Malaysian Journal of Microbiology, Vol 20(1) 2024, pp. 1-6
DOI: http://dx.doi.org/10.21161/mjm.230256

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

A study of prodigiosin extracted from *Serratia marcescens* and its purification using the preparative-HPLC technique

Fatima Riyadh Mohammad* and Ghada Abdulrazzaq Mohammad

Department of Biology, College of Science, University of Mosul, Mosul, Iraq.
Email: fatimariyad95@gmail.com

Received 9 April 2023; Received in revised form 11 August 2023; Accepted 30 August 2023

ABSTRACT

Aims: The aim of this study was to determine which natural and inexpensive materials induced the highest production of prodigiosin pigment in local *Serratia marcescens* isolates. Furthermore, this study focused on purifying and identifying a single red pigment among several pigments in the crude extract of *S. marcescens* by HPLC.
Methodology and results: Two isolates of *S. marcescens* (S1 and S2) were isolated from urine and a urinary catheter. Isolates were identified based on the red color of colonies when growing on nutrient agar medium incubated at 28 °C, which gave an adverse reaction to Gram stain; the diagnosis was completed with several biochemical tests. The highest yield of this pigment was investigated using Luria-Bertani (LB) medium supplemented with available materials (sesame, peanut, and coconut meat seed powders). Results showed that LB containing sesame powder medium induced the highest prodigiosin production in S1 and S2 isolates (179.398 and 107.280 unit/cell, respectively). On the other hand, S1 and S2 isolates on LB supplemented with peanut medium produced 150.492 and 93.970 units/cell of prodigiosin, respectively. However, coconut meat supplement through LB failed to induce bacteria to synthesize the pigment. The pigment was identified in a retention time equal to 2.2 min through crude extraction and prodigiosin (with red color) was purified successfully by the preparative-HPLC technique.
Conclusion, significance and impact of study: This study successfully showed that natural and inexpensive products were able to induce prodigiosin pigment production from local *S. marcescens* isolates. Results showed that sesame seed powder was the best carbon source that induced prodigiosin, followed by peanut seed powder. Prodigiosin was identified and purified successfully by the preparative-HPLC technique. Research findings suggest that low-cost materials could be used to reduce the cost of prodigiosin production.

Keywords: Coconut meat, peanut, preparative-HPLC technique, prodigiosin, *Serratia marcescens*, sesame

INTRODUCTION

Serratia marcescens is Gram-negative rod-shaped bacterium. It belongs to the Enterobacteriaceae family, displaying a high genetic plasticity that allows it to adapt and persist in multiple niches, including soil, water, plants, and hospital environments (Procop *et al*., 2017). This species is differentiated from other enteric bacteria by its ability to produce a red pigment. However, many species of *Serratia* are non-pigmented and others vary widely in their pigmentation production levels (Krithika and Geetha Ramani, 2013). Prodigiosin is a natural red pigment that can be produced by various bacterial species with *S. marcescens* being the main producer (Santos *et al*., 2021; Sebastian *et al*., 2022). Prodigiosin is a secondary metabolite with a tripyrrole structure and has the chemical formula $C_{20}H_{25}N_3O$, which exhibits a broad spectrum of biological properties (Liu *et al*., 2021). Prodigiosin has three pyrrole rings, two of which are linked together and the third is coupled to methane, thus creating a connection between pyrrolopyrrole and methane (Ibrahim *et al*., 2014). A cluster of operonic genes known as pig A-N regulates the production of prodigiosin in *S. marcescens* (Williamson *et al*., 2005).

Prodigiosin has several biological activities, such as antibacterial, antifungal, immunosuppressive, anticancer and antioxidant (Giri *et al*., 2004). In addition, prodigiosin has been applied as a natural colorant of candles, textile materials, soap, and paper, and also as a biodegradable ink (Venil *et al*., 2013; Ren *et al*., 2021; da Silva *et al*., 2022; Liu *et al*., 2022).

