THE ROLE OF RED PIGMENT PRODUCED BY *Serratia marcescens* AS ANTIBACTERIAL AND PLASMID CURING AGENT.

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ABSTRACT

The local isolate *Serratia marcescens* SM2 was selected in this study for its high productivity of the red pigment (prodigiosin).Which was estimated 255.21 (unit/cell).

The optimum conditions for pigment production were determined and it was noticed that the powder of peanut seeds medium was the best medium which reveales the higher production of pigment at 28 ºC and pH 8 for 72 hr.

The pigment was extracted using acetone and ethyl acetate and purified by organic solvents and thin layer chromatography (TLC), and it was found that prodigiosin has two peaks the higher peak was at 535.5 nm.

The inhibitory effect of prodigiosin on Gram positive and Gram negative bacteria was studied and it was found that it has a higher inhibitory effect on Gram positive bacteria than that on Gram negative.

Prodigiosin pigment was found to be a successful curing agent on plasmids of *E.coli* HB101 and *S.aureas* and failed to cure plasmids of *Proteus mirabilis* and *Enterococcus avium.*

INTRODUCTION

Gram negative bacteria of the *Serratia* are opportunistic human,plant and insect pathogens and are members of Enterobacteriaceae .*Serratia marcescens* has been isolated from soil , water , air , foodstuff , plant surface and animals.(17), it produce a spectrum of virulence factors including: chitinases,proteases,lipases,nucleases, bacteriocin which are capable of damaging human cells and tissues then cause infections of the respiratory tract , urinary tract , wounds , bloodstream and nosocomial infections that are clinically problematic because multidrug resistance is widespread within this species. (3,5,30)Most wild-type strains of *S.marcescens* produce a characteristic secondary metabolite,the red pigment prodigiosin(PG),which is a linear tripyrrole pigment have a low molecular weight(323.4)dalton appearing only in the late stages of bacterial growth.This pigment was isolated from other species such as:*Serratia plymuthica*, *Serratia rubidaea*,*Pseudomonas magnisiorubra* , *Hahella chejuensis*,*Vibrio gazogenes* and *Vibrio psycroerythreus*(1,20).The pigment has no defined role in the physiology of producing strains, but have been reported to have antifungal, antibacterial, algicidal, antiprotozoal / antimalarial activities , immunosuppressive and anticancer activities (8,19,25, 26, 29) .

Prodigiosin ($C_{20}H_{25}N_3O$)biosynthesis is a bifurcated process in which the monopyrrole 2-methyl-3-n-amylpyrrole (MAP) and bipyrrole 4- methyl -2, 2-bipyrrole -5- carboxyaldehyde (MBC) precursors are synthesized separately and then the final step involves the (PG) condensing enzyme (PCE) which condenses the (MAP) and (MBC) together, genes encodes (PG) biosynthesis located on either cell chromosome (9) or plasmids or both of them (15) .

MATERIALS AND METHODS

Bacterial isolates:Two isolates of *S.marcescens* isolated from milk were used in this study and identified according to(7,18),the other bacterial isolates used in this study included *Staphylococcus aureus*,*Bacillus subtilus*,*Staphylococcus saprophyticus*, *Streptococcus pyogenes* , *Enterococcus avium*, *Escherichia coli*,*Klebsiella pneumoniae*, *Aeromonas hydrophila*,*Pseudomonas aeruginosa*,*Proteus mirabilis* and *Escherichia coli* HB101, were obtained from Biotechnology Department/ Science College/Baghdad University .

