

Production of Prodigiosin using *Serratia Marcescens* from Tilapia Scale Hydrolysates: Influence of Stirring Speed and NaCl Concentration

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Prodigiosin is a bacterial pigment with therapeutic and antimicrobial properties. Although traditionally produced by *Serratia marcescens*, alternative methods based on agroindustrial waste are being explored. This research focused on determining the feasibility of obtaining prodigiosin using hydrolysates of Tilapia scales by *Serratia marcescens* QSC23, isolated from the La Libertad region in Peru. Wastewater from a treatment plant was collected and enriched in a Luria-Bertani medium to isolate colonies of the *Serratia* genus. These colonies were characterized morphologically, biochemically, and using molecular techniques, including 16S rDNA gene sequencing. Tilapia scales were obtained and subjected to alkaline hydrolysis to produce hydrolysates, which served as a culture medium for prodigiosin production. Different conditions of stirring speed (100 - 250 rpm) and NaCl (0.5 - 2.5%) were evaluated for prodigiosin production over a 72 h incubation period at an initial pH of 7 and 25 °C. QSC23 was able to produce prodigiosin, with a maximum production of 516.03 ± 15.84 units per cell under conditions of 150 rpm and 1.5 % NaCl from the fermentation of tilapia scale hydrolysates. The chemical characterization of the pigment was confirmed using UV-VIS spectroscopy methods. *Serratia marcescens* QSC23 demonstrated the ability to produce prodigiosin using fermentation residues from tilapia scales. This sustainable and cost-effective production offers a promising avenue for the global market of natural pigments.

1. Introduction

Prodigiosin (PG) is a red alkaloid tripyrrole-structured bacterial pigment that typically accumulates in cell membranes and intracellular granules (Hu et al., 2016). It possesses strong antioxidant activity against UV radiation and in food preservation. Moreover, research has revealed its ability to counteract oxidative stress and its pharmacological properties, including anti-inflammatory, antifungal, antibacterial and antitumor effects (Araujo et al., 2022). Additionally, it has demonstrated antimicrobial activity against various oxacillin-resistant strains such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter sp.* and *Streptococcus pyogenes* (Palacio-Castañeda et al., 2019).

PG is primarily produced by *Serratia marcescens* and has been obtained using commercial culture media, such as nutrient broth, yeast extract, Luria Bertani broth, casein, soy tryptone or peptone-glycerol (Cediél et al., 2022). However, traditional PG production faces economic and environmental challenges. Nevertheless, alternative low-cost culture media have been employed, such as cassava, sesame, peanut, and coconut oils, as well as copra, sesame, and peanut seeds (Bhagwat and Padalia, 2020). PG has also been obtained from feather waste and peanut frying oil, offering a cost-effective and sustainable approach to managing these waste products (Asitok et al., 2022). It is true that PG productivity is enhanced by the availability of organic nitrogen sources like tryptophan, proline, serine and glycine, which serve as key precursors in its biosynthesis (Xiang et al., 2022). Therefore, it is essential to identify new, accessible and economical sources of organic nitrogen.

The fishing industry generates 28 million tons of waste annually, and there has been growing interest in reusing these waste products to produce bioactive compounds through microbial fermentation (Venugopal, 2021). Wang et al. (2012) used squid feathers as the primary source of carbon and nitrogen for microbial PG production, achieving significant yields. Several studies have utilized fishery byproducts, especially squid feathers and shrimp byproducts, obtaining PG yields of up to 5910 mg/L (Nguyen et al., 2021). Together, fishery byproducts prove to be promising sources for PG production.

Tilapia highly valued in aquaculture for its adaptability and prolificacy, generates byproducts during processing, with tilapia scales standing out as rich in commercial proteins such as collagen and other protein concentrates rich in amino acids (Hernández-Ruiz et al., 2023). These agro-industrial waste products, especially tilapia scales, emerge as a viable source for obtaining high-value compounds, aligning with the principles of circular economy and sustainability. However, they have been insufficiently explored for prodigiosin (PG) production (Nguyen et al., 2022). In this research, we aim to assess the feasibility of producing prodigiosin from tilapia scale hydrolysates by *Serratia marcescens*, a bacterium isolated from the La Libertad region in Peru, and to study the impact of variables such as agitation speed and sodium chloride (NaCl) concentration on production.