Some researchers have used commercial media such as nutrient broth, beef extract, Luria-Bertani (LB) broth and yeast extract for prodigiosin biosynthesis (Lin *et al*., 2019; Paul *et al*., 2022). Industrial commercialization is still limited because of the high cost of production, mainly due to the use of expensive substrates. To lower the cost of prodigiosin production, some researchers have used

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

cheap organic materials for fermentation (Luti *et al*., 2018; Santos *et al*., 2021; da Silva *et al*., 2022).

The pathway for prodigiosin production, as like other pathways for bacterial pigment production, is affected by the type of carbon and nitrogen source added in the medium (Arivizhivendhan *et al*., 2015; Mohammad and Daod, 2019; Valentina *et al*., 2019). Recent studies have shown that adding different oils and seeds to the medium stimulates pigment production (Islan *et al*., 2022). In addition, sodium chloride at specific concentrations has been shown to stimulate several pathways for pigment production in bacteria (Wei and Chen, 2005).

The aim of this study was to determine which natural and inexpensive materials induced the highest production of prodigiosin pigment from local *S. marcescens* isolates. Furthermore, this study focused on purifying and identifying a single red pigment among several pigments present in the crude extract of *S. marcescens* by HPLC.

MATERIALS AND METHODS

Bacterial isolates

Two isolates of *S. marcescens* (S1 and S2) were isolated from the urinary catheter and urine. These isolates were diagnosed by culturing the samples on nutrient agar and MacConkey agar, Gram stain of pure bacterial isolates, as well as many biochemical tests including catalase, oxidase, indole, Voges-Proskauer, citrate utilization, TSI, urease, motility, phenylalanine deaminase, arginine dihydrolase and ornithine decarboxylase (Tille *et al*., 2017; de la Maza *et al*., 2020).

Prodigiosin production

Luria-Bertani broth (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L) was used for the cultivation of two *S. marcescens* isolates. Available, inexpensive natural materials were added to the medium separately to stimulate the production of prodigiosin pigment. Powders obtained from sesame, peanut and coconut meat were added to LB with a percentage of 2% (Wei and Chen, 2005). The pH of the medium was adjusted to 7.2 before autoclaving. After sterilization and cooling, each flask of LB broth with a particular material was inoculated with 2% of each bacterial isolate and then incubated in a shaker incubator at 30 °C and 200 rpm for 72 h.

Prodigiosin extraction

Serratia marcescens in Luria-Bertani broth was centrifuged at 6000 rpm for 20 min. The supernatant was discarded and the pellet was suspended in 95% methanol to extract the pigment from the cells. The mixture was vortexed for 2 min and re-centrifuged for 20 min at 6000 rpm. The extract was filtered by using sterile medical gauze. The filtrate was placed in an electric oven at 60 °C to obtain the pigment as a dry powder and the extract was stored in a glass tube covered with aluminum foil (Chen *et al*., 2013).

Presumptive test

Before being oven-dried, 2 mL of filtered pigment was placed in two test tubes. One of them was acidified with a drop of concentrated HCl and the other was alkalinized with a drop of concentrated NaOH solution. A red or pink color in the acidified solution and a yellow color in the alkaline solution indicates a positive result for the detection of prodigiosin (da Silva *et al*., 2022).

Determination of prodigiosin concentration

The concentration of prodigiosin was estimated using the following equation according to Mehta and Shah (2015).

Prodigiosin unit/cell
$= [OD_{534}-(1.381 \times OD_{620})] \times 1000/OD_{620}$

Where: OD_{534} = pigment absorbance, 1.381 = constant, OD_{620} = bacterial cell absorbance.

Pigment purification

Crude prodigiosin was purified by preparative-HPLC. The parameters of C18 column (4.6 × 100 mm), mobile phase: acetonitrile/water (60:40, v/v), flow rate 0.8 mL/min were achieved according to Faraag *et al*. (2017) who obtained a retention time equal to 2.062 min of prodigiosin, which consequently was employed to compare our findings. The supernatant was subjected to a UV-visible spectrum scanning in the 200-800 nm range.