ISOLATES SCREENING FOR PRODIGIOSIN PRODUCTION

The best isolate for (PG) production was determined by counting the amount of (PG) produced after the growth of the isolates in brain heart infusion medium for 24 hours at 37˚C by using visible spectrophotometer (Baush and Lamb) as described by (14) and according to the following equation

$$\text{Prodigiosin Unit/Cell} = \frac{[\text{O. D}_{499} - (1.3831 \times \text{O.D}_{620})] \times 1000}{\text{O. D}_{620}}$$

O . D : optical dencity
O . D_{499} : pigment absorbance
O . D_{620} : bacterial cells absorbance
1.3831 :constant
1000 : to avoid working with numbers smaller than one

OPTIMUM CONDITIONS FOR PRODIGIOSIN PRODUCTION

*** Media composition**: six different types of media were used which included Peptone glycerol broth, Nutrient broth,Powdered peanut seeds medium, Powdered sunflower seeds medium,Powdered sesame seeds medium and liquid minimal medium they were prepared as described by(13) to determine the optimum medium for (PG)production .

*** Temperature value** : to determine the optimum temperature for pigment production.The following temperatures were used 20,25,28,37,40˚C .

*** PH value** : The PH value used included 5,6,7,8 and 9.To determine the optimum PH for (PG) production .

*** Incubation period** : to determine the optimum incubation period for pigment production, The incubation periods used included 18,24,36,48 and 72 hours .

*** Extraction and purifiaction of prodigiosin**: (PG) was extracted using organic solvents ethyl acetate and aceton. This method was easy, fast, and gave a good yield of the pigment (13). Organic solvents were also used for pigment purification with TLC and the RF value was 0.65 (21,24).

*** Antibacterial activity of prodigiosin pigment**: antibacterial activity of (PG) was studied against different species of gram positive and gram negative bacteria.

*** Extraction of plasmid DNA**:plasmid DNA was isolated using salting-out method as described by(27).

*** Gel Electrophoresis**: plasmid profile of bacterial isolates was monitored using (0.8%)agarose gels as described by (23).

*** Plasmid curing**: the effect of (PG) pigment was tested as a curing agent for the first time to cure plasmids of different bacteria as described by(32).

RESULTS AND DISCUSSION

Identification of bacterial isolates: The two isolates *S.marcescens* SM1 and *S.marcescens* SM2 were identified depending on morphological and biochemical characteristics as shown in table (1) these results confirm that the two isolates are *S.marcescens***(18)** .

Table (1):- Morphological and Biochemical characteristics of *S.marcescens* isolates

Characteristics	*S.marcescens* sm1	*S.marcescens* sm2
Colony color	**red**	**red**
Cell shape	**rod**	**rod**
Gram stain	**–**	**–**
Growth on MacConkey	**+**	**+**
Catalase production	**+**	**+**
Oxidase production	**–**	**–**
Ureas production	**–**	**–**
DNase production	**+**	**+**
Gelatinase production	**+**	**+**
Motility	**+**	**+**
Production of prodigiosin	**230.42 unit/cell**	**255.21unit/cell**

(+): positive , (–): negative

Prodigiosin Production :

Results shown in figure (1) revealed that the isolate *S.marcescens* SM2 gave a high productivity of prodigiosin 255.21unit/cell as compared with the amount produced by *S.marcescens* SM1which was 230.42unit/cell (14).

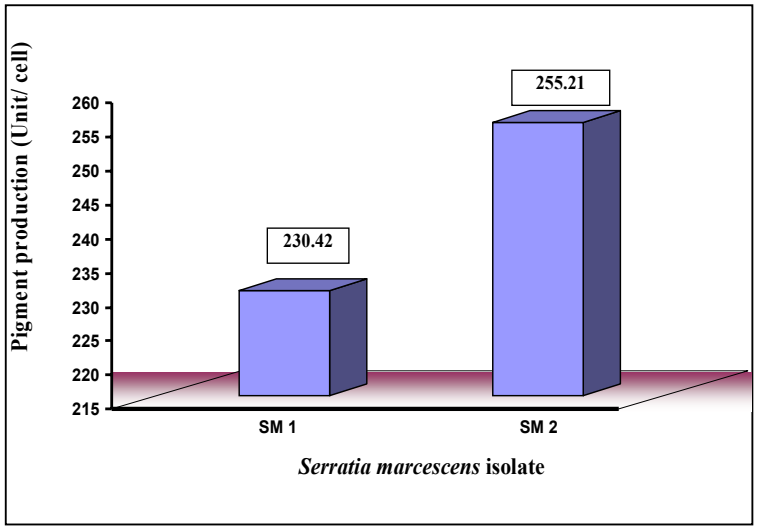

Fig (1):- prodigiosin production by Serratia marcescens isolates SM1and SM2

Optimization Of Growth Conditions For Prodigiosin Production :

