Determination of Parameters Required to Enhance the Production of Prodigiosin by Serratia Marcescens with The Antimicrobial Activities Evaluation

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Abstract
Prodigiosin has been recognized as an important subject of numerous extensive studies, due to its antibacterial, antifungal, antiprotozoal, antimalarial, cytotoxic, antitumor, antioxidant, and nonsteroidal anti-inflammatory activities. The aim of this study investigation and reorganization the effect of different chemical and physical factors on the production of prodigiosin by Serratia marcescens and antimicrobial activity of prodigiosin were studied. The results showed the importance of some components such as (1.5% peptone, 3% sucrose, 0.3% glycerol, 0.1% Fe2(SO4)3 and 0.1 % BaCl2) to obtain an abundant amount of prodigiosin pigment by S. marcescens. Antimicrobial test revealed that prodigiosin pigment has an inhibitory role on S. mutans, S. aureus and B. subtilis, and this effect can be increased with increasing acidity at 35°C. In conclusion, Prodigiosin has antimicrobial activity that can be used to treat some of bacterial and fungal infections and the researcher can use many compound to enhance its bioactive activity.

Keywords: Serratia marcescens, prodigiosin, biochemical tests, optimized media, antimicrobial.

1. Introduction

S. marcescens is a Gram-negative bacterium that belongs to the Enterobacteriaceae family. It differs from other members of the family by producing prodigiosin and three unique enzymes: DNase, lipase, and gelatinase [1]. Various bacteria, notably S. marcescens, produce prodigiosin, a non-diffusible red pigment. This pigment is a secondary metabolite of these species, which appears clearly as a red color on starchy foodstuffs. Usually this pigment is formed at room temperature only in the presence of oxygen [2]. Prodigiosin inhibits the growth of various fungi, bacteria, protozoa, trypanosomes and viruses; also induces cell apoptosis in cancer cell lines [3, 4].

Plants and microbes are the two main sources of Prodigiosin [5, 6]. Since microorganisms can grow easily, faster, cheaper and less defective compared to plants regardless of environmental conditions, they are used in various fields such as research, medicine, and a variety of industrial applications [7]. This pigment is photosensitive and water insoluble, however it is soluble in chloroform, methanol, acetonitrile, dimethyl sulfoxide, and alcohol and ether in modest amounts. The production of secondary metabolites is usually influenced by physicochemical parameters such as temperature, oxygen, pH, and phosphate and metallic ion concentrations [8]. Several previous studies [9-11] have shown that several types of bacteria can resist the antibiotics currently in use, necessitating the development of an effective alternative treatment with fewer side effects. The aim of this research is to develop a culture medium for S. marcescens to produce a large amount of prodigiosin pigment and to investigate its antibacterial properties.

2. Material and Methods

Isolation and identification

Microbial isolation and identification were done in Microbiology Laboratory of Pharmacy Department in Asool Al-Deen University College in Baghdad during the period between December 2020 to March 2021. S. marcescens was identified based on biochemical tests such as (nitrate reduction, motility, indole, oxidase, methyl red, citrate utilization, hydrogen sulfide production, urea hydrolysis, Voges Proskauer and gelatin hydrolysis), and confirmed by studying the molecular characteristics by classical PCR using S. marcescens-specific primers UB-1492R (TACGGYTACCTTGTTACGACTT) and Sm-456F (GGTGAGCTTAATACGTTCATCA).

The PCR mixture was prepared in a total volume of 25 µL, which contains 5X master mix (Solis Biodyne, Tartu, Estonia), 1 µL of 10 pmol Sm-456F and 1 µL of 10 pmol UB-1492R primers, 3 µL DNA and 15 µL milli Q water.

The thermal cycler (BIORAD thermocycler T100, USA) was programmed into: Initial denaturation for 5 minutes at 95°C, followed by 30 cycles of denaturation for 1 minute at 95°C, annealing for 30 seconds at 58°C, extension for 90 seconds at 72°C, and a final extension of 7 minutes at

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72°C. TAE buffer was used to evaluate the PCR generated product on a 1.2 percent agarose gel electrophoresis. The generated DNA patterns were captured and evaluated using a gel documentation system under UV light and a transilluminator (Herolabs, Germany).

