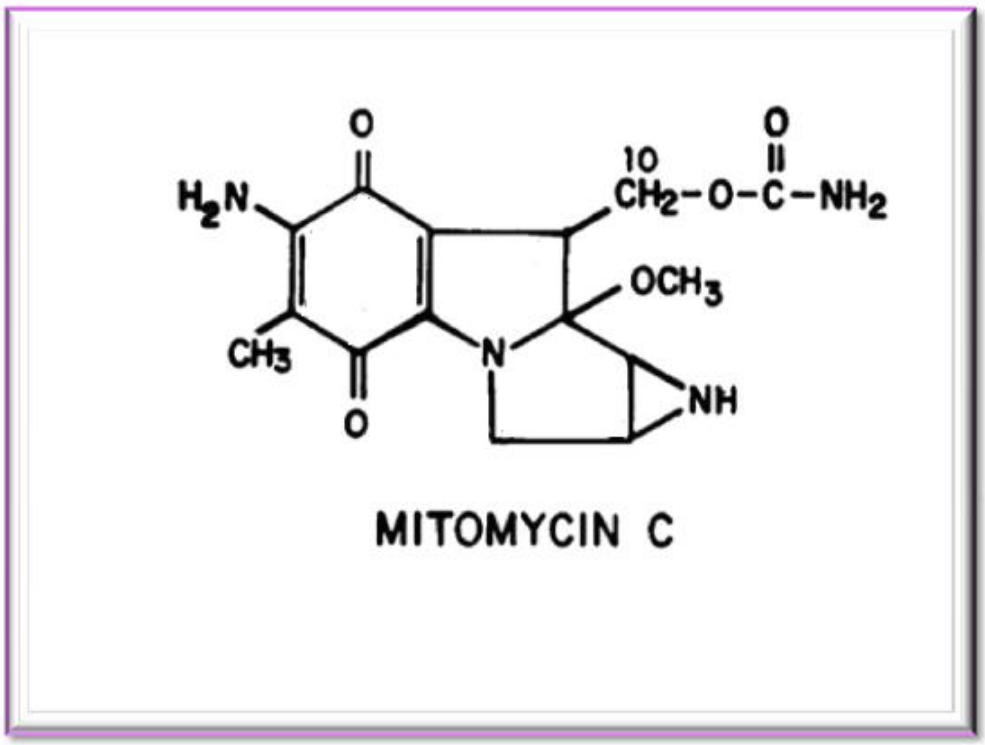


Figure (1-3): Mitomycin C structure (Mercado and Tomasz, 1972).

Chapter Two

Materials And Methods

3. Results and Discussion

3.1 Isolation of *Serratia* species

In order to isolate *S.marcescens*, fifty seven samples were collected from different environments in Baghdad governorate during the period from 2/2008 to 4/2008. Results mentioned in table (3-1) showed that from these samples, 30 isolates were obtained from water, sewage, and soil samples.

Table (3-1): Local isolates from different environmental samples.

Source of samples	No. of Samples	No. of Isolates	Presence of red pigmented colonies
Water	11	4	4
sewage	26	16	7
Soil	20	10	4
Total	57	30	15

Among the total isolates, only 15 isolates were able to produce red pigment, which give an indicator that these isolates are belong to *Serratia spp*. These 15 isolates were further characterized and identified according to their cultural, morphological characteristics, and biochemical tests.

From the results mentioned in table (3-1) it can be concluded that there are other 15 isolates from environmental samples may belong to other pathogenic or nonpathogenic bacteria from different genera.

3.2 Identification of bacterial isolates

Local isolates that were able to produce red pigment, were further identified according to their cultural and morphological characteristics and biochemical tests. For the former, colonies of each isolate that were plated on nutrient agar medium showed different morphological characteristics of *S.marcescens* such as red round, convex colonies with smooth, shiny surface and has fishy–urinary odor. Microscopical examination of each isolate showed that they are all having single cells, non-spore forming, gram negative and rod shape.

Some biochemical tests were done to ensure that these 15 isolates are *S.marcescens*. Results listed in table (3-2) showed that only five of these isolates are belong to *S.marcescens* symbod S3, S5, S7, S11, and S13. These isolates are able to grow at 40ºC, positive for catalase and oxidase production, and positive for methyl red and voges-proskaure tests, and they are motile and positive for DNase production. Results also showed that these isolates are able to utilize sucrose as a sole source for carbon and energy, while they are unable to produce acid from the fermentation of lactose, xylose, raffinose, and arabinose. These results are confirmed that these five isolates are belong to *S. marcescens* and as it was described by Atlas *et al*., (1995). From the other results mentioned in table (3-2), it can be concluded that there are other 10 isolates may belong to other different genera.

Table (3-2): Biochemical tests of the locally isolated *Serratia* spp.

Isolate / Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Growth at 40°C	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-
Motility	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+
DNase	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+
Voges–proskauer	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-
Acid production from:															
lactose	+	+	-	-	-	-	-	+	+	-	-	+	-	+	+
sucrose	-	-	+	+	+	-	+	-	+	+	+	-	+	+	+
xylose	+	+	-	+	-	+	-	-	-	+	-	-	-	+	-
raffinose	+	-	-	+	-	+	-	+	+	+	-	+	-	-	+
arabinose	+	-	-	+	-	+	-	-	+	+	-	-	-	+	+

3.3 Screening of *S.marcescens* isolates for prodigiosin production

In order to select the efficient isolate in prodigiosin production, the ability of these local isolates in pigment production was assayed using LB Medium by determining pigment activity (U/cell) in culture filtrate using pigment assay procedure mentioned in (2.2.5). As it was shown in table (3-3), *S.marcescens* S11 gave the maximum production of prodigiosin according to the pigment activity (200 U/cell) in its culture medium, while the pigment activity in the culture medium of S3, S5, S7, S13 isolates are 103.4, 89.5, 78.9, and 98.1 U/cell respectively. From these results *S.marcescens* S11 regarded the efficient isolate in prodigiosin production. This isolate was selected to study the optimum conditions for prodigiosin production.

Table (3-3): Ability of locally isolated *S.marcescens* in prodigiosin production after incubation with shaking at 150 rpm in LB medium for 24hr at 28ºC.

Isolate	Prodigiosin activity (U/cell)
S.marcescens S3	103.4
S.marcescens S5	89.5
S.marcescens S7	78.9
S.marcescens S11	200
S.marcescens S13	98.1

3.4 Optimum conditions for prodigiosin production

Optimum conditions for prodigiosin production by the locally isolated *S.marcescens* S11 were studied under the effect of different growth factors and as follows:

3.4.1 Effect of Carbon Source

Six carbon sources (fructose, glucose, sucrose, lactose, olive oil, and sunflower oil) were used as a sole source of carbon and energy to determine the optimum in prodigiosin production by the locally isolated *S.marcescens* S11, these carbon sources were added to the production medium in a concentration of 2%.

Results indicated in figure (3-1) showed that the maximum production of prodigiosin was obtained when the culture medium was supplemented with olive oil, by using this carbon source, the prodigiosin activity in culture medium was 440.3 U/cell, while the productivity of prodigiosin using glucose, sunflower oil, fructose, sucrose, and lactose under the same conditions are 82.1, 91.8, 152, 169.1, and 343.4 U/cell respectively.

From these results it can be concluded that olive oil was the best carbon source because it contain many nutrients, fatty acids, growth factors and variety of vitamins that supplements the growth requirements for the microorganism, further more olive oil contain several mineral salts (K, Mn, Ca, P, Mg, S, Zn, Na, Fe, and Cu), and vitamin such as vitamin A, K, E, B6, B12, and C (Jama, 2008). It was well known that *S.marcescens* has lipase activity and thereby it was capable for hydrolyzing oil substrates to liberate fatty acids as a sole source for carbon and energy (Wei and Chen, 2005). Glucose may inhibit prodigiosin production due to catabolic repression or by lowering the medium pH during growth and fermentation (Sole *at el.*, 1997).