RESULTS AND DISCUSSION

S1 and S2 bacterial isolates carrying characteristics of *S. marcescens* were identified by observing the red color of the colonies on nutrient agar, as shown in Figure 1. The cells were Gram-negative rods; in addition, the results of biochemical tests for diagnosing *S. marcescens* are shown in Table 1.

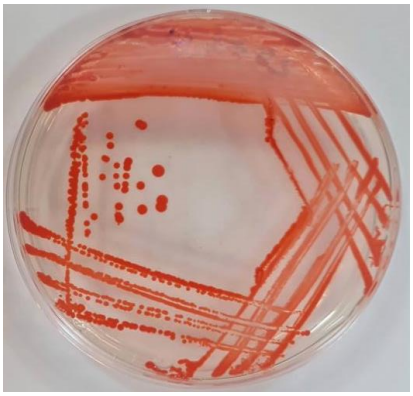

Figure 1: *Serratia marcescens* growth on nutrient agar medium after incubation at 28 °C for 72 h.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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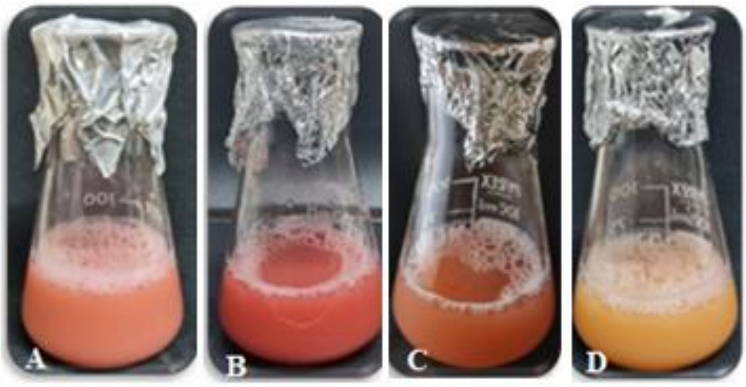

Figure 2: The color intensity of *S. marcescens* (S1) pigment in LB medium containing different materials after 72 h of incubation at 30 °C. A: LB; B: LB + Sesame; C: LB + Peanut; D: LB + Coconut meat.

Table 1: Biochemical tests for *S. marcescens* diagnosis.

Tests	Results
Catalase	+Ve
Oxidase	-Ve
Indole	-Ve
Voges-Proskaeur	+Ve
Citrate utilization	+Ve
TSI	K/A, -Ve H_2S, -Ve gas
Urease	+Ve
Motility	+Ve
Phenylalanine deaminase	-Ve
Arginine dihydrolase	-Ve
Ornithine decarboxylase	+Ve

Table 2: Concentrations of prodigiosin produced from *S. marcescens* grown on LB medium with different supplements at 30 °C for 72 h.

Medium with natural materials	Prodigiosin concentration (unit/cell)	
	S1	S2
LB	65.036	12.566
LB + Sesame seeds	179.398	107.283
LB + Peanut seeds	150.492	93.970
LB + Coconut meat seeds	0	0

Prodigiosin's production

Adding sesame and peanut powder to the LB medium produced a prodigiosin pigment with a deeper color than the LB medium without additives. However, in the medium to which coconut meat was added, no color was observed, no prodigiosin was created (Figure 2).

The results indicated that all-natural substances used except coconut meat positively increased pigment production from *S. marcescens*. Table 2 shows the superiority of isolate S1 over isolate S2 in all the tested materials added to the LB medium. The sesame seed powder added to the medium apparently gave the highest value for prodigiosin production. Pigment production reached 179.398 unit/cell for isolating S1 while isolating S2 gave 107.280 unit/cell. For peanuts, the pigment concentration values for both isolates were 150.492 and 93.970 units/cell, respectively. The medium supplemented with coconut meat failed to induce prodigiosin production.