3. Optimization of the Media

Selection of the media

Different media were prepared (Nutrient agar, blood agar, peptone glycerol agar, glycerol beef extract agar, bile salt agar, gelatin agar, glycerol yeast extract agar and tryptone soy bean agar). S. marcescens was inoculated on all agar media and incubated at 25°C for 24-48h. After the incubation period a red pigment was formed on agar media. The pigment was scraped off with a scraper and dissolved in 95% methanol in labeled centrifuge tubes. Centrifugation was performed at 4°C, 5000 rpm for 20min and the supernatants were transferred to sterile tubes. Using methanol as a blank, the optical density (O.D) of the samples was determined at a wavelength of 490nm.

Selection of the best wavelength for reading the O.D of prodigiosin production

The supernatant of the selected medium was re-measured at different wavelengths (450-500nm) using methanol as a blank.

Selection of carbon source

The selected medium was prepared and equally distributed in 8 bottles. Then, 1% of different carbon sources were added to the respective bottles (glucose, fructose, galactose, maltose, sucrose and lactose), while the remaining parameters were fixed.

Effects of varying carbon source concentrations on prodigiosin production

The selected medium was prepared with different concentrations of the selected carbon sugar (0.0 control, 0.5, 1.0, 1.5 and 3.0% w/v), while the remaining parameters were fixed.

Selection of nitrogen source

The selected medium was prepared with the effective concentration of the selected carbon source and equally distributed in 10 bottles. Then, 1% of different nitrogen sources were added to the respective bottles (peptone, yeast extract, tryptone, beef extract, NH4Cl, (NH4)2SO4 and NaNO3), while the remaining parameters were fixed.

Effects of varying carbon source concentrations on prodigiosin production

The selected medium was prepared with different concentrations of the selected nitrogen sugar (0.0 control, 0.5, 1.0, 1.5 and 3.0% w/v), while the remaining parameters were fixed.

Effects of various glycerol concentrations on prodigiosin production

The optimized medium was prepared with the optimum concentrations of the best carbon and nitrogen sources. Then, different concentrations of glycerol (0.0 control, 0.1, 0.3, 0.5, 1.0, 1.5 and 3.0% v/v) were used, while the remaining parameters were fixed.

Effect of various Iron sources on prodigiosin production

The optimized medium was prepared and 1% of different iron sources were added to the respective bottles (ferrous sulphate, ferric sulphate, ferric chloride and ferric tartrate), while the remaining parameters were fixed.

Effect of sodium chloride (NaCl) on prodigiosin production

The optimized medium was prepared with different concentrations of NaCl (0.0 control, 0.1, 0.3, 0.5, 1.0, 1.5 and 3.0% w/v), while the remaining parameters were fixed.

Effect of heavy metals on prodigiosin production

The optimized medium was prepared with 1% of different heavy metals (lead acetate, barium chloride, magnesium chloride, mercuric chloride, zinc chloride and copper sulphate), while the remaining parameters were fixed.

Effect of different temperatures on prodigiosin production

S. marcescens was inoculated on the optimized medium and incubated at different temperatures (20, 23, 25, 28, 30, 33, 35, 38 and 40°C) for 24-48h.

Effect of different periods of incubation on prodigiosin production

S. marcescens was inoculated on the optimized medium and incubated at 25°C for different incubation periods (24, 48, 72, 96 and120h).

Prodigiosin pigment Extraction and purification

Extraction

Prodigiosin pigment was extracted by scraping the pigment from the agar medium using a scraper and dissolving it in 95% methanol before being centrifuged at 4oC, 5000rpm for 20min. Centrifugation was repeated twice.

Purification

A separating funnel was used to purify the prodigiosin pigment. Briefly, equal volumes of extracted pigment and petroleum ether were poured in a separating funnel and the mixture was shaken for 20min. Later, the precipitate was collected in plates and some amount of chloroform was added to the plates, and left to dry completely at 50°C. Then, the dried pigment was re-suspended in chloroform and stored at 4°c.

Antimicrobial activity

The antibacterial effect of prodigiosin pigment on several bacterial and fungal species was investigated using the agar well diffusion technique (chloroform was used as a control). Moreover, the purified pigment was adjusted at different pH (4, 7 and 9) and treated with different temperatures (20, 35 and 50oC) to study the effects of different pH and temperatures on the antimicrobial activities of prodigiosin

pigment.