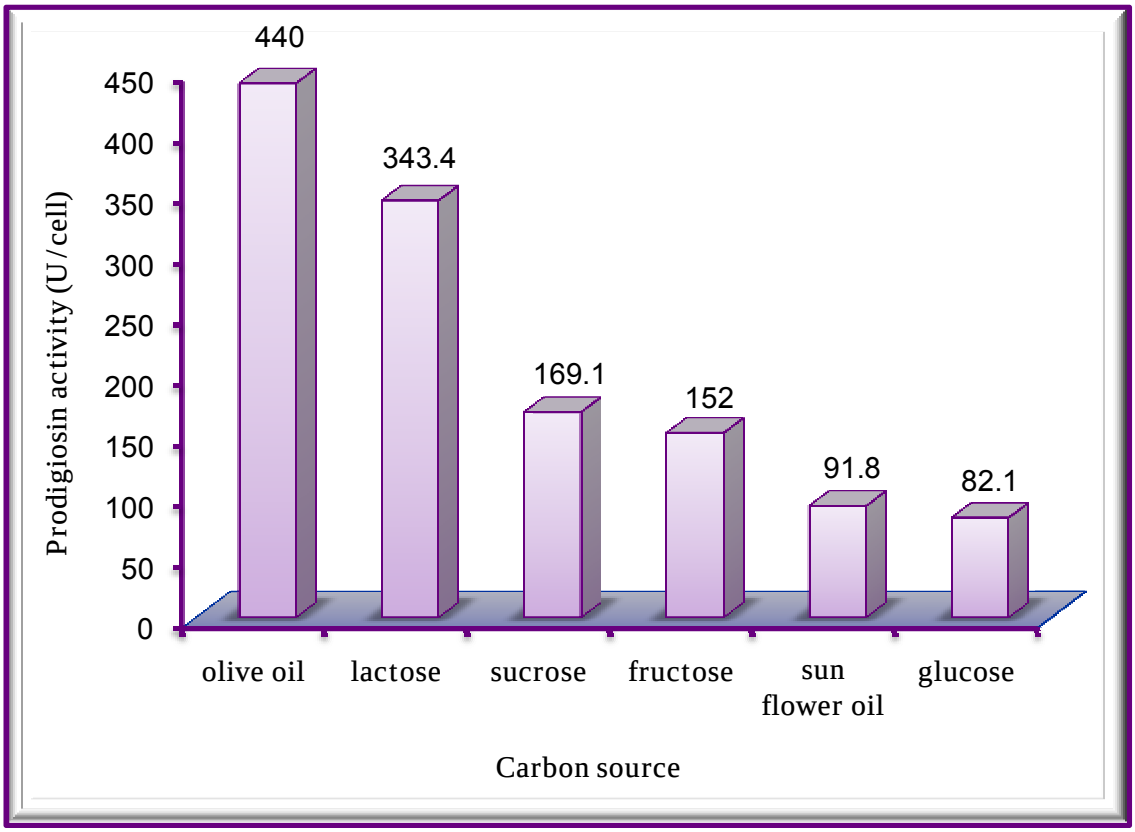


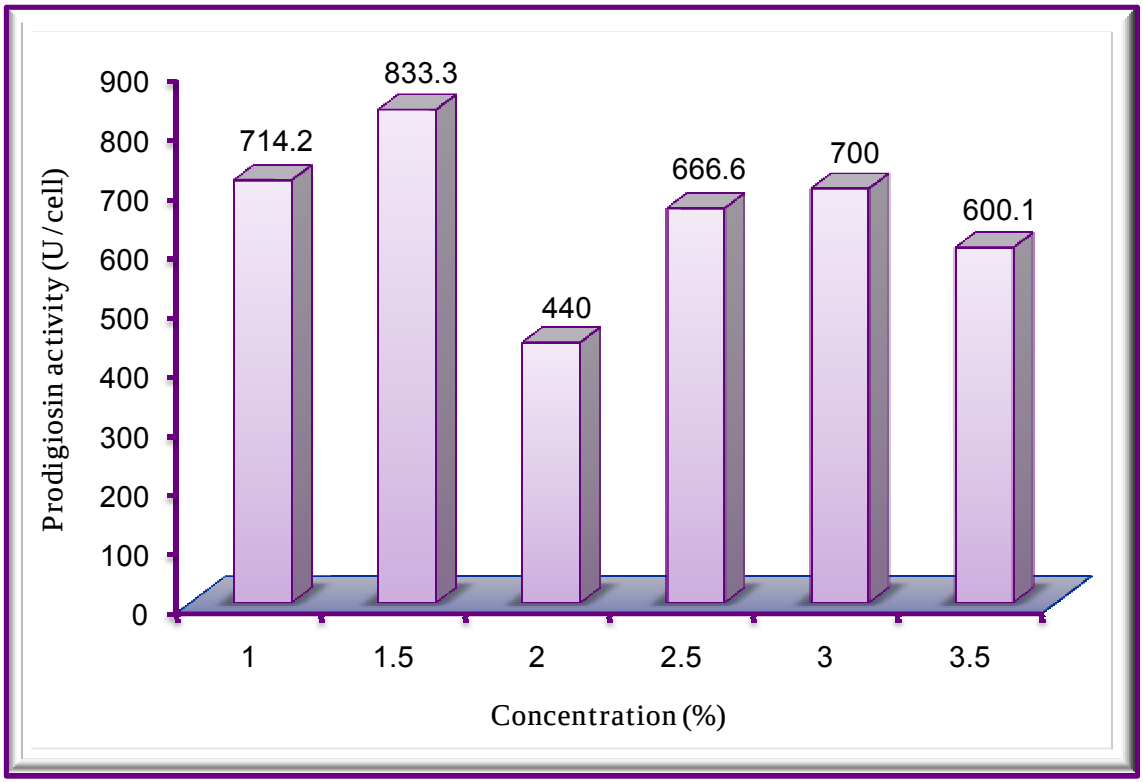
Figure (3-1): Effect of carbon source on prodigiosin production by locally isolated *S.marcescens* S11 after incubation in shaker incubator at 150 rpm, for 24hr at 28°C.

The optimum carbon source (olive oil) was used in the next experiments of optimization for prodigiosin production.

3.4.2 Effect of concentration of carbon source

The optimum carbon source (olive oil) was used to supplement the production medium in a concentrations of 1, 1.5, 2, 2.5, 3, and 3.5% (v/v) to determine the optimum for production of prodigiosin by the locally isolated *S.marcescens* S11.

As it was shown in figure (3-2),the maximum production of prodigiosin was obtained when olive oil was used in a concentration of 1.5%, at this concentration the prodigiosin activity in culture medium was 833.3 U/cell which may indicate that this concentration of carbon source is the best for



Figure(3-2): Effect of different concentrations of olive oil on prodigiosin production by the locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm, for 24hr at 28°C.

providing the microorganism with the needed energy for growth and maximum production of the pigment. Other studies indicated that the optimum carbon and energy source for the pigment production varies between different concentration of olive oil, for example Yamashita *et al.*, (2001) referred that the optimum carbon source for prodigiosin production at 4% (w/v) of *S.marcescens* SMΔR.

The optimum concentration of olive oil (1.5%) was used in the next experiments of optimization for prodigiosin production.

3.4.3 Effect of nitrogen source

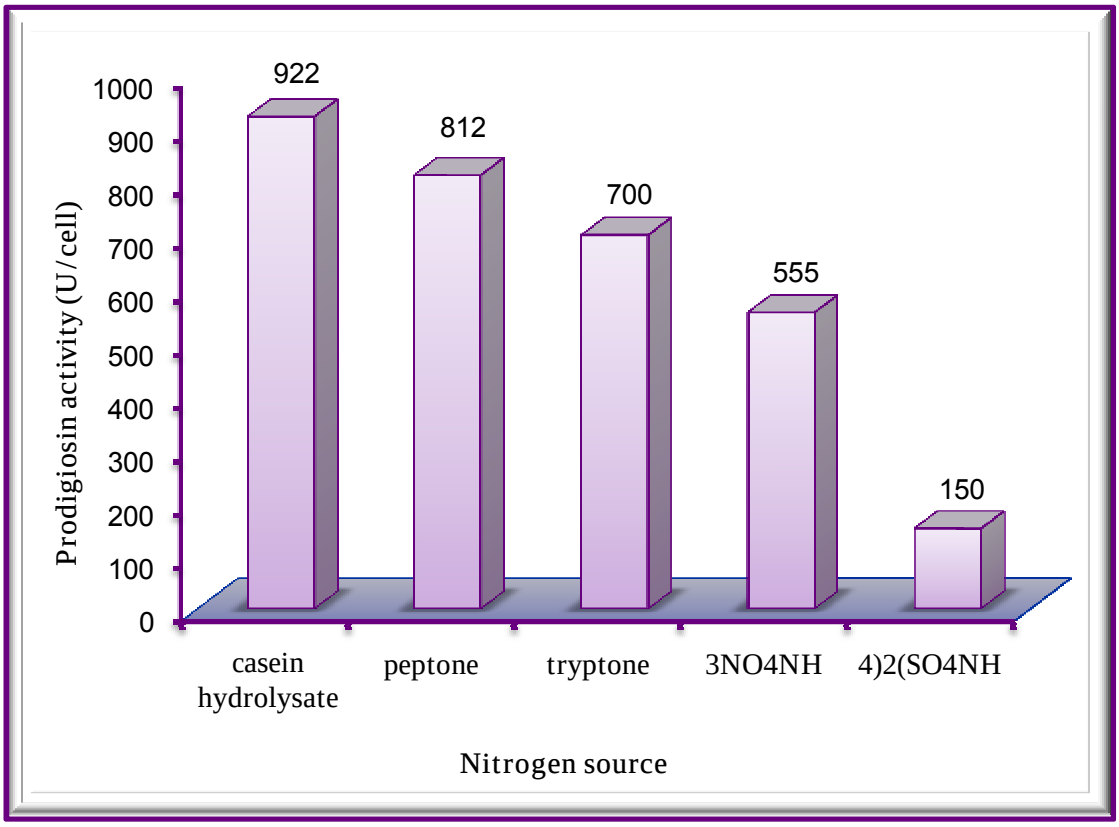
Five nitrogen sources were used to supplement the production medium to enhance prodigiosin production by the locally isolated *S.marcescens* S11, three of these nitrogen sources are organic (peptone, tryptone, and casein hydrolysate), and two inorganic (ammonium nitrates and ammonium sulphate).

These sources were added to the production medium instead of tryptone and yeast extract in a concentration of 1.5%. Results mentioned in figure (3-3) showed that the maximum production of prodigiosin in culture medium was obtained when the production medium was supplemented with casein hydrolysate as an organic nitrogen source. The prodigiosin activity in culture medium using this nitrogen sources was 922 U/cell. This result may be attributed to the type of nitrogen source and its growth factors contents that supplements the bacterial requirements for growth, production and secretion of prodigiosin to culture medium and as it was mentioned by Kim *at el*., (1999).