2. Method

2.1 Sampling and Microbial Isolation

A total of 1000 ml of wastewater was collected from a wastewater treatment plant (WWTP) located in La Libertad, Peru (8°11'18.8"S 78°59'51.3"W). 10 mL of the sample was enriched in 90 mL of Luria-Bertani (LB) culture medium with the following composition (g/L): NaCl 10, yeast extract 5 and tryptone 10. The enrichment was incubated at 30 °C for 24 - 48 h. Subsequently, serial dilutions were prepared and surface plating on nutrient agar Petri dishes was performed. After incubation at 30 °C for 24 h, colonies with characteristic morphology of the *Serratia* genus were selected and purified on nutrient agar tubes. Finally, the resulting subcultures were stored at 4 °C (Montero et al., 2016).

2.2 Morphological, Biochemical and Molecular Characterization

From a pure 24 h culture, the macroscopic characteristics of colonies were determined and Gram staining was conducted for microscopic analysis. Biochemical tests were performed using the colorimetric and enzymatic MicroScan system (WalkAway 96, USA) for bacterial identification (Ombelet et al., 2021).

For a more precise species determination, total DNA was extracted using the DNA extraction kit (InnuPREP DNA kit, AnalytikJena, Germany). The extracted DNA segment was amplified by PCR targeting the 16S rDNA segment following the methodology described by Cueva-Almendras et al (2022) using primers 27F and 1942R. The PCR product was sent to Macrogen (Seoul, South Korea) for sequencing using Ion Torrent technology. The partial sequence of the 16S rDNA gene was aligned using Mega X software for the subsequent construction of the phylogenetic tree; nucleotide sequences were compared in the National Center for Biotechnology Information (NCBI) database.

2.3 Obtaining and conditioning of Tilapia scales

Specimens of 9-month-old Tilapia (*Oreochromis niloticus*) were collected from the Experimental Genetics Center of the Faculty of Biological Sciences, National University of Trujillo (Peru). The specimens were subjected to a non-painful cold shock (-20 °C), after 30 min, they were rinsed with clean water and the scales were then removed (Reátegui-Pinedo et al., 2022). The scales were washed with 100 ppm NaClO for 10 min and rinsed three times with distilled water. They were then dried to a maximum weight of 2 Kg at 60 °C for 6 h. Subsequently, they were ground and sieved to a 0.45 mm pore size.

2.4 Characterization of Tilapia Scales

Infrared spectra (IR) were determined from a 0.5 g sample using a Thermo Nicolet IS50 instrument (USA) in the diffuse reflectance mode (ATR) with a diamond-type crystal. The analysis was carried out in the range of 4000-500 cm⁻¹ (Trilokesh and Uppuluri, 2019).

2.5 Production of prodigiosin from TSH

Tilapia scales (TS) underwent an alkaline hydrolysis process to obtain scale hydrolysates (TSH), following the method described by Boarin-Alcalde and Graciano-Fonseca (2016). Dry scale material was added to 100 mL of 1 % NaOH aqueous solution (at a ratio of 1:8, w/w) with agitation at 250 rpm at 50 °C for 3 h; subsequently, the material was filtered and the obtained filtrate was neutralized with 2 M HCl to a neutral pH.

A culture medium was prepared using the hydrolysates of Tilapia scales as the sole nutrient source and its supplementation with NaCl (0.5 - 2.5 %) was evaluated at different stirring speeds (100 - 250 rpm). It was

fermented with a working volume of 20 mL of culture medium and 5 % of the enriched broth with bacteria having an optical density of 1.0 at 600 nm at pH 7, previously grown in BHI broth for 24 h, was inoculated. Incubation was carried out at 25 °C for 72 h.