4. Results

The results of biochemical tests of *S. marcescems* are shown in (Table 1).

Table 1: Results of biochemical tests

TESTS	RESULT
Gram stain	-
Oxidase	-
Indole production	-
Methyl Red	-
Voges-Proskaeur	+
Citrate (Simmons)	+
H2S production	-
Urea hydrolysis	-
Nitrate reduction	+
Motility	+
Gelatine hydrolysis	+
Prodigiosin pigment	+
Acid from lactose	-
Acid from glucose	+
Acid from mannitol	+
Acid from sucrose	+

Selection of the media

Among all selected media, peptone glycerol agar and glycerol beef extract agar clearly supported bacterial growth more than other media (Figure 1).

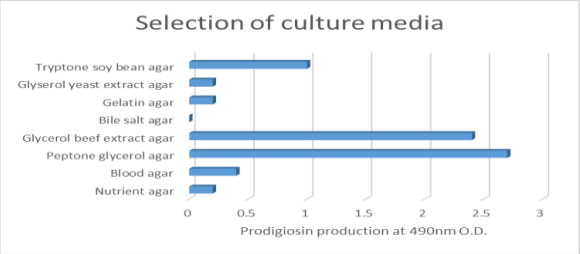

Figure 1: Selection of the best medium

Selection of the best wavelength.

All the different wavelengths used to measure the OD of the sample were almost close. Therefore, 490nm was chosen as the best wavelength (Figure 2).

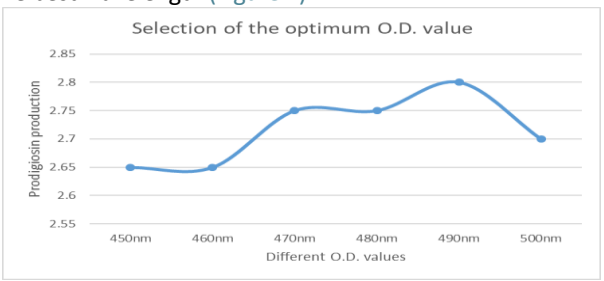

Figure 2: Selection of the absorption maxima

Selection of carbon source

Sucrose, fructose and lactose were giving the best readings respectively, while the glucose inhibited the pigment production (Figure 3).

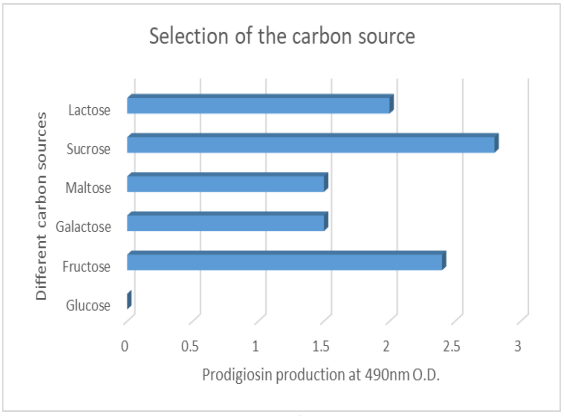

Figure 3: Selection of the carbon source

Effects of different concentrations of carbon source

The pigment productions were increased respectively with the increasing of the concentration of the sucrose (Figure 4).

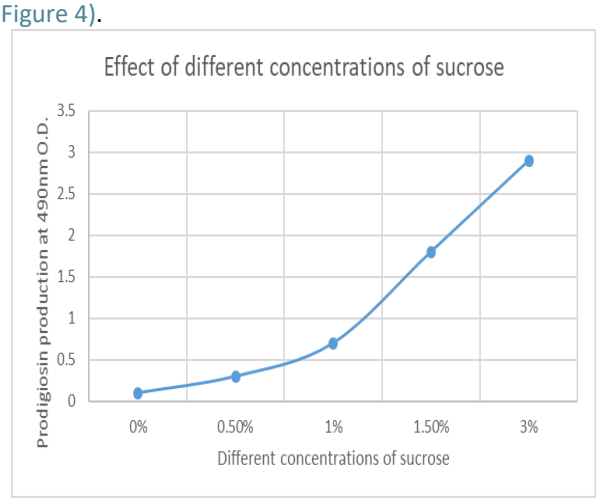

Figure 4: Effects of different concentrations of carbon source

Selection of nitrogen source

Among the organic nitrogen sources; Peptone, beef extract, tryptone and yeast extract showed effects on pigment production respectively, while inorganic nitrogen sources did not support pigment productions (Figure 5).