From the other results mentioned in figure (3-3), it was shown that the production of prodigiosin was lower when peptone, tyrptone, ammonium nitrate, ammonium sulfate were used respectively as a nitrogen sources in the production medium. Furthermore, the production of prodigiosin in culture medium by *S.marcescens* S11 using organic nitrogen sources (peptone, tryptone, casein hydrolysate) was better than the prodigiosin production using inorganic nitrogen sources (ammonium sulfate, ammonium nitrate) under the same condition, these results were agreed with Brivonese and Sutherland, (1989).

The increase in the production of prodigiosin using the casein hydrolysate may be attributed to its natural component that provide the medium with nitrogen source which contributed in the supporting of bacterial biomass, also it contains minerals such as Ca, Mg, and carbohydrates that provide the optimum condition for pigment activities especially those enzymes responsible for biosynthesis of prodigiosin, and as it was mentioned by Kim *at el.*, (1998).

The optimum nitrogen source (casein hydrolysate) was used in the next experiments of optimization for prodigiosin production.

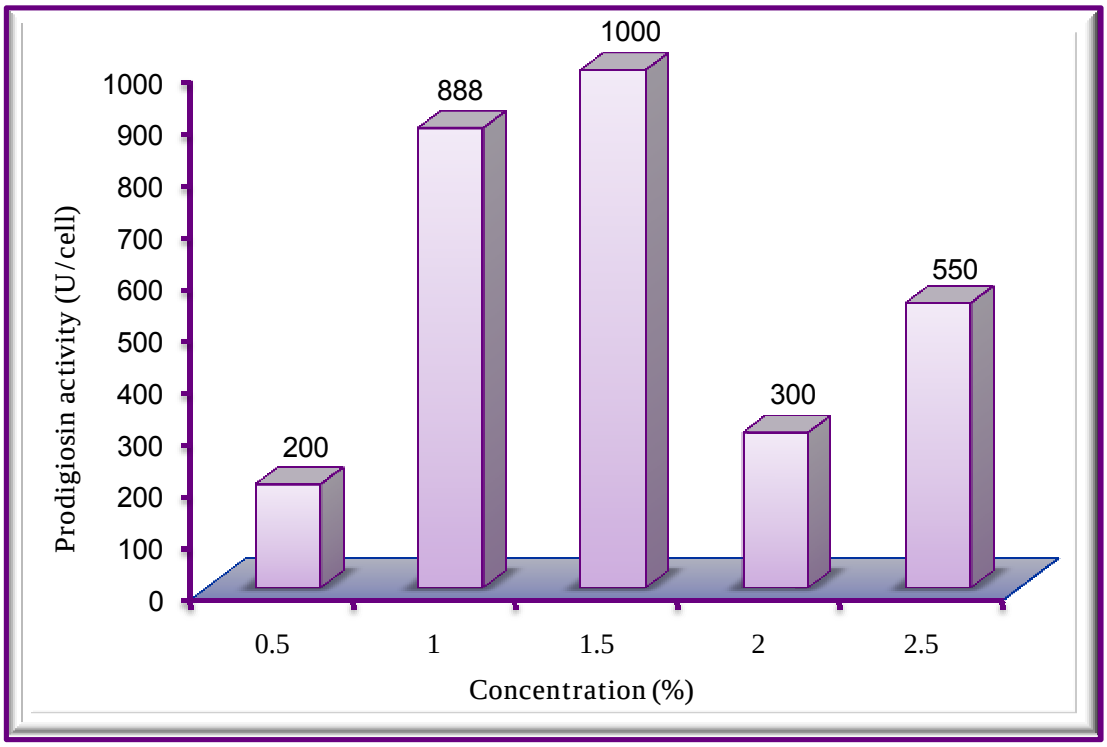


Figure(3-3): Effect of nitrogen source on prodigiosin production by the locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm, for 24hr at 28°C.

Effect of nitrogen source concentration

To determine the optimum concentration of casein hydrolysate as it was the optimum nitrogen source for prodigiosin production, five concentrations (0.5, 1, 1.5, 2, and 2.5 % w/v) were used to supplement the production medium to examine the ability of prodigiosin production by the locally isolated *S. marcescens* S11.

Results indicated in figure (3-4) showed that the maximum production of prodigiosin was obtained when casein hydrolysate was added to the production medium in a concentration of 1.5%, the prodigiosin activity in culture medium was 1000 U/cell, on the other hand the increase or decrease in the concentration of casein hydrolysate above or below the optimum concentration causing



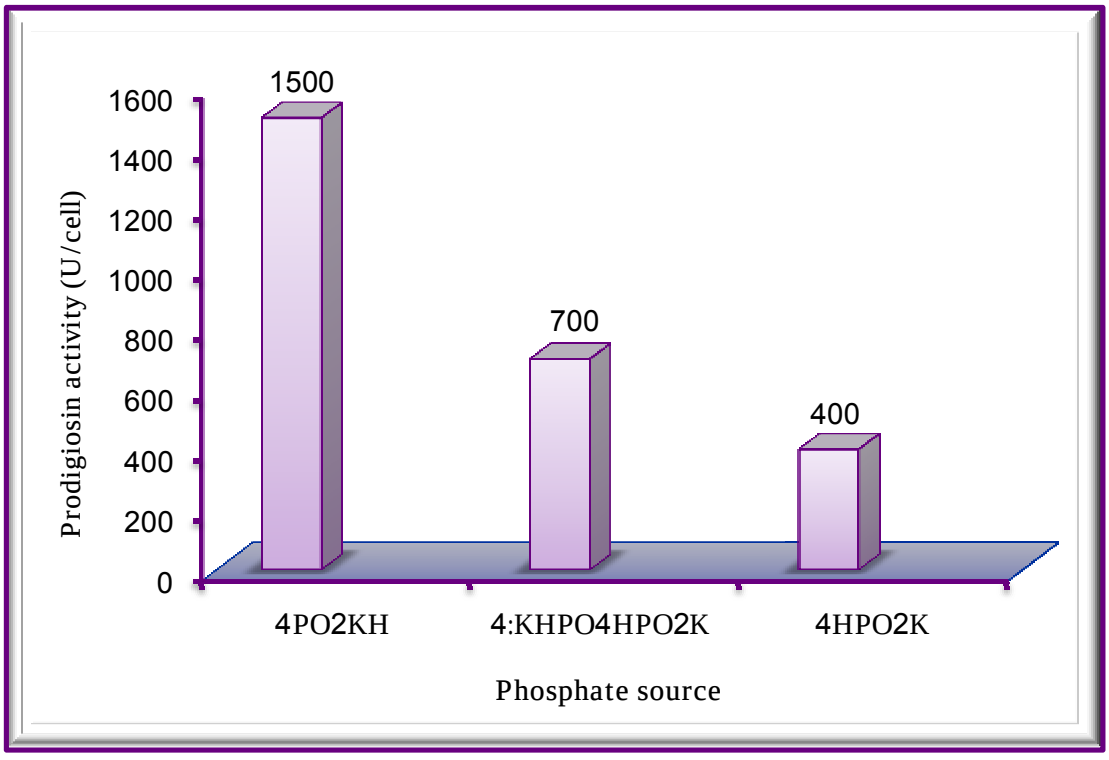
Figure(3-4): Effect of casein hydrolysate concentration on prodigiosin production by the locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm, for 24hr at 28°C.

a decrease in prodigiosin production, this may be due to the change in the C/N ratio in production medium that affects different secondary metabolites pathways especially those responsible for prodigiosin production and as it was mentioned by Kim *at el*., (1999).

The optimum concentration of casein hydrolysate (1.5%) was used in the next experiments of optimization for prodigiosin production.

3.4.5 Effect of phosphate source

Different phosphate sources were also studied to determine the optimum for prodigiosin production by the locally isolated *S.marcescens* S11. Two types of phosphate sources (KH_2PO_4 and K_2HPO_4) were added to the production medium



Figure(3-5): Effect of phosphate source on prodigiosin production by the locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm for 24hr at 28°C.

at a concentration of 0.1% and also a mixture of them (0.07% of KH_2PO_4 and 0.03% of K_2HPO_4) was used. Results indicated in figure(3-5) showed that the maximum production of prodigiosin was obtained when the production medium was consists of KH_2PO_4, which causes an increase in prodigiosin production, the prodigiosin activity in culture medium was 1500 U/cell in comparison with K_2HPO_4 or the mixture of them, this is may be due to the synthesis of two precursors of the pigment 4-methoxy-2, 2-bipyrrole-5-carbaldehyde (MBC) and 2-methyl-3-n-amyl-pyrrole (MAP) which was inhibited by concentration of these compounds (K2HPO4 and K2HPO4:KH2PO4) as it was mentioned by Frank *et al.*, (1997). The presence of phosphate in culture medium works as a buffering capacity when the medium become alkaline due to the biosynthesis of prodigiosin (Frank *et al.*, 1997).

The optimum phosphate source (KH_2PO_4) was used in the next experiments of optimization for prodigiosin production.

3.4.6 Effect of Temperature

In order to determine the optimum incubation temperature for prodigiosin production by the locally isolated *S.marcescens* S11, different incubation temperatures (24, 28, 32, 36, and 40ºC) were used for this purpose.

As it was shown in figure (3-6), it was found that the maximum production of prodigiosin was obtained when the temperature of growth medium was 28˚C. At this temperature, the prodigiosin activity in culture medium was 2714 U/cell, this is may be due to the effect of this temperature on growth of the microorganism and production of prodigiosin, on otherhand some studies indicated that the best temperature for prodigiosin production was 30˚C (Robert *et al.*, 1971; Greenwood *et al.*, 2002).