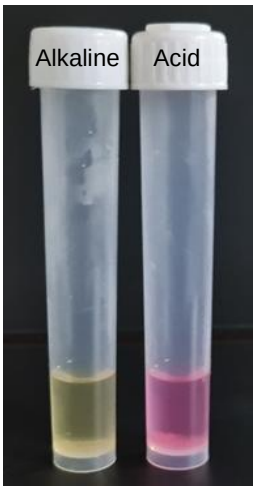

Figure 3: Presumptive test for prodigiosin pigment**.**

The results of our study showed that the powder of sesame and peanut seeds added to the LB medium gave the best production of prodigiosin pigment by *S. marcescens* compared to the LB medium alone. These results are consistent with the results of previous studies (Shahitha and Poornima, 2012; Al-Bakri and Al-Bederi, 2016; Bhagwat and Padalia, 2020). Seeds consist of vitamins and saturated and unsaturated fatty acids and these components differ from seed to seed (Srimathi *et al*., 2017). The medium containing fatty acids is considered a rich medium as it contains minerals, vitamins, saturated fatty acids and unsaturated fatty acids since *S. marcescens* is capable of producing lipase enzyme, which in return degrades these fatty acids and uses them as a carbon source (El-Bialy and El-Nour, 2015). The reason for not producing prodigiosin in coconut meat powder medium is that it contains 50%

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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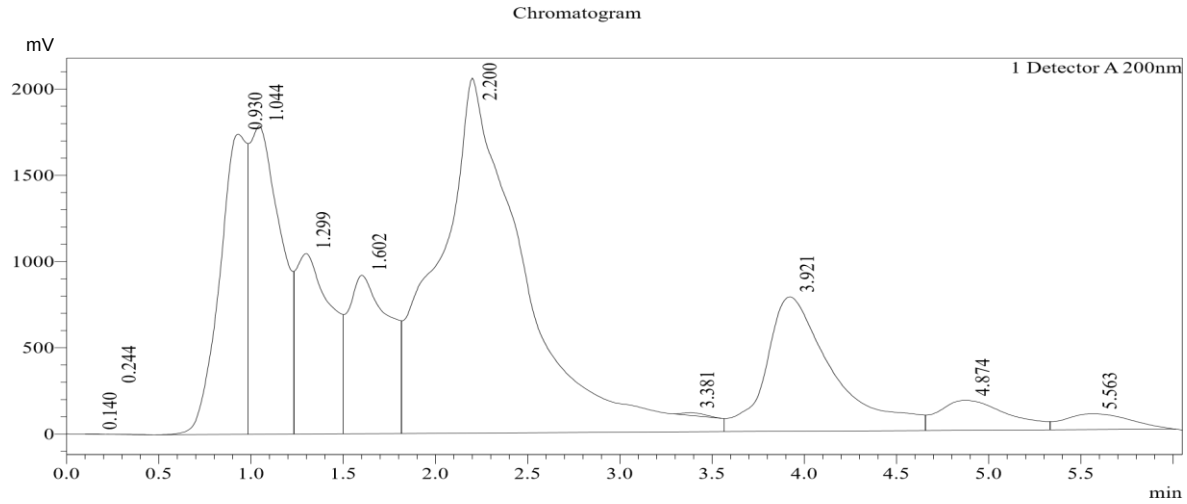

Figure 4: HPLC chromatogram for the crude prodigiosin pigment.

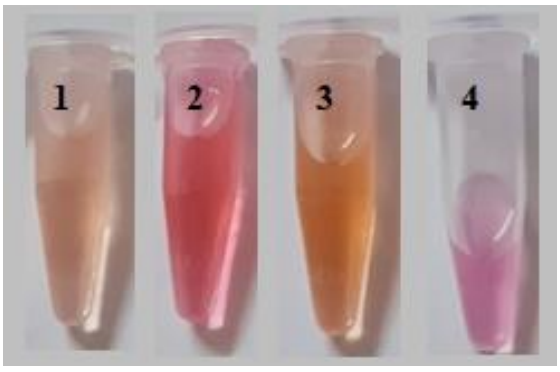

Figure 5: The intensity of the colors of the solutions separated by HPLC according to retention time. The number on each tube (1-4) indicates the type of pigment extracted from the crude prodigiosin extract.

capric acid and 70% lactic acid, which have an inhibitory effect against bacteria and thus is reflected in the metabolism of the pigment (Widianingrum *et al*., 2019; Mohammad and Daod, 2021).