2.6 Analytical Measurements

Microbial growth was determined by spectrometry at a wavelength of 620 nm for each sample taken at specified time intervals. Total protein quantification was performed according to the method described by Rakh et al. (2017) using the Biuret method at 545 nm. Prodigiosin levels under these conditions were estimated after 24, 48, and 72 h. The crude prodigiosin was extracted using acidified ethanol (96 mL of ethanol 95 % and 4 mL HCl 37 %). The dissolved prodigiosin was estimated using the following equation (1).

$$(\text{Prodigiosin Unit}) \text{ PU/cell} = \left(\frac{(\text{OD}_{499} - (1.381 \times \text{OD}_{620})) \times 1000}{\text{OD}_{620}} \right) \quad (1)$$

Where OD_{499} is the pigment absorbance, OD_{620} is the bacterial cell absorbance and the constant is 1.381.

2.7 Characterization of the Microbial Pigment Prodigiosin

Pigmented cells were treated with 95 % acidified ethanol. Subsequently, cellular residues were removed by centrifugation at 5000 rpm for 15 min. The cell sediment was resuspended in 1 mL of acidified ethanol (95 %) and vortexed for 15 min before being subjected to centrifugation at 5,000 rpm again. The cell-free supernatant was read in a single-beam UV-visible spectrophotometer (UVILINE 9400, Germany) in the range of 300 - 800 nm (Palacio-Castañeda et al., 2019).

2.8 Statistical Analysis

Under different experimental conditions, comparative analyses of the results were performed using ANOVA tests with the statistical software Design Expert v13. A significance level of $p < 0.05$ was determined.

3. Results and discussion

Out of a total of 12 microbial isolates obtained from wastewater at a WWTP, only one microbial culture exhibited morphological characteristics belonging to the genus *Serratia* (designated as QCS23). QCS23 displayed bright, convex colonies with regular edges and a red color pigment. Additionally, under microscopy, the cells adopted a bacillary shape and were Gram-negative (Figure 1). Biochemically, it was determined that QCS23 fermented glucose, sorbitol, inositol and o-nitrophenyl-beta (ONPG). However, the isolate tested negative for raffinose, urease, rhamnase, H_2S , maltose, arabinose, indole and oxidase. These results are consistent with those reported by Yehia et al. (2016). Furthermore, the biochemical and physiological characteristics identified in this study align with the description of *Serratia marcescens* in Bergey's Manual, according to Zhang et al. (2020). From the pure culture QCS23, molecular characterization was performed through the sequencing of the 16S rRNA gene, resulting in a 1571 base pair (bp) fragment. Sequence alignment analysis revealed a 93.53 % similarity with *Serratia marcescens* ATCC13880 (NR_041980). The phylogenetic analysis based on the 16S rRNA gene sequence, was compared to 9 closely related nucleotide sequences from the *Serratia* genus and an external control group, showing a close relationship within the group of *S. marcescens* species with a minimum pairwise distance of 0.2, indicating a higher similarity proximity (Figure 1).

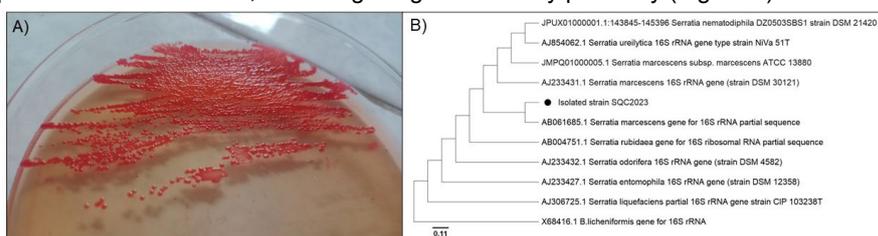


Figure 1: Pure culture isolation QCS23. (A) Macroscopic observation of colony-forming units after 24 h of incubation on nutrient agar; and B) The phylogenetic tree was constructed using the Neighbor-Joining method based on 16S rRNA gene sequences. *Bacillus licheniformis* X68416 was used as an external control.