A block in prodigiosin production was occur above 30°C in culture medium, while the presence of fatty acids in culture medium supported prodigiosin production up to 42°C (Giri *et al.*, 2004).

The increase in temperature to 40°C led to decrease the bacterial growth rate and made the conditions unsuitable for prodigiosin production and finally led to repress the expression of genes responsible for prodigiosin with less effect on bacterial growth (Haddix and Werner, 2000), or may repress the genes responsible for Prodigiosin Condensing Enzyme (PCE) which are sensitive to high temperature (Furstner, 2003).

The optimum temperature (28°C) was used in the next experiments of optimization for prodigiosin production.

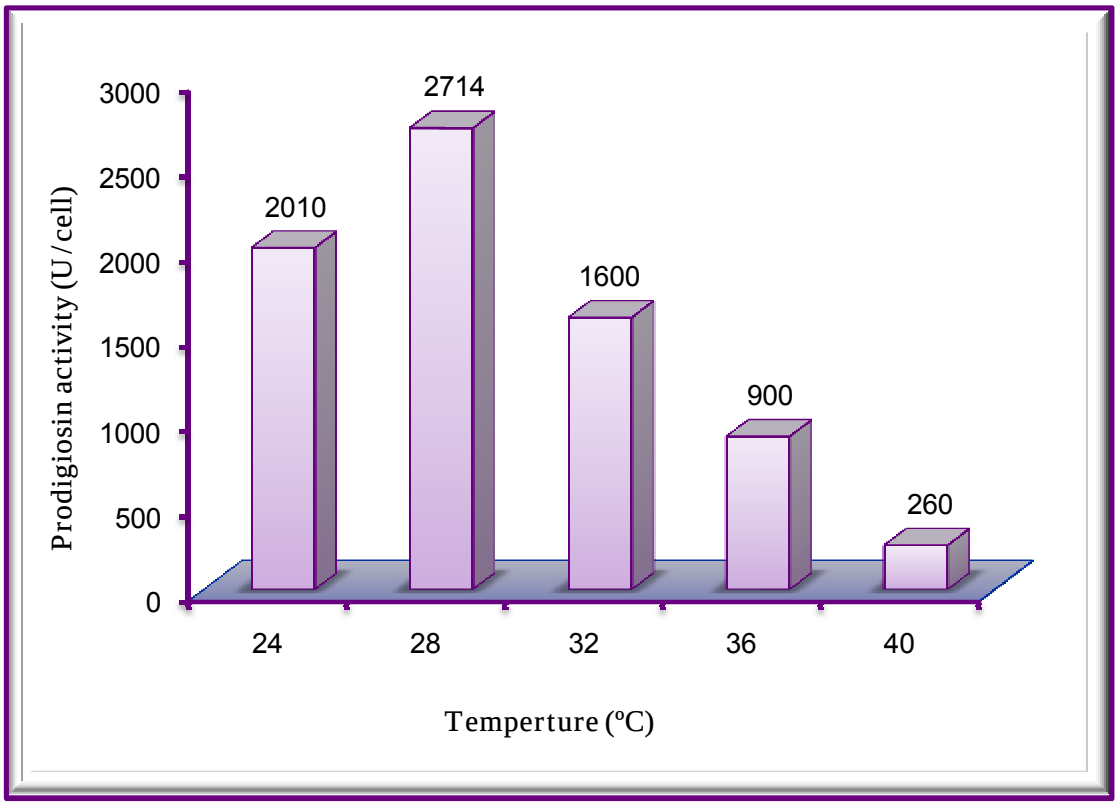


Figure (3-6): Effect of different incubation temperature on prodigiosin production by locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm, for 24hr at 28°C.

3.4.7 Effect of pH

Different pH values were used to determine the optimum for prodigiosin production by the locally isolated *S.marcescens* S11, the following pH values were used for this purpose (7.0, 7.5, 8.0, 8.5, and 9.0).

As it was shown in figure (3-7), the maximum production of prodigiosin was obtained when the pH value of the growth medium was adjusted to 8.0, at this pH the prodigiosin activity in culture medium was 3000 U/cell. This result was agree with Giri *et al.*, (2004) who noticed that the maximum production of prodigiosin from *S.marcescens* was obtained when the production medium was adjusted to alkaline pH.

pH8 may work to inhibit the activity of proline oxidase, this enzyme causes proline inhibition which are the important amino acid precursor to produce MBC (Solem *et al.*, 1994).

Results mentioned in figure (3-7) showed that the increase or decrease in the pH value of the production medium above or under the optimum pH causes a significant decrease in prodigiosin production, this may be because of the alteration of the activities of all genes responsible for prodigiosin biosynthesis as it was mentioned by (Sole *et al.*, 1997).

The effect of medium pH value on pigment productivity and activity is due to two reasons, (Bull and Bushnel, 1976).

- Its effect on the properties of the culture medium including the solubility of the nutrients molecules, transport and ionization.

- pH value affects the stability of the pigment.

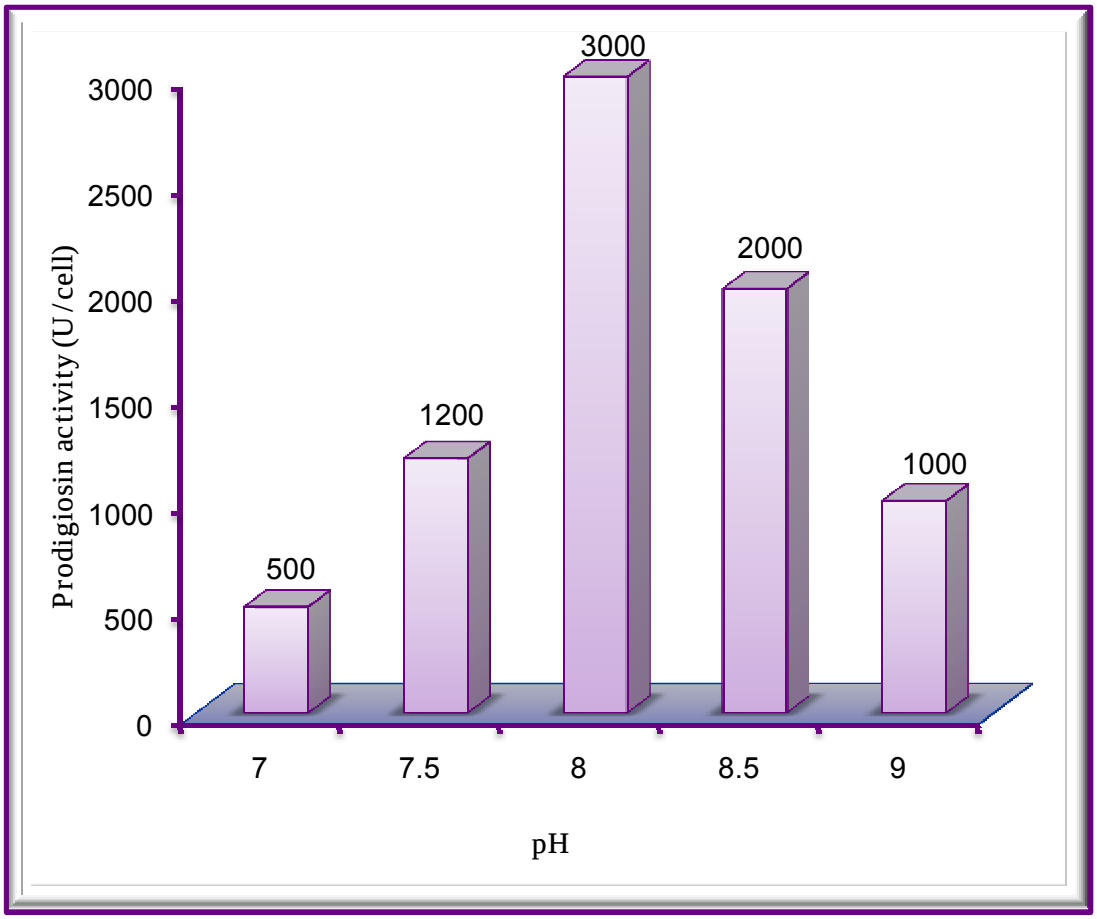


Figure (3-7): Effect of different pH values on prodigiosin production by the locally isolated *S.marcescens* S11 after incubation in shaking incubator at 150 rpm, for 24hr at 28°C.

3.5 Mutagenesis of *S. marcescens* S11

In order to enhance the ability of locally isolated *S.marcescens* S11 in prodigiosin production, it was subjected to mutagenesis by different types of mutagens includes physical mutagens (UV radiation) and chemical mutagens (Mitomycin C).

According to Abbas *et al*., (2004); colonies screened to investigate the genetic alteration after mutagen treatments caused 90% death (10% survival) which may lead to over production mutants. By observing the morphological differences, the mucoidal growth and colony size, thirty five mutant colonies were selected after each mutagenic treatment. The prodigiosin activity was determined as in (2.2.7) for each mutant.

3.5.1 Physical mutagenesis by UV radiation:

The results mentioned in figure (3-8) showed that the mutagenic and lethal effect UV irradition on *S. marcescens* S11, there was a reduction of total viable count of the bacterial cells from $288x10^9$ CFU/ml in the zero time to $199x10^9$ CFU/ml after the exposure to the first dose of UV radiation (1 j/m^2), after subjection to this dose the survival percentage was 60%, followed by sever reduction to 30%, 15%, and 1.6% after subjection to next doses of UV irradiation (2, 3, and 4 j/m^2).