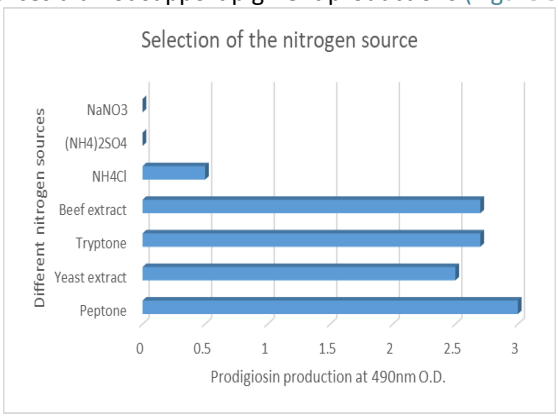

Figure 5: Selection of the nitrogen source

Effects of different concentrations of nitrogen source

Differences in peptone concentration showed slight variance in prodigiosin production (Figure 6).

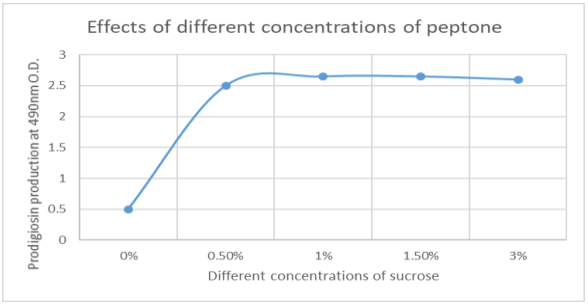

Figure 6: Effect of different concentrations of nitrogen source

Effects of different concentrations of glycerol

There was a slight increasing in prodigiosin production when the glycerol concentration was less than 0.5%. However, increasing the concentration of glycerol by more than 0.5% showed no significant effect on prodigiosin production (Figure 7).

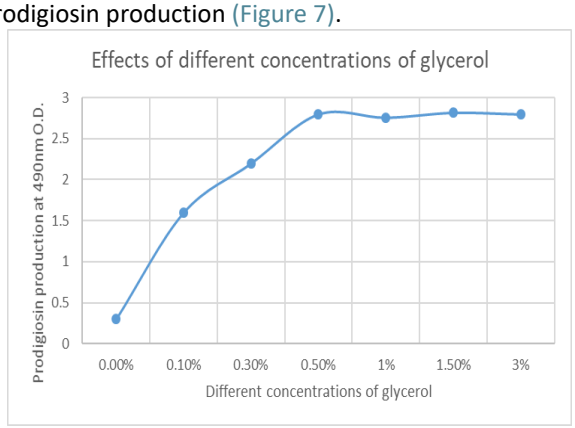

Figure 7: Effect of different concentrations of glycerol

Effects of different iron sources on prodigiosin production

Prodigiosin production was inhibited when ferrous sulphate was used as an iron source. On the contrary, ferric sulphate enhanced the production of prodigiosin. Moreover, ferric chloride and ferric tartrate also enhanced prodigiosin production, but less than ferric sulphate (Figure 8).

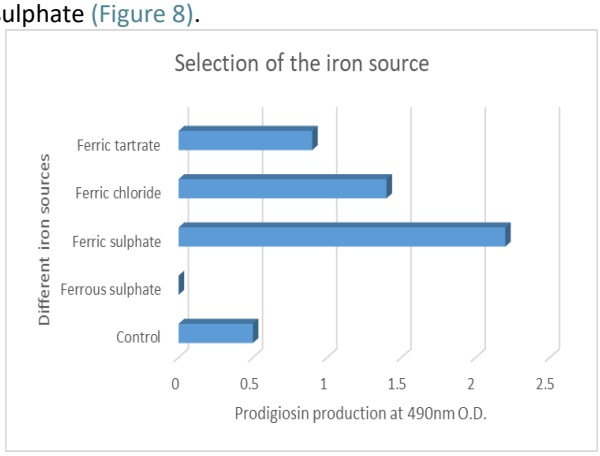

Figure 8: Effect of different iron sources on prodigiosin production

Effects of sodium chloride (NaCl) on prodigiosin production

The result showed that increasing the concentration of NaCl reduces the production of prodigiosin by S. marcescens (Figure 9).