Presumptive test

A presumptive test was conducted to verify that the pigment produced in the media is actually prodigiosin. A pink color appeared when adding acid while a yellow color was observed in alkaline conditions (Figure 3). This indicates a positive result for prodigiosin production, and this finding agrees with other studies (Rakh *et al*., 2017; da Silva *et al*., 2022).

Purification of prodigiosin

After performing the Preparative-HPLC technique for the crude pigment under the specified conditions (Faraag *et al.*, 2017). Our result showed multiple peaks from 0.9-5.6 min, as shown in Figure 4.

Due to the absence of a prodigiosin standard, the method of Faraag *et al*. (2017) was adopted. In our study, to collect prodigiosin in pure form, the injection of crude solution in HPLC was repeated and then the fluid obtained from the device was collected in Eppendorf tubes with great accuracy based on the retention time in discrete parts separately, as shown in Figure 5.

The appearance of several peaks in the HPLC graph with the color gradation of the fluids indicates the presence of other prodigiosin-like pigments produced in the fermentation cycle (Lin *et al.*, 2019). Another study (Lee *et al*., 2011) also mentioned that prodigiosin-producing strains could produce other pigments similar to prodigiosin in their cultures.

In order to make sure that the fluid of red color in Eppendorf tube No 2 is the prodigiosin pigment, its absorption value was determined by conducting an ultraviolet-visible (200-800 nm) scan. The result showed that the pigment recorded the highest absorption peak at the wavelength of 535 nm as shown in Figure 6. This confirms that HPLC-extracted and purified pigment is prodigiosin. The absorbance of the isolated pigment is consistent with many studies conducted worldwide (Song *et al.*, 2006; Lee *et al*., 2011; Lin *et al*., 2019).

CONCLUSION

Research findings suggest that sesame and peanut seeds powder significantly induce prodigiosin production when added to *S. marcescens* grown in an LB medium. This study also showed the effectiveness of the preparative-HPLC technique in purifying prodigiosin from crude extracts of *S. marcescens* cultures. Further research is required to explore the possibility of prodigiosin production on a large scale.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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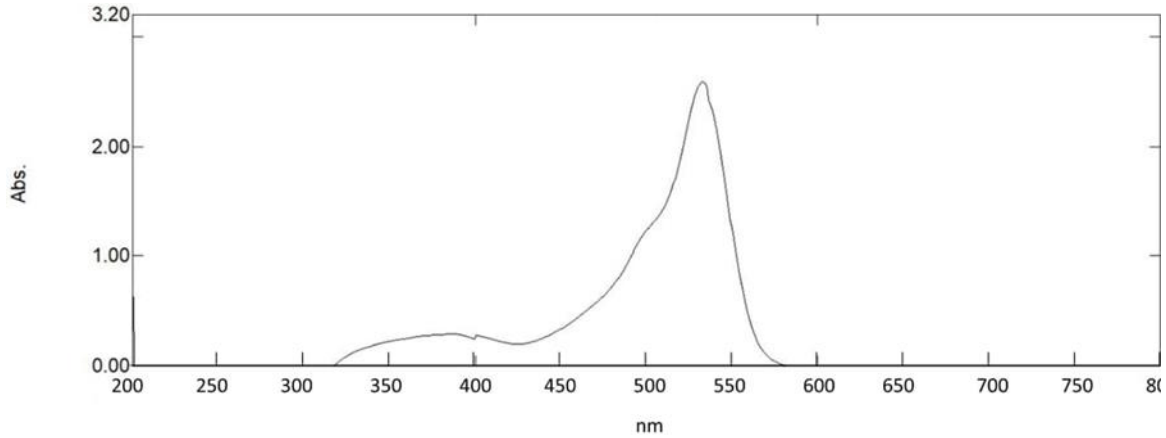

Figure 6: The highest absorbance value of the purified prodigiosin pigment in 535 nm.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538