The best media for prodigiosin production was powdered peanut seeds culture broth as shown in figure (2)since the amount of prodigiosin produced by the isolate SM2 in this culture was 255.21unit/cell the highest as compaired with other media these results were in agreement with those obtained by (13). This medium is a rich medium because it contains minerals, vitamins, saturated and unsaturated fatty acids (13). *S. marcescens* is also capable to produce lipase enzyme which degrade these fatty acid and could be used by the bacteria as a carbon source (13).

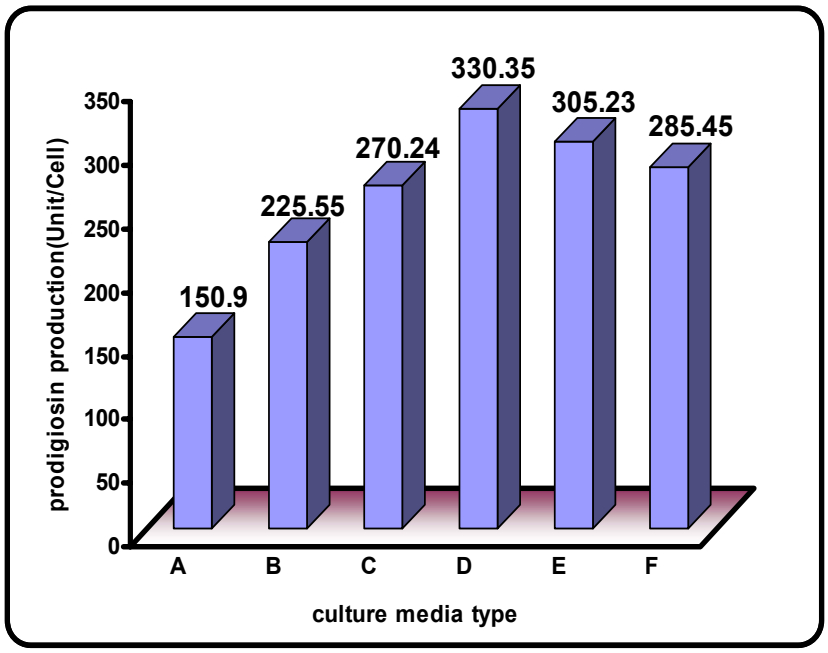

Fig (2):- Pigment produced from *S.marcescens* SM2 grown indifferent media at 37ºC for 24 hoours.
A-Peptone glycerol broth
B-Pigment production media
C-Nutrient broth
D-Powdered peanut media
E-Powdered sunflower seeds media
F-Powdered seasem seeds media

Regarding other optimum growth conditions studied ,the results showed that the optimum temperature was 28˚C at pH 8 and incubation period of 72 hours with good aeration.These results were in agreement with those obtained by (4).PG is a secondary metabolite appearing only in the late stages of bacterial growth specially when good aerobic conditions are available (12,22).

Antibacterial activity of prodigiosin pigment :

Results shown in table(2),and figures(3and4) indicated indicated that the prodigiosin antibacterial activity was higher against gram positive bacteria, including *Staphylococcus aureus , Staphylococcus saprophyticus , Bacillus subtilus , Enterococcus avium* and *Streptococcus pyogenes* as compared with gram negative bacteria such as *Echerichia coli , Pseudomonas aeruginosa , Aeromonas hydrophila , Proteus mirabilis* and *Klebsiella pneumoniae*.The antibacterial activity of prodigiosin (PG) is the result of their ability to pass through the outer membrane and to their capacity for inhibiting target enzymes , such as DNA gyrase and topoisomerase IV,which inhibit the cell growth (2).