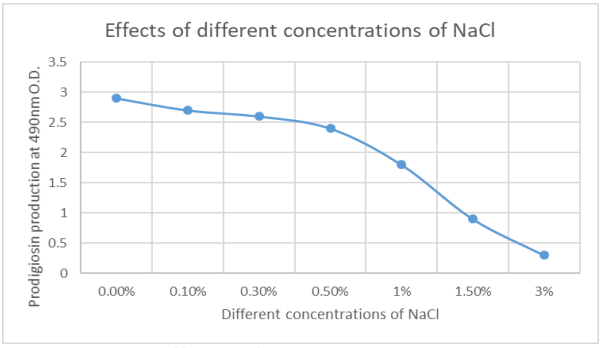

Figure 9: Effects of sodium chloride (NaCl) on prodigiosin production

Effects of heavy metals on prodigiosin production

Heavy metals such as (barium chloride, magnesium chloride and copper sulfate) showed significant effects on pigment production. Moreover, mercuric chloride and zinc chloride inhibited the pigment production (Figure 10).

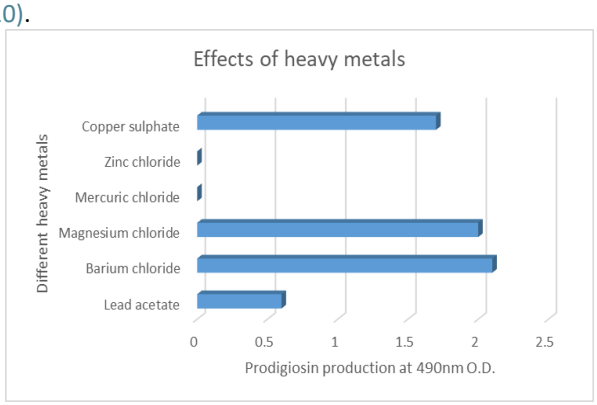

Figure 10: Effects of heavy metals on prodigiosin production

Effects of different temperatures on prodigiosin production

The result showed that S. marcescens produces prodigiosin pigment at 20-30°C with an optimum temperature of 25°C, while increasing the temperature to more than 30°c inhibits pigment production (Figure 11).

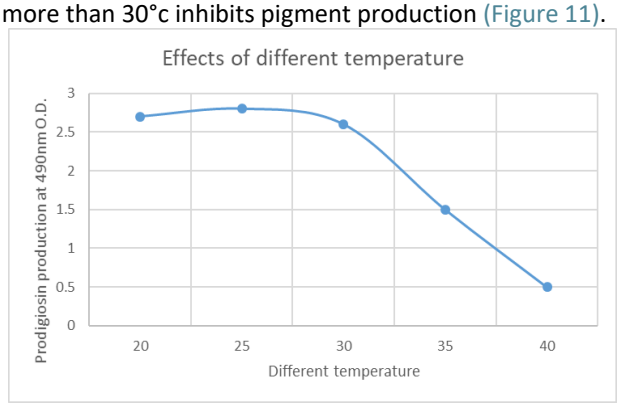

Figure 11: Effect of different temperatures on prodigiosin production

Effect of different incubation period on prodigiosin production

There was a slight increase in the pigment production with increasing incubation period (Figure 12).

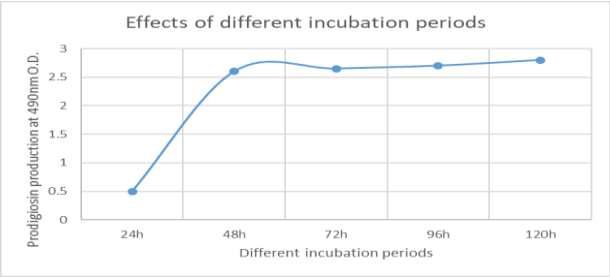

Figure 12: Effect of different incubation period on prodigiosin production

Optimal medium composition (gram / liter)

The required ingredients for optimal production of prodigiosin from S. marcescens are shown in (Table 2).