The FTIR spectrum (Figure 3) of untreated Tilapia scale waste showed peaks around 3290 cm^{-1} due to NH-stretching (Nadeem et al., 2008); peaks in the $1,452 - 1,392 \text{ cm}^{-1}$ range were attributed to the amino alkyl group. Peaks around $1,635$, $1,548$ and $1,243 \text{ cm}^{-1}$ were assigned to amide I, II and III bands, respectively, indicating the presence of proteins inherent to collagen polypeptides (Nkansah et al., 2022). Another band, around $1,079.33 \text{ cm}^{-1}$, was assigned to phosphate stretching, which is found in fish scale apatites (Mohd et al., 2019).

After treatment with NaOH, it is evident that the TS showed significant differences in the transmittance intensities in the 3,600-3,000 cm^{-1} and 1,600 – 1,200 cm^{-1} regions, which can be attributed to the removal of proteins and mineral residues during hydrolysis. This is confirmed in the TSH, where a total protein content of 3.830 ± 0.008 g/100 g was found.

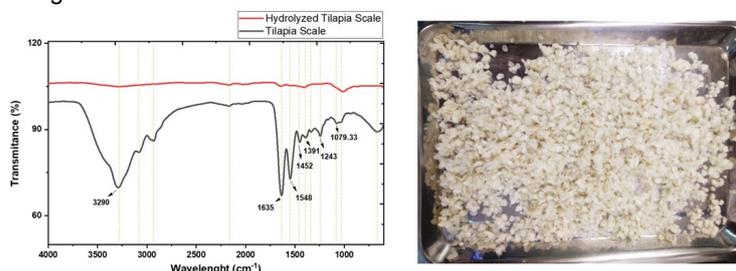


Figure 3. Comparative FTIR spectra of untreated and NaOH-hydrolyzed Tilapia scale waste.

The production of PG by *S. marcescens* QCS23 was examined under various rpm and NaCl, using Tilapia scale hydrolysates as a nutritional source. Three essential criteria were measured: the amount of prodigiosin, the percentage of total consumed proteins, and cell density (Table 1). The maximum PG production was observed in trial 8 with 516.03 ± 15.84 PU/cell at 150 rpm and 1.5 % NaCl, while the minimum production was observed in trial 12 with 143.03 ± 16.35 PU/cell at 250 rpm and 2.5 % NaCl. The maximum amount recorded differs by 40 % from what Elkenawy et al. (2017) reported, where they achieved 870 PU/cell using crude glycerol supplemented with 1 % peptone as a commercial nitrogen source. Furthermore, this result varies by 42 % compared to what was reported by Yip et al. (2021), who obtained 892 PU/cell using a commercial medium composed of 5 g/L of peptone and 10 mL/L of glycerol. It is worth noting that in our study, TSH was the sole nutrient employed, yielding over 50 % higher yields. The results exceeded by 61 % the values obtained in a Luria Bertani commercial medium, which were around 200 PU/cell, as described by Gulani et al. (2012).

Table 1: Production of prodigiosin, protein consumption and biomass under different stirring speed and NaCl.

Run	Rpm (X1)	% NaCl (X2)	PG Unit/cell	% Total protein consumption	Cell biomass (OD ₆₂₀)
1	100	0.5	453.54 ± 52.41	31.45 ± 0.18	2.94 ± 0.04
2	150	0.5	477.50 ± 3.38	36.19 ± 0.03	3.02 ± 0.07
3	250	0.5	213.38 ± 41.57	37.34 ± 0.07	2.85 ± 0.03
4	100	1	347.42 ± 57.70	31.56 ± 0.03	2.94 ± 0.03
5	150	1	400.35 ± 21.11	31.74 ± 0.08	3.00 ± 0.04
6	250	1	159.25 ± 7.89	39.69 ± 0.35	2.84 ± 0.03
7	100	1.5	384.90 ± 31.50	31.92 ± 0.01	2.90 ± 0.02
8	150	1.5	516.03 ± 15.84	32.21 ± 0.01	3.01 ± 0.05
9	250	1.5	158.52 ± 21.75	43.86 ± 0.11	2.83 ± 0.03
10	100	2.5	365.22 ± 42.08	30.80 ± 0.01	2.82 ± 0.03
11	150	2.5	322.44 ± 15.37	33.38 ± 0.02	2.97 ± 0.06
12	250	2.5	143.03 ± 16.35	42.56 ± 0.14	2.78 ± 0.03