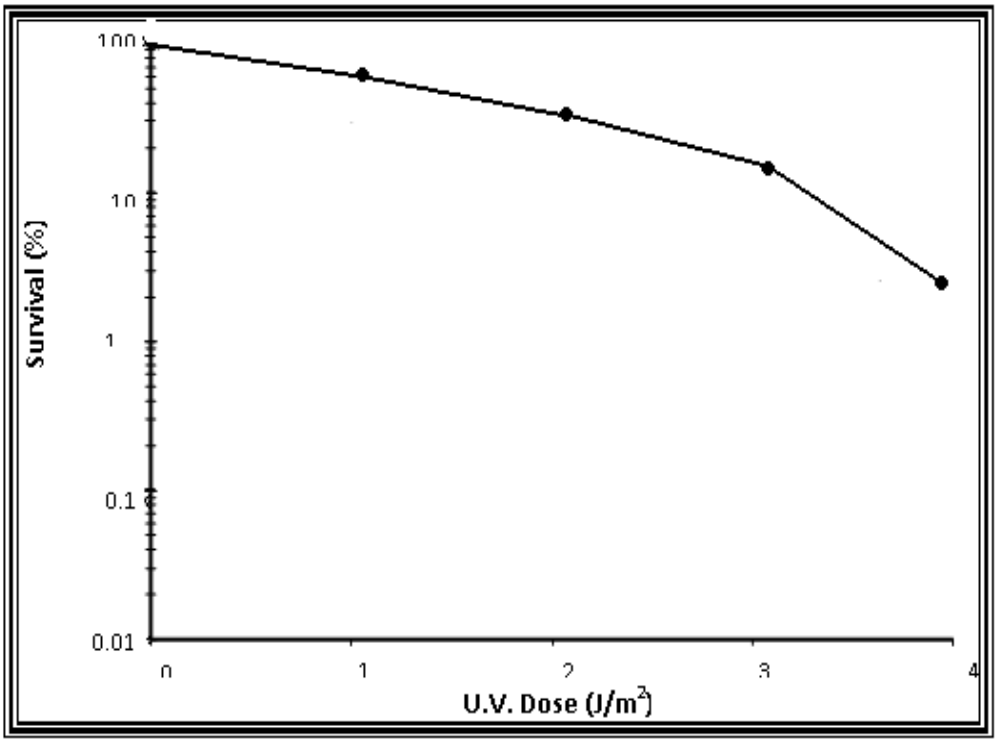


Figure (3-8) Effect of different doses of UV. Radiation on the survivals of locally isolated *S.marcescens* S11.

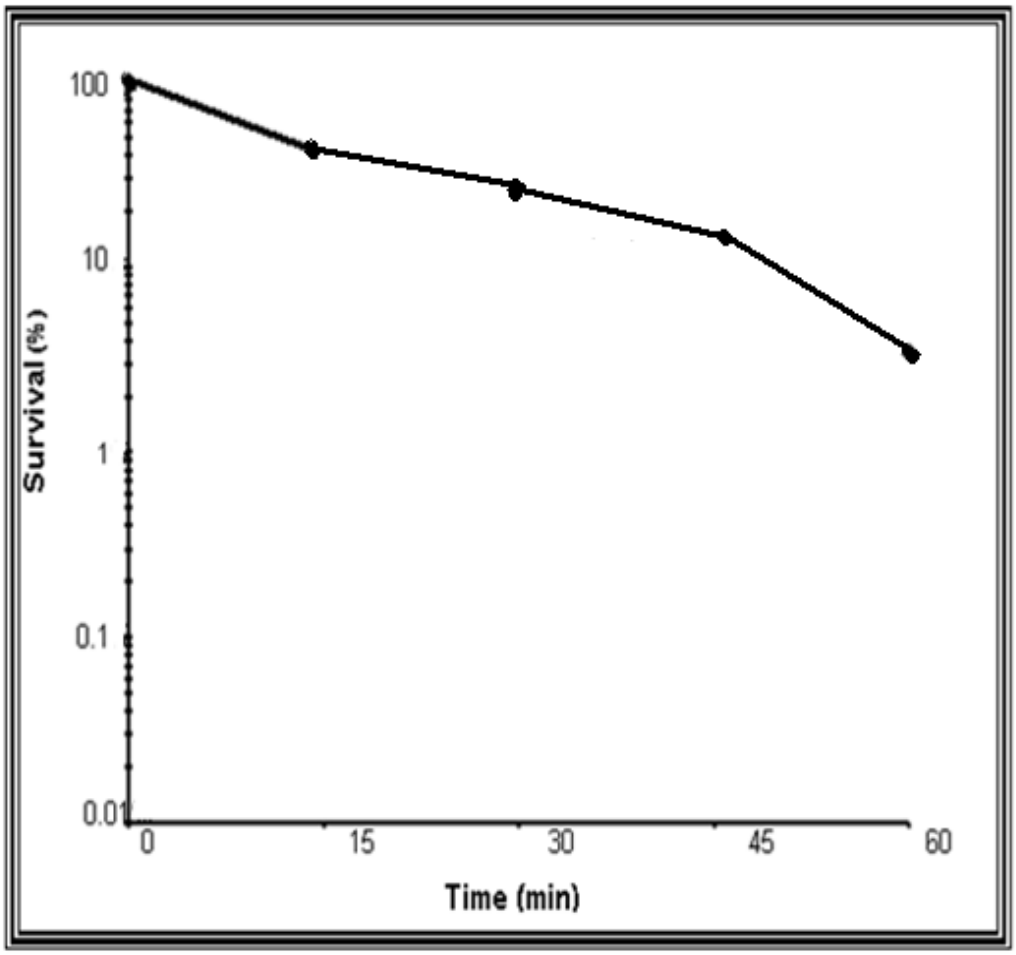
Results mentioned in table (3-4) showed that after UV radiation sixteen mutants out of thirty five (45.7%) showed an increase in prodigiosin production to 220U/cell for mutants S11H3, S11H10, S11H17, and S11H33 to 350U/cell for mutant S11H7 compared with the productivity of the wild type. (200 U/cell). On the other hand there are another ten mutants 28.5% with lower prodigiosin production than the wild type. The prodigiosin activity produced by these mutants ranged between 30U/cell for (mutant S11H4) to 90U/cell (mutants S11H9, S11H12, S11H22, S11H25, and S11H28). UV irradiation is affected via miss repair of damaged DNA by SOS repair system and termed indirect mutagen it was applied as mutagens for the halotolerant *Micrococcus* sp., cell survival and mutability of *P. aeruginosa* and *P. syringae* and the survival of *Shewanella oneidensis* were determined after UV radiation (Qiu *et al*., 2004).

Table (3-4): Prodigiosin production by mutants of *S. marcescens* S11 after irradiation with UV ray.

Mutants	Prodigiosin activity (U/cell)
S.marcescens **S11H1**	200
S.marcescens **S11H2**	255
S.marcescens **S11H3**	220
S.marcescens **S11H4**	30
S.marcescens **S11H5**	200
S.marcescens **S11H6**	66
S.marcescens **S11H7**	350
S.marcescens **S11H8**	200
S.marcescens **S11H9**	90
S.marcescens **S11H10**	220
S.marcescens **S11H11**	290
S.marcescens **S11H12**	90
S.marcescens **S11H13**	300
S.marcescens **S11H14**	320
S.marcescens **S11H15**	200
S.marcescens **S11H16**	280
S.marcescens **S11H17**	220
S.marcescens **S11H18**	40
S.marcescens **S11H19**	200
S.marcescens **S11H20**	250
S.marcescens **S11H21**	200
S.marcescens **S11H22**	90
S.marcescens **S11H23**	290
S.marcescens **S11H24**	340
S.marcescens **S11H25**	90
S.marcescens **S11H26**	310
S.marcescens **S11H27**	200
S.marcescens **S11H28**	90
S.marcescens **S11H29**	245
S.marcescens **S11H30**	333
S.marcescens **S11H31**	200
S.marcescens **S11H32**	80
S.marcescens **S11H33**	220
S.marcescens **S11H34**	200
S.marcescens **S11H35**	70
S.marcescens **S11**wild type	200

3.5.2 Chemical mutagenesis by Mitomycin C

Another type of mutagens (Mitomycin C) was used to generate over producer mutants of prodigiosin from *S.marcescens* S11. Results in figure (3-9) indicated that this mutagen has significant mutagenic and lethal effect on the bacterial cells of *S.marcescens* S11. This can be noticed from the reduction in the total viable count of bacterial cells from $15.1x10^8$ CFU/ml in the zero time to $7.4x10^8$ CFU/ml after the incubation with the mutagen for 15 min, after subjection to this period the survival percentage was 54%, followed by sever reduction to 26.4%, 17.8%, and 5% after subjection to next period of incubation (30, 45, and 60 min).



Figure(3-9): Survival of locally isolated *S.marcescens* S11 after incubation with Mitomycin C (30µg/ml) for different periods.

After Mutagenesis with Mitomycin C, the illustrated results in table (3-5) appeared: nineteen mutants out of thirty five (54.2%) with higher prodigiosin production ranged between 230 U/cell for (mutants S11H38, S11H42, S11H59, S11H64 and S11H69) to 400 U/cell for (S11H54 mutant), than the productivity of wild type (200U/cell). Another ten mutants (28.5%) showed decreasing in prodigiosin production. The prodigiosin activity produced by these mutants ranged between 60U/cell for (mutant S11H49) to 120U/cell (mutants S11H36, S11H44, S11H52, S11H56, S11H61 and S11H66). Six mutants (17.1%) have no changed in prodigiosin production (200U/cell for the mutants S11H37, S11H40, S11H48, S11H58, S11H63 and S11H70).