Table 2: The ingredients of optimized medium	
Ingredients	**Composition (gram / liter)**
Peptone	15 gm
Sucrose	30 gm
Glycerol	3 ml
Fe2(SO4)3	1 gm
BaCl2	1 gm
Distilled water	1000 ml
pH	7.2
Agar	20 gm

Antibacterial activity

Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans, S. aureus ATCC 25923, Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, and Pseudomonas aeruginosa were employed to test prodigiosin pigment's antibacterial activity. The result was recorded by measuring the diameter of the inhibition zone. Prodigiosin pigment was found to be more efficient against Gram-negative bacteria than Gram-positive bacteria (Table 3).

Table 3: Antibacterial activity			
Bacterial culture	**Inhibition zone/mm Chloroform (Control)**	**Inhibition zone/mm Prodigiosin**	**Inhibition zone/mm Pure effect of Prodigiosin**
B. subtilis	9	11	2
S. aureus	0	8	8
S. mutans	9	18	9
S. aureus ATCC 25923	8	15	7
E. coli	0	0	0
P. mirabilis	6	11	5
K. pneumonia	7	13	5
P. aeruginosa	6	11	
Effects of pH on the antibacterial activity of prodigiosin pigment			

The variation in the pH value affects the antibacterial activity of prodigiosin; The higher the acidity, the greater the effect. In addition, prodigiosin pigment treated at 35°C showed effective antibacterial activity compared to 20°C and 50°C (Table 4).

Table 4: Effect of pH and Temperature on the antibacterial activity of prodigiosin pigment							
Bacterial culture	**Control**	**pH**			**Temperature**		
		4	**7**	**9**	**20oC**	**35oC**	**50oC**
B. subtilis	10 mm	13 mm	13 mm	10 mm	12 mm	14 mm	10 mm
S. aureus	7 mm	22 mm	18 mm	14 mm	12 mm	16 mm	10 mm
S. mutans	7 mm	19 mm	18 mm	10 mm	18 mm	20 mm	13 mm
S. aureus ATCC 25923	6 mm	12 mm	12 mm	9 mm	10 mm	14 mm	8 mm
E. coli	0 mm	7 mm	0 mm	0 mm	10 mm	10 mm	11 mm
P. mirabilis	6 mm	12 mm	10 mm	0 mm	13 mm	12 mm	10 mm
K. pneumonia	6 mm	14 mm	13 mm	9 mm	11 mm	12 mm	12 mm
P. aeruginosa	7 mm	17 mm	11 mm	13 mm	11 mm	10 mm	m

Antifungal activity

Fungal species such as (Candida albicans, Candida parapsilosis, Trichophyton mentagrophytes, Trichophyton rubrum, Cryptococcus neoformans, Aspergillus oryzae, Aspergillus flavus and Aspergillus niger) were used to study the antifungal activity of prodigiosin pigment. The result was recorded by measuring the diameter of the inhibition zone and compared with the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The results revealed that Candida spp. are more sensitive to prodigiosin pigment than others, while T. mentagrophytes and A. flavus showed resistance to the pigment (Table 5).

Table 5: Antifungal activity			
Fungal cultures	**Chloroform (Control)**	**Prodigiosin**	**Pure effect of Prodigiosin**
C. albicans	10 mm	15 mm	5 mm
C. parapsilosis	15 mm	20 mm	5 mm
T.mentagrophytes	0 mm	0 mm	0 mm
T. rubrum	10 mm	12 mm	2 mm
C. neoformans	10 mm	12 mm	2 mm
A. oryzae	6 mm	8 mm	2 mm
A. flavus	0 mm	0 mm	0 mm
A. niger	6 mm	10 mm	mm
Effects of pH and temperature on the antifungal activity of prodigiosin pigment			

Variation in the pH value and temperature of the prodigiosin pigment showed no effect on the antifungal activity of the pigment.

5. Discussion

Prodigiosin is a red pigment belonging to the tripyrrole family that has been found to have antimicrobial, immunomodulating, antitumor, and antimalarial properties. The current study focused on modifying the optimal medium for efficient production of prodigiosin from S. marcescens and evaluation of the antimicrobial activities of the pigment. Biochemical tests of the isolate were identical with several previous studies [12-17], which showed that the isolate was

S. marcescens. Previous studies [18, 19], revealed that the primers Sm-456F and UB-1492R are specific for S. marcescens, which were identical with the results of the current study and confirmed the results of biochemical tests. Several studies [20-23], revealed that when glycerol is used as a carbon source, the production of prodigiosin from S. marcescens is clearly increased. In our study different media were used to produce prodigiosin from S. marcescens, among which media containing glycerol showed greater production of the pigment than other media.