According to Table 2, the stirring speed and % NaCl have a significant impact on prodigiosin PG, as evidenced by their low p-values (1.11×10^{-16} and 4.03×10^{-7} , respectively). Furthermore, the interaction between these two factors is also significant, with a p-value of 8.88×10^{-4} , indicating that the influence of agitation speed on prodigiosin production varies with NaCl concentration, and vice versa. Therefore, it is essential to consider both factors and their interaction when studying prodigiosin production by *S. marcescens* QCS23 in TSH.

Table 2: ANOVA statistical analysis of prodigiosin production at different NaCl and stirring speed.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Stirring speed	2	4.431	2.215	247.133	1.11×10^{-16}
NaCl	3	0.599	0.199	22.297	4.03×10^{-7}
Interaction	6	0.304	0.050	5.657	8.88×10^{-4}
Model	11	5.335	0.485	54.099	3.34×10^{-14}

In a recent study, it was identified that NaCl concentrations of up to 4 % induced high prodigiosin production (182 mg/L), partly determined by the tolerance capacity of certain microorganisms, such as *Vibrio gazogenes* PB1 (Vijay et al., 2022). However, Ghosh and Patel (2020) achieved a maximum PG production (1,500 units/cell) at 1 % NaCl using *Serratia nematodiphila* S04K and found minimal amounts when increasing to 2.5 % NaCl, indicating the NaCl effect and coinciding with our study. Meanwhile, agitation speed has also been shown to

influence PG production, in agreement with Miglani et al. (2023), who observed an increase in PG production (0.795 CVU per mg) by increasing the agitation speed from 100 to 200 rpm during the fermentation of rice straw hydrolysate using *Serratia marcescens* CMS-2 at pH 6.5. The preliminary identification of the extracted pigment was achieved through UV-VIS spectrum scanning between 300 - 800 nm, with a maximum of 535 nm within the 520 - 560 region (Figure 4). These results align with Tunca-Koyun et al. (2022), who confirmed the presence of PG at 500 - 538 nm with a peak maximum at 538 nm in 95 % ethanol; similarly, Abdul et al. (2020) determined a peak maximum of 539 when dissolved in methanol. It is suggested that variations in the peak absorbances of the PG pigment are influenced by the different solvents used. The findings of this study align with the information previously reported in the literature.

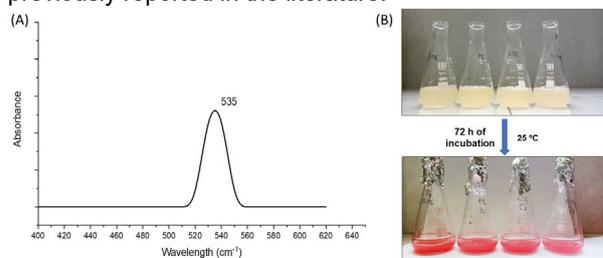


Figure 4. UV-VIS spectrum of (A) the spectrophotometry analysis of the pigment extract obtained from (B) the fermentation of *Tilapia scale hydrolysates*.

4. Conclusions

A strain of *Serratia marcescens* QCS23 was identified and cultured, demonstrating the ability to grow and produce prodigiosin pigment from *Tilapia scale hydrolysate* waste as the sole nutrient source. During the course of the experimental design, a production of 516.03 PU/cell was obtained at 150 rpm with 1.5 % NaCl at an initial pH of 7 after 72 h. Furthermore, the QCS23 pigments exhibited typical characteristics of the prodigiosin group based on their UV-VIS spectrum at 534 nm. The findings indicate that *Serratia marcescens* QCS23 has potential for industrial prodigiosin production using *Tilapia scale* waste. This method offers a cost-effective alternative to reduce dependence on expensive nutrients and mitigate environmental pollution. It contributes to the feasibility of scaling up and its potential incorporation into the global prodigiosin market.

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