Table (2):- Antibacterial activity of prodigiosin against Gram positive and Gram negative bacteria.

Bacterial Name	Diameter Of Inhibition Zones(Mm)
Staphylococcus aureus	14
Staphylococcus saprophyticus	10
Bacillus subtilus	12
Enterococcus avium	9
Streptococcus pyogenes	9.5
Echerichia coli	8.5
Klebsiella pneumoniae	7
Proteus mirabilis	4.5
Aeromonas hydrophila	6.5
Pseudomonas aeruginosa	6

Fig (3):- prodiogisin pigment inhibitory effect against *S.aureus*

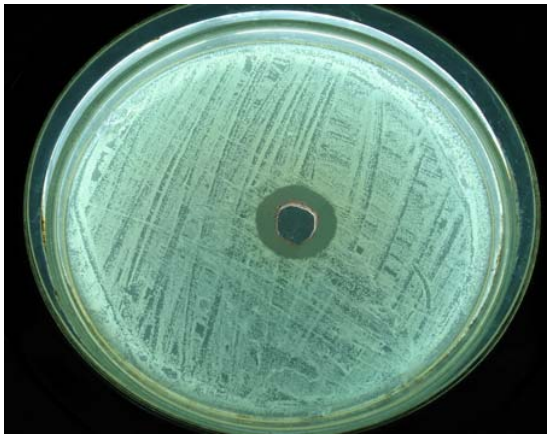

Fig (4):- prodiogisin pigment inhibitory effect against *E.coli*

Plasmid profile :The results are shown in figure (5) which indicate that the two isolates contain two small plasmid DNA bands , and these plasmid bands were approximately of the same size as compaired with pBR322 plasmid (4.3) kb,while other studies showed that, *S.marcescens* strains contain number of plasmids with differnt molecular weights ranged between 2-66 Megadalton (10) .

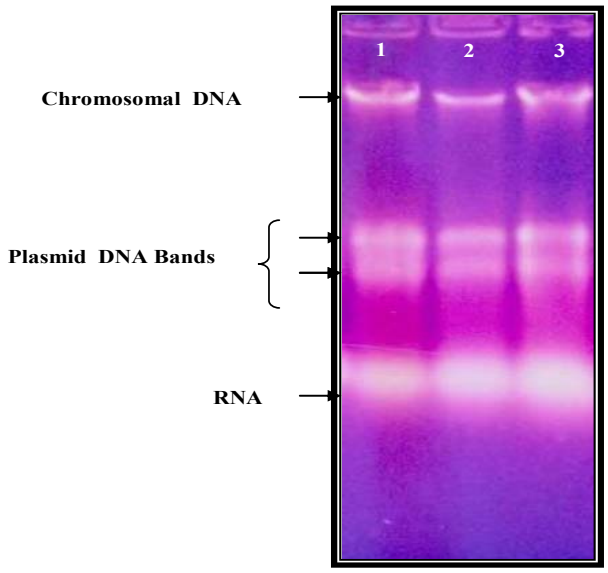

Fig (5):- 0.8% Agarose gel electrophoresis
Lane (1) whole genomic DNA of *E.coli* HB101
Lane (2) whole genomic DNA of *S.marcescens* SM1
Lane (3) whole genomic DNA of *S.marcescens* SM2

Plasmid curing :The results showed that prodigiosin is a powerful agent in eliminating plasmids of *E.coli* HB101 and *S.aureus* as shown in figures(6 and 7), and since prodigiosin is an intercalating agent , it cleavage DNA and cause complete inhibition of plasmid replication.(11,31,32).Curing experiments were failed when *Proteus mirabilis* and *Enterococcus avium* were used this may be releated to the large plasmids of these species and may be cured by using other curing agent (6).