The molecular basis of Mitomycin C bioactivity derived mainly from its propensity to covalent interact with DNA sequences, causing lethal intra and interstrand cross-links as well as alkylation and formation of reactive oxygen species (Sheldon *et al.*, 1999).

Table (3-5): Prodigiosin production by mutants of *S.marcescens* S11 after mutagenesis with Mitomycin C.

Bacterial Isolate	Prodigiosin activity (U/cell)
S.marcescens **S11H36**	120
S.marcescens **S11H37**	200
S.marcescens **S11H38**	230
S.marcescens **S11H39**	250
S.marcescens **S11H40**	200
S.marcescens **S11H41**	330
S.marcescens **S11H42**	230
S.marcescens **S11H43**	370
S.marcescens **S11H44**	120
S.marcescens **S11H45**	266
S.marcescens **S11H46**	100
S.marcescens **S11H47**	278
S.marcescens **S11H48**	200
S.marcescens **S11H49**	60
S.marcescens **S11H50**	320
S.marcescens **S11H51**	330
S.marcescens **S11H52**	120
S.marcescens **S11H53**	360
S.marcescens **S11H54**	400
S.marcescens **S11H55**	290
S.marcescens **S11H56**	120
S.marcescens **S11H57**	295
S.marcescens **S11H58**	200
S.marcescens **S11H59**	230
S.marcescens **S11H60**	246
S.marcescens **S11H61**	120
S.marcescens **S11H62**	268
S.marcescens **S11H63**	200
S.marcescens **S11H64**	230
S.marcescens **S11H65**	90
S.marcescens **S11H66**	120
S.marcescens **S11H67**	340
S.marcescens **S11H68**	80
S.marcescens **S11H69**	230
S.marcescens **S11H70**	200
S.marcescens **S11**wild type	200

From the preceded results, UV radiation successfully enhanced prodigiosin production from *S. marcescens* S11 since its productivity 1.8 fold higher than the prodigiosin produced of the wild type.

Also, the chemical mutagens Mitomycin C successfully developing the prodigiosin productivity about 2.0 fold for the mutant S11H54 compared with productivity of the wild type. By comparing the highest prodigiosin concentration 350 U/cell for mutant (S11H7) that obtained after UV radiation and 400 U/cell for mutant (S11H54) after treatment with Mitomycin C, the chemical mutagens Mitomycin C was better than UV radiation in enhancing prodigiosin productivity. The mutation either occurred in the structural genes or in the regulatory genes that negatively regulate prodigiosin production.

To explain the obtained results after mutagenesis with the physical mutagens and the chemical mutagens, the increment in prodigiosin production by these mutants may be due to the effect of the mutagens which lead to inactivate the regulatory genes responsible for negative regulation of prodigiosin production. The high productivity of prodigiosin by locally isolated *S. marcescens* mutants after exposure to different mutagens may be due to genetic mutations that inactivates negative regulatory gene which causes an increasing in prodigiosin biosynthesis, Ying *et al*., (1998).

Reduction in prodigiosin production by the mutants may be due to the genetic mutations induced in the structural genes responsible for prodigiosin biosynthesis pathway (Williamson *et al*., 2006), or due to the mutations that may occur in the regulatory genes which compromise the main switch controlling the conversion to the mucoidal growth (prodigiosin production) (Ying *et al* ., 1998).

Finally, unchangeable in prodigiosin level expected due to genetic mutations in different genes of the chromosomal DNA of locally isolated *S. marcescens* other than those responsible for production and regulation of prodigiosin biosynthesis pathway, as it was mentioned by Ying *et al.*, (1998).

Chapter Three

Results and Discussion

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

The following equipments and apparatus were used in this study:

Equipment	Company
Autoclave	Karl Kolb (Germany)
Compound Light microscope	Olympus (Japan)
Laminar air flow	Memmert (Germany)
Distillator	Gallenkamp (England)
Incubator	Gallenkamp
Electrical Oven	Gallenkamp
pH-meter	Gallenkamp sanyo (U.K.)
Sensitive balance	Mettler (Swizerland)
Shaker incubator	Sartorius (Germany)
Visible spectrophotometer	Baush and Lamb (England)
Micropipettes	Volac (Germany)
Vortex	Stuart scientific (U.K.)
Refrigerator	Ishtar (Iraq)
Portable Centrifuge	Hermle labortechnik(German)
Ultraviolet transilluminator	Ultraviolet product (USA)
Millipore filter unit (0.45 µm)	Millipore Corp.(U.S.A.)

2.1.2 Chemicals

The following chemicals were used in this study:

Chemicals	Company(origin)
Trypton, pepton	Fluka (Switzerland)
Absolute ethyl alcohol	BDH (England)
HCl	BDH
NaOH, KOH	Merck (Germany)
NaCl	Merck
Sunflower oil, olive oil	Fruee (Turkey)
Mitomycin C	Kyowa Hakko Kogyo (Japan)
KH_2PO_4 , K_2HPO_4, Glucose, Sucrose, Lactose, Raffinose, Arabinose , Xylose, Fructose, Ammonium sulphate.	BDH (England)
Phenol, Ammonium chloride, Casein hydrolysate.	Riedel-Dehaeny-(Germany)
Yeast extract	Biolife (Italy)
Agar-Agar	Oxoid (England)
Glycerol	Riedel-DeHaen (Germany)
Hydrogen peroxide, , N,N,N,N-tetramethyl-p-phenylene-diamine dihydrochloride	Difco (U.S.A)
Toluidine blue	Difco
α- naphthol	Sigma (USA)

2.1.3 Media

2.1.3.1 Ready to Use Media

These media were prepared as recommended by manufacturing Companies, pH was adjusted to 7.0 and sterilized by autoclaving.

- Brain heart infusion agar (Difco–USA).
- Brain heart infusion broth (Difco).
- Semi – solid agar (Difco).
- DNase agar (Difco).
- Methyl red-Voges Proskauer broth (Oxoid–England).
- Nutrient agar (Oxoid).

2.1.3.2 Synthetic Media

- **Luria- Bertani medium** (Maniatis *et al*., 1982)**.**

This medium consists of the following components:

Component	Concentration(g/L)
Tryptone	10
Yeast Extract	5
NaCl	10

All components were dissolved in 950 ml of distilled water, pH was adjusted to 7.0, then volume was completed to 1000 ml with D.W. and sterilized by autoclaving.

- **Luria- Bertani Agar** (Maniatis *et al*., 1982).

Agar (15%) was added to LBB and autoclaved.

Motility medium (Macfaddin, 2000).

This medium was consist of the following components :

Component	Concentration(g/L)
Tryptone	1
NaCl	0.5
Agar – Agar	o.5

All components were dissolved in 950 ml distilled water, pH was adjusted to 7.2 then volume was completed to 1000 ml and sterilized by autoclaving .

Sugar fermentation medium (Macfaddin, 2000).

This medium was consist of the following components :

Component	Concentration(g/L)
Peptone	1
NaCl	0.5
Phenol red	0 .08

All components were dissolved in 950 ml distilled water, pH was adjusted to 7 then volume was completed to 1000 ml and sterilized by autoclaving.

Sugars (Lactose, sucrose, xylose, raffinose and arabinose) were prepared separately by dissolving 1g of sugar to 100 ml D.W and sterilized by autoclaving for 9 min.

2.1.4 Reagents

- **Catalase reagent** (Atlas *et al*., 1995):

  This reagent was prepared to be consists of (3%) hydrogen peroxide.

- **Oxidase reagent** (Atlas *et al*., 1995):

  This reagent is composed of 1g of N, N, N; N-tetramethyl-p-phynylene-diamine dihydrochloride dissolved in 100 ml distilled water and kept in dark bottle at 4°C.

- **Methyl red reagent** (Collee *et al.,* 1996):

  It was prepared by dissoliving 0.1g of methyl red in 300ml of ethanol (99%) then 200ml of D.W. was added, mixed thoroughly and kept at 4ºC until use.

- **Vogas- Prosker reagent** (Collee *et al*., 1996):

  Solution (A): This solution was prepared by dissolving 40g of potassium hydroxide (KOH) in 80 ml D.W., then volume was completed to 100ml with D.W.

  Solution (B): This solution was prepared by dissolving 5g of α-napthol in 100 ml D.W.

  Equal volumes of solution A and B were mixed thoroughly and kept at 4ºC until use.

2.1.5 Buffer and Solution

Phosphate Buffer Solution (pH7)

This solution was prepared by dissolving 9.52g of Na_2HPO_4 and 6g of NaH_2PO_4 in 950ml of D.W., pH was adjusted to 7, then volume was completed to 1000ml with D.W., and sterilized by autoclaving(Cruikshank *et al.*, 1975).

Mitomycin C stock solution

Stock solution of Mitomycin C (1000 µg/ml) was prepared by dissolving 10 mg of Mitomycin C in 10 ml of distilled water.

2.2 Methods

2.2.1 Sterilization methods

Moist heat sterilization

Media and solutions were sterilized by autoclaving at 121°C for 15min.

Dry heat sterilization

Glasswares were sterilized using electric oven at 180°C for 3 hrs.

2.2.2 Isolation of *S.marcescens*

2.2.2.1 Samples collection

Different samples were collected from different sources in order to isolate *S.marcescens* and as follows:

Environmental Samples

Water, soil, and sewage samples were collected from different locations in Baghdad governorate during 2 – 4 /2008.