A study of [24], revealed the negative effect of glucose on prodigiosin production, suggesting that glucose inhibits cAMP production, which is a positive regulator of pigment production. However[25] indicated that cAMP has negative effect on prodigiosin production, and that glucose inhibits prodigiosin production in a cAMP-independent manner. Furthermore[25-29] proposed that HexS may be the major regulator involved of the inhibition of prodigiosin by glucose. Whatever the reason, all studies pointed to the negative effect of glucose on the production of the pigment prodigiosin, which is completely consistent with the result of this study. Although, other carbon sources (sucrose, fructose and lactose) were effective in pigment production, sucrose was chosen as the best carbon source, consistent with previous studies [25-30].

The type and concentration of the nitrogen source plays an important role in the growth and pigment production of S. marcescens. In this study, all the organic nitrogen sources exhibited a positive effect on bacterial growth and pigment production, while the inorganic nitrogen sources inhibited the pigment production. These results were in harmony with previous studies [23, 31], which indicate that ammonium is a poor nitrogen atom donor and that organic nitrogen sources such as peptone contain different amino acids that increase the production of prodigiosin.

Glycerol can act as a carbon source for bacterial growth and also as a 'prodigiosin inducer', as it can increase prodigiosin production 7 times [21, 31]. The present study suggests that 0.5% is an appropriate concentration of glycerol that enhances prodigiosin production.

Previous studies[32-34] revealed that iron enhances prodigiosin production. In this study, ferric sulphate, ferric chloride and ferric tartrate enhanced pigment production, while ferrous sulphate inhibited pigment formation by S. marcescens. Suggesting that only ferric irons can enhance the production of prodigiosin by S. marcescens.

Previous studies [33, 35, 36], suggested that NaCl inhibited the synthesis of prodigiosin either partially or completely. In addition [33] indicated that high concentrations of NaCl may inhibit synthesis of the condensing enzyme or may permit synthesis of the condensing enzyme but inhibit its activity. Which was compatible with our study.

Previous studies [37-39], revealed the effect of heavy metals on prodigiosin production by S. marcescens. In the same manner, the present study detected that BaCl2, MgCl2, and CuSO4 have a positive effect on proigiosin production, while HgCl2 and ZnCl2 have a negative effect. However [37] suggested that heavy metal may accelerate the aging process in individual cells.

[40] found that biosynthesis of prodigiosin pigment by S. marcescens occurred over a relatively narrow range of temperatures with maximal production being between 24 - 28ºC, although the bacteria grow over a broad range of temperature. Similarly, in the present study, temperatures between 20-30°C were found to be the optimal range for prodigiosin synthesis, while at temperatures above 30°C the pigment formation was clearly decreased. This study also revealed that S. marcescens can grow at 37˚C or above, as white colonies with complete inhibition of pigment formation, the similar result was obtained [27, 40].

The incubation period may slightly affect the amount of the pigment to be synthesized. The longer the incubation period, the greater the pigment formation. However, since the pigment production increases slightly with respect to the incubation period, 48 hours may be sufficient.

An antimicrobial test showed that the prodigiosin pigment has an inhibitory effect on the tested Gram-positive bacteria at room temperature when the pH is neutral, while the tested Gram-negative bacteria are resistant to the pigment. However, increasing the acidity of the pigment showed a positive effect on the antimicrobial activity of the pigment on both Gram-positive and Gram-negative bacteria at an optimum temperature of 35°C. In addition, the pigment showed an inhibitory role on the growth of some fungi such as C. albicans, C. parapsilosis and A. niger, while the variation in pH value and temperature did not show any significant effect. The findings of this study were in harmony with previous studies [32], which revealed the inhibitory role of prodigiosin pigment on Gram-positive bacteria and some fungi.

6. Conclusion

Prodigiosin is a secondary metabolite produced by S. marcescens bacterium, which appears clearly in red on starchy foods. The present study showed the required components to obtain the optimum amount of this pigment and revealed that prodigiosin pigment has an inhibitory role on S. mutans, S. aureus and B. subtilis, and this effect can be increased with increasing acidity at 35°C. Further studies are needed to determine the effective ingredient of this pigment, which can be as a treatment for some bacterial and fungal infections.

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