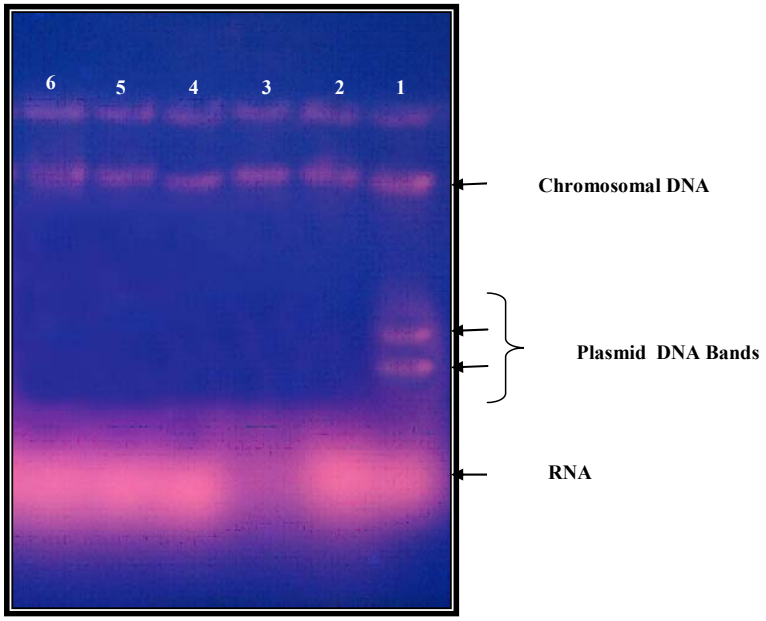

Fig (6):- 0.8% Agarose gel electrophoresis

Lane (1) whole genomic DNA for the standard strain E.coli HB101 (not cured)
Lane (2,3) whole genomic DNA of the cured *E.coli* HB101 treated with prodiogisin

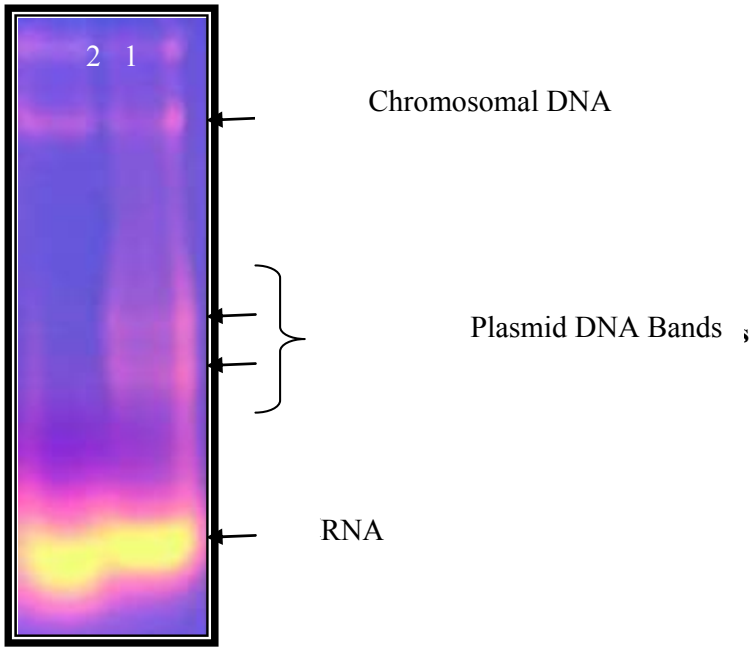

Fig (7):- 0.8% Agarose gel electrophoresis
Lane (1) whole genomic DNA of *S.aureus* (not cured)
Lane (2) whole genomic DNA of cured *S.aureus* treated with prodiogisin

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