2.2.2.2 Samples preparation

Environmental samples from water, soil, and sewage were diluted using sterilized distilled water and as follows:

Water and Sewage Samples

Serial dilutions of water and sewage samples were done by taking 1ml aliquots of each sample and diluted with 9 ml of sterilized distilled water, mixed thoroughly, then 100 µl aliquots from the appropriate dilution were taken and spread on Nutrient agar plates and incubated at 28°C for 18 hours.

Soil Samples

One-gram of each soil sample was added to test tubes containing 9ml of sterilized distilled water, mixed thoroughly and serial dilution for each tube were done separately, then 100µl aliquots from the appropriate dilution were taken and spread on Nutrient agar plates and incubated at 28°C for 18 hours. After incubation, the growing colonies were selected and streaked on Brain heart infusion agar plates and incubated at 28°C for 24 hour.

2.2.3 Identification of *Serratia* spp.

Different isolates from environmental samples were taken and grown on Brain heart infusion agar medium to make sure that this type of bacteria may belong to the genus of *Serratia*, then these isolates were identified according to their cultural and morphological characteristics (staining ability, shape, color, size, production of pigment, transparency and mucoidal properties), of the growing colonies on nutrient agar and Brain heart infusion agar plates, then some biochemical tests were achieved to confirm results of identification and as follows:

2.2.3.1 Cultural and Morphological characteristics

- **Colonial morphology**

Shape, color and viscous growth of the colonies were studied on Brain heart infusion agar plates medium after incubation at 28°C for 24 hr.

- **Gram's stain (**Harely and Prescott, 1996**)**

Single colony of each isolate was transferred and smeared on a clean slide and fixed by heat. The smear was stained with crystal violet (for 1 minute) and excess stain was washed then treated with iodine (for 1 min.) and the excess was washed with distilled water, then decolorized with absolute alcohol and the excess was washed, and then counterstained with safranine (for 30 second) and the excess was washed, then examined under light microscope.

- **Growth at 40̊C** (Grimont *et al*., 1984)

One of the tests used for differentiation between species of the genus *Serratia* is achieved by growing bacterial isolates at 40°C. Bacterial isolates (or the suspected *Serratia* isolates) were grown on Brain heart infusion agar plates and incubated at 40°C for 24 hr. Presence of bacterial growth indicates a positive result.

2.2.3.2 Biochemical testes

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

Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide to water and gaseous oxygen, this test was done by adding few drops of 3% hydrogen peroxide on a single colony smeared on clean glass side. Production of gaseous bubbles indicates a positive result.

Oxidase test (Atlas *et al*., 1995)

Filter paper was saturated with oxidase reagent (tetra methyl-p-phenylene diamine dihydrochloride), then single colony of each bacterial isolate was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result.

Methyl red test (Collee *et al*., 1996)

This test is employed to detect production of sufficient acid during fermentation of glucose.

The MR-VP media was prepared and divided in 5 ml test tubes and sterilized by autoclaving, then test tubes were inoculated with each bacterial isolate and incubated at 28ºC for 48hr. 5drops of methyl red reagent were added to each tube and mixed gently. Appearance of red color represents positive result.

Voges – proskauer test (Collee *at el*., 1996)

This test was achieved to detect the production of acetoin as an intermediary metabolite. Acetoin is also known as acetyl methyl carbinol.

MR–VP medium was prepared and distributed into sterile test tubes (5ml/tube), then test tubes were inoculated with fresh cultures (100µl aliquots) of bacterial isolates and incubated at 28ºC for 48hr, after incubation 0.5ml of reagent A and 0.5 ml of reagent B were added and shaking vigorously for 30 second. Appearance of red color represent a positive result.

DNase test (Atlas, 1995)

DNase catalyzes the hydrolysis of deoxyribonucleic acid (DNA). DNA agar plates were inoculated with fresh culture of bacterial isolates by streaking, then incubated at 28ºC for 24hr.

After incubation plates were flooded with 0.01% of toluidine blue. Presence of pink halo around bacterial growth indicates a positive results.

Sugar fermentation (Macfaddin, 2000)

This test was employed to detect acid and gas production from sugar fermentation by bacterial isolates.

Sugar fermentation medium was prepared and distributed into sterile test tubes, sugar used for this purpose are lactose, sucrose, xylose, raffinose, and arabinose (5ml/tube), then test tubes were inoculated with fresh cultures of bacterial isolates and incubated at 28ºC for 48hr. Appearance of yellow color represents a positive result.

Motility test (Macfaddin, 2000)

In semi–solid agar medium, motile bacteria (swarm) and give diffuse spreading growth that is visually recognized.

This medium was prepared by adding 0.5% of agar to nutrient broth, then 10 ml aliquots were transferred to test tubes and left in vertical position. All tubes were inoculated with bacterial isolates by stapping in straight line and incubated at 28ºC for 24hr, motile bacteria would grow and diffuse around the line of stapping.

2.2.4 Maintenance of Bacterial Isolates

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

(a) Short-term storage

Bacterial isolates were maintained for period of few weeks on brain heart infusion agar plates. The plates were tightly wrapped in parafilm and stored at 4°C.

(b) Medium-term storage

Bacterial isolate were maintained in stab culture for period of few months. Such cultures were prepared in screw-capped bottles containing (5-8 ml) of agar medium and stored at 4°C.

(c) Long-term storage

Bacteria can be stored for many years in brain heart infusion broth 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 bacterial isolates in a screw-capped bottle with final volume (10 ml) and stored at -20°C.

2.2.5 Ability of bacterial isolates in prodigiosin production

Ability of locally isolated *S.marcescens* in prodigiosin production was examined in liquid medium by inoculating brain-heart infusion broth with fresh cultures of bacterial isolates and incubated in shaker–incubator (150 rpm) at 28ºC till the optical density of the growth medium was 0.75 at 620nm.

At the same time the optical density of the growth medium was measured at 499nm.

Prodigiosin activity (U/cell) produced by the local isolates of *S.marcescens* was determined according the following equation (Haddix *et al*., 2000) using the uninoculated growth medium as blank:

$$\textbf{Prodigiosin (U/Cell)} = \frac{[\ O.\ D_{499} - (1.3831 \times O.D_{620}\ )] \times 1000}{O.\ D_{620}}$$

$O.D_{499}$: represent pigment absorbance.

$O.D_{620}$: represent bacterial cell absorbance.

1.3831 : constant.

1000 : this factor are included to avoid working with numbers smaller than one.

2.2.6 Optimum conditions for prodigiosin production

In order to determine the optimum conditions for prodigiosin production, the selected isolate which was the best among the different isolates in pigment production, was used for this purpose. Optimum conditions include type and concentration of carbon source, type and concentration of nitrogen source, type of phosphate source, temperature, and pH.

2.2.6.1 Effect of Carbon source

Six different carbon sources (fructose, glucose, sucrose ,lactose, olive oil, and sunflower oil) were used to determine the optimum for prodigiosin production.

These carbon sources were added to the production medium in a concentration of 2% w/v and 2% v/v for olive oil at 28ºC, pH 7 for 24 hr.

The optimum carbon source was used to supplement the production medium in the next experiments of optimization.

2.2.6.2 Concentration of carbon source

The optimum carbon source was used for prodigiosin production by the selected isolate was used in different concentrations (1%, 1.5%, 2%, 2.5% , 3%, and 3.5%) to determine the optimum for production of the pigment after incubation in a shaker incubator (150 rpm) at 28 ºC, pH 7 for 24hr.

The optimum concentration was used to supplement the production medium in the next experiments of optimization.

2.2.6.3 Effect of nitrogen source

Five nitrogen sources were used to determine the optimum in prodigiosin production [Tryptone, Peptone, Casein hydrolysate, NH_4NO_3, and $(NH_4)_2SO_4$]. These nitrogen sources were added to the medium at a concentration of 1.5% w/v at 28 ºC, pH 7 for 24hr.

The optimum nitrogen sources was used to supplement the production medium in the next experiments of optimization.

2.2.6.4 Concentration of nitrogen source

Different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5%) of the optimum nitrogen source were used to determine the optimum for prodigiosin production by the selected isolate after incubation in a shaker incubator (150 rpm) at 28 ºC, pH 7 for 24hr.

The optimum concentration was used to supplement the production medium in the next experiments of optimization.

2.2.6.5 Effect of phosphate source

In order to determine the optimum phosphate source for prodigiosin production, two types of phosphate sources (KH2PO4 and K2HPO4) and a mixture of them (0.07% of KH2PO4 and 0.03% of K2HPO4) were used in a concentration of (0.1% w/v), to determine the optimum for prodigiosin production by the selected isolate after incubation in a shaker incubator (150 rpm) at 28 ºC, pH 7 for 24hr.

The optimum phosphate source was used to supplement the production medium in the next experiments of optimization.

2.2.6.6 Effect of temperature

In order to determine the optimum incubation temperature for prodigiosin production by the selected isolate, the production medium was inoculated with the selected isolate and incubated at different temperatures (24, 28, 32, 36, and 40°C) in a shaker incubator (150 rpm) for 24hr.

The optimum temperature was fixed in the next experiments of optimization.

2.2.6.7 Effect of pH

In order to determine the optimum pH for prodigiosin production ,initial medium pH was adjusted to different pH values(7.0, 7.5, 8.0, 8.5 and 9.0), then inoculated with selected isolate and incubated in a shaker incubator (150 rpm) at 28ºC for 24hr.

2.2.7 Mutagenesis of *S.marcescens*

In order to study the ability of the locally isolated *S.marcescens* in prodigiosin production, it was subjected to random mutagenesis using physical and chemical mutagens to induce genetic mutations leading to increase prodigiosin production by the selected isolate and as follows:

2.2.7.1 Physico – chemical mutagenesis

Serratia marcescens was subjected to physical mutagenesis using UV radiation according to Kidambi *et al.*, (1996). This was done, first by inoculating luria broth medium with fresh culture of the selected isolate and incubated at 28ºC till mid log phase, then cells were pelleted by centrifugation at 6000 rpm for 15 min, washed twice with phosphate buffer and resuspended in 5ml of the same buffer, and subjected to UV

irradiation in a dark place using UV photoelectric cell [flou-link FLX], by transferring 5ml aliquots of the cell suspension into clean and sterile petri dishes in a very thin layer. The distance between UV source and the surface of the cell suspension was 11 cm.

Irradiation was achieved by subjection the suspension of bacterial cells to different doses (0, 1, 2, 3 and 4 J/m^2) of UV ray in a UV transiluminator, then 0.1 ml aliquots was taken from the irradiated cell suspension after each dose of irradiation, diluted properly and spread on LB agar plates and incubated in a dark place at 28ºC for 24 hrs. Viable counts and survivals of *S.marcescens* were determined after each dose treatment of incubation.

2.2.7.2 Chemical mutagenesis

Serratia marcescens was subjected to chemical mutagenesis using Mitomycin C. Mutagenesis was achieved according to Bowring and Morris, (1985) first by inoculating luria broth medium with fresh culture of the selected isolate of *S.marcescens* and incubated at 28ºC till mid log phase, then cells were pelleted by centrifugation at 6000 rpm for 15 min, washed twice with phosphate buffer and resuspended in 5ml of the same buffer and incubated with Mitomycin C, by adding 0.15 ml of Mitomycin C stock solution to sterile test tubes containing 5 ml of cell suspension to give final concentration of 30µg/ml then 0.1 ml aliquots of cell suspension was taken every 15 min. of incubation with mutagen, diluted properly and spread on LB agar plates and incubated at 28ºC for 24 hr. Viable counts and survivals of *S.marcescens* were determined after each treatment of irradiation.

Committee certification

We, the examining committee certify that we have read this thesis " Determination of the Optimum Conditions for Prodigiosin Production by the Locally Isolated *Serratia marcescens"* and examined the student "Israa Hasan Mahmood Dhahee" in its contents and that in our opinion , it is accepted for the Degree of Master of Science in Biotechnology.

(Chairman)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name:
Scientific Degree:
Date:

(Member)
Signature:
Name: Dr. Hameed Majeed
Scientific Degree: Assistant Prof.
Date:

(Member)
Signature:
Name: Dr. Abdul Kareem Jasim
Scientific Degree: Assistant Prof.
Date:

I, hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Laith Abdul-Aziz AL-Ani
Scientific Degree: Assist. Prof.
Title: Dean of the College of Science
Date:

Conclusion and Recommendation

Conclusions

- *Serratia marcescens* isolated from different environmental samples were able to produce prodigiosin.

- Among different isolates, *S.marcescens* S11 was the best in prodigiosin production.

- Optimum conditions for prodigiosin production by the locally isolated *S. marcescens* S11 involved the use of olive oil (1.5%), casein hydrolysate (1.5%), KH2PO4, at pH 8.0, and incubation at 28ºC.

- Physical and chemical mutagens may lead to prodigiosin over producer mutants for *S. marcescens* S11.

- Chemical mutagens using Mitomycin C are better than physical mutagens using UV radiation in increasing the prodigiosin productivity of *S. marcescens* S11.

Recommendations

- Large scale production of prodigiosin by *S.marcescens* S11 under the optimum conditions for production.

- Determine the optimum conditions for prodigiosin production by the most over-producer mutants raised after subjection to physical and chemical mutagens.

- Studying the effect of prodigiosin production by *S.marcescens* S11 as an antitumor agent.

- Genetic study on *S.marcescens* S11 responsible for prodigiosin production.

List of Abbreviations

Abbreviations	Full name
ATP	Adenosin Tri Phosphate
D.W.	Distilled Water
HBC	4-hydroxy-2,2′-bipyrrole-5-carbaldehyde
HBM	4-hydroxy-2,2′-bipyrrole-5-methanol
IC%50	Inhibitory Concentration %50
J/m^2	Joule/ meter square
LB	Luria Bertani
MMC	Mitomycin C
O. D.	Optical Density
PCE	Prodigiosin Condensing Enzyme
Pig	Pigment
rpm	Round per minute
Sp.	Species
Spp.	Specieses
UV	Ultraviolet
UVR	Ultraviolet Radiation

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الاهــداء

الــى الامــي الذي علم الامة ... الــى معلم الخلق وأمــام التقوى ... الرسول الاعظم مـحمـد (صلى الله عليه وسلم)

الــى نبراس دربي ... الــى الشـفاه التي اكثرت لـي الدعاء كلما نطقت ... الــى القـلب الطيب .... الــى معلمي و قدوتي ومثلي الاعلى في حياتي ... الــى من كان صبري حين عجزت ان اصبر...الــى نـهر العطاء وقدوة المربين والاباء...

أبــي العزيز

الــى مـن حملتني وهناعلى وهـن ... الــى نبع الحنان ... الــى مـن فرشت أيام عـمرها طريقاً لأيصالي ... الــى من سهرت فصارت طلباً لآمالي ... الــى من أضاءت لي عمرها لتنير لي كل الأزمان...الــى من طوقتني بالحب والحنان ...الى من لولاها لما كنت هـذا الأنسان...

أمــي العزيزة

الى القلوب الطيبة التي تنبض بالوفاء ... الى عزي و سندي في الحياة ...

أخــواتي
شيماء واسماء

الى رفيقة دربي ...الى من شاطرتني افراحي واحزاني...

أخـتـي رشـا

اهدي لكم ثمرة جهدي المتواضع..... اسـراء حـسن

بسم الله الرحمن

الرحيم

اللَّهُ نورُ السماواتِ والاَرضِ مثلُ
نُورهِ كِمشكاتٍ فيهَا مصَباحٌ
المصباحُ في زُجاجة الزُجاجةُ
كَأنها كوكبٌ دُريٌ يُوقدُ من
شَجَرةٍ مُباركة زَيتُونة لَا
شَرقية ولَا غَربية يكادُ زَيتُهَا
يُضيء ولوَ لَم تمَسسهُ نارٌ
نورٌ علي نُور يَهدي اللَّهُ لنُوره
من يشاءُ ويضربُ اللَّهُ الامثالَ
للنَاس واللَّهُ بكُل شيءٍ عليمٌ ۝

صدق الله العظيم

سورة النور
الاية{35 }

الخـــلاصـــــــة

جمعت57عينة من مختلف المناطق البيئية (مياه, مياه المجاري والتربة) ومن مواقع مختلفة في محافظة بغداد. وقد تم الحصول على 30 عزلة شخصت 15 عزله منها على إنها *Serratia* spp بعد التحري عن قابليتها على انتاج الصبغة الحمراء. بعد إجراء الفحوصات الكيموحيوية على هذه العزلات (15عزلة) تم تشخيص 5 عزلات منها على انها *S.marcescens* .

اختبرت قابلية العزلات المشخصة على إنتاج البرودجيوسين وقد أظهرت النتائج أن العزلة البكتيرية *S.marcescens* S11 كانت هي الأكفأ في الإنتاج، إذ بلغت كمية البرودجيوسين المنتج منها 200 وحدة/خلية.

درست الظروف المثلى لإنتاج البرودجيوسين بوساطة العزلة البكتيرية *S.marcescens* S11، وقد أظهرت النتائج أن الظروف المثلى للإنتاج تضمنت استخدام الوسط الإنتاجي الحاوي على زيت الزيتون بتركيز1.5 ٪ (مصدرا كاربونيا)، مستخلص الحليب الذائب بتركيز 1.5٪ (مصدرا نايتروجينيا)، فوسفات البوتاسيوم ثنائية الهيدروجين (مصدرا فوسفاتيا)، برقم هيدروجيني 8 وحضن بدرجة حرارة 28 درجة مئوية، وقد بلغت إنتاجية البرودجيوسين تحت هذه الظروف 3000 وحدة/ خلية.

تمت عملية التطفير لخلايا بكتيريا *S.marcescens* S11 لغرض زيادة انتاجية البرودجيوسين. انجز التطفير العشوائي باستعمال تطفير فيزيائي (الاشعة فوق البنفسجية) وتطفير كيميائي (Mitomycin C). اظهرت النتائج وذلك بتعريض *S.marcescens* S11 الى التطفير الفيزيائي (الاشعة فوق البنفسجية) والتطفير الكيميائي (Mitomycin C) للحصول على عدة مطفرات تميزت بقدرتها العالية على انتاج البرودجيوسين. وصلت انتاجية البرودجيوسين بعد التطفير الفيزيائي للطافرة (S11H7) الى 350 وحدة/خلية، بينما ارتفعت انتاجية البرودجيوسين بعد التطفير الكيميائي للطافرة (S11H5) الى 400 وحدة/خلية بالمقارنة بالنوع البري الذي كانت انتاجيته 200 وحدة/خلية.

جمهورية العراق
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كلية العلوم

تعيين الظروف المثلى لانتاج صبغة البرودجيوسين من بكتيريا *Serratia marcescens* المعزولة محليا

رسالة
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كجزء من متطلبات نيل درجة الماجستير في علوم/
التقانة الاحيائية

من قبل
اسراء حسن محمود ضاحي
بكالوريوس علوم تقانة احيائية (جامعة النهرين 2006)

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