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SUSTAINABLE PIGMENT BIOPRODUCTION: *SERRATIA MARCESCENS* ISOLATED FROM THE LOCAL ENVIRONMENT AS A SOURCE OF FOOD-GRADE PRODIGIOSIN

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Abstract

The growing demand for natural, eco-friendly and sustainable colorants has drawn attention to microbial pigments as substitute to chemical-based ones. This study evaluated *Serratia marcescens* isolated from greywater, as a sustainable source for food-grade prodigiosin production. The bacterium was isolated and characterized based on morphological and biochemical attributes, and its prodigiosin production was assessed under optimized fermentation conditions. Bioactive compositions of the pigment were identified and its safety as food-grade pigment was assessed using the rat model. The findings revealed that the organism yielded optimally in peptone-glycerol medium at 30°C, 150 rpm agitation, and after 72 hours incubation. Gas Chromatography-Flame Ionization Detection (GC-FID) analysis revealed the presence of varying quantities of bioactive compounds in the pigment including methoxy prodigiosin (16.68 %), prodigiosin precursor (56.75 %) and prodigiosin dimer (11.39 %), with the precursor as the predominant component. Safety evaluation of the prodigiosin in rats showed insignificant histopathological changes compared to the severe histological damage observed in rats treated with the synthetic colorant, Tartrazine (E102). Thus, results in this study highlight *Serratia marcescens* as a promising sustainable source for natural red pigment production, and thus, can serve as safer and eco-friendly substitutes for synthetic colorants in food applications.

Keywords: prodigiosin, food-grade, *Serratia marcescens,* optimization, prodigiosin precursor

Introduction

Colour plays an essential role in the aesthetics of food products. Commonly, synthetic colorants are applied in the food industry to meet the consumer appeal and

demands. However, the extensive use of synthetic colorants has raised significant health and environmental concerns. They are associated with allergenicity, carcinogenicity and diverse levels of toxicity (Amchova *et al*., 2024). Owing to the recalcitrance of the synthetic colours, they tend to persist in the environment, contributing to pollution of the lithosphere and hydrosphere (Kumari, 2024). These problems contribute to the drift towards exploration of pigments of natural origin as sustainable substitutes. Amongst the microbially sourced pigments, prodigiosin, a secondary metabolite, released by microbial genera *Serratia* (Anaukwu *et al*., 2024), *Vibrio* (Vijay *et al*., 2022), *Hahella* (Kim *et al*., 2023, *Streptomyces* (Alam *et al*., 2022) and *Pseudoalteromonas* (Wang *et al.,* 2021), has attracted considerable interests due to its broad applications in food, pharmaceutical and cosmetics industries (Guryanov and Naumenko, 2024; Han *et al*., 2021).

Serratia marcescens, a Gram-negative bacterium belongs to the Enterobacteriaceae family, and has been reported for red pigment production. Pigment from *Serratia* has been characterized as prodigiosin. *S. marcescens* grows well on ordinary and synthetic media comprising various kinds of carbon sources, under aerobic and anaerobic conditions, at pH 9 and temperatures between 20-37°C. Found in diverse environments, including soil, water, plants, insects, animals, and humans, the prodigiosin producing *Serratia* includes *marcescens, plymuthica,* and *odorifera* (Al-Ghanem, 2018). The chemical structure of prodigiosin ($C_{20}H_{25}N_3O$; molecular weight 323.44 Da) consists of a tripyrrole ring system which is responsible for its characteristic red hue and lipophilic nature, making it soluble in organic solvents like methanol and chloroform (Kimata *et al*., 2017). Prodigiosin provides an opportunity for the food industry to meet the safe pigment demand because it is naturally derived, biologically produced, and can be obtained through scalable microbial fermentation systems using inexpensive substrates (Han *et al*., 2021).

Regardless of the potential of *Serratia marcescens* for prodigiosin production, its utilization in large scale production remains limited by cost, identification of the suitable conditions for growth and production, and safety concerns. The use of waste-derived isolates, for instance from grey water, presents not only viable recovery of the biopigments, but an eco-friendly sustainable approach for low-cost production. Moreover, safety testing such as subchronic, chronic, Absorption Distribution Metabolism and Excretion (ADME), genotoxicity and safe-level determination are conducted on bioproducts to validate their safe-for-consumption or generally regarded as safe (GRAS) status. A short animal study can also serve to determine the ability of the biopigment to cause histological changes (Darshan and Manonmani, 2015). This study aimed to evaluate *Serratia marcescens* isolated from grey water for its potential for prodigiosn production under optimized conditions and to assess its safety as a natural food-grade colorant.

Materials And Methods

Grey Water Collection of Samples:

Five grey water samples (containing 100 ml each) were collected from the discharge points of five different bathing rooms in student hostels at Nnamdi Azikiwe University, Awka Campus, Anambra State, Nigeria, following the method described by Anaukwu *et al*. (2024). The samples were adequately transported to the Microbiology Laboratory of Nnamdi Azikiwe University Awka for analysis. The Awka Campus of Nnamdi Azikiwe University is located in the Southeast along the Onitsha-Enugu Expressway and between longitude 7.115°

to 7.121°E and latitude 6.245° to 6.283°N. The campus contains several hostels that are inhabited by male and female undergraduate and postgraduate students and that comprise bathrooms on their floors.

Isolation And Identification of *Serratia Marcescens*

Caprylate thallous agar (CTA), a selective medium, was used for the isolation of *S. marcescens* from grey water samples. One milliliter of the sample was serially diluted 10-fold and inoculated using the pour plate technique onto sterile CTA agar plates. Incubation of the agar plates lasted for 24 hours at a temperature of 25°C. The developed colonies were counted, sub-cultured onto sterile CTA plates, and stored on sterile nutrient agar slants at a temperature of 4°C. Following the protocols for microbial identification described by Cheesbrough (2010), the stored isolates were identified based on their cultural and biochemical characteristics. Alongside the Gram reaction, the qualitative biochemical tests conducted included tests for catalase, coagulase, and oxidase enzyme production; citrate utilization; the ability to release indole in the presence of amino acid (indole test); methyl red; Voges-Proskauer; motility; and the ability to ferment monosaccharides (glucose) and disaccharides (lactose, sucrose, maltose and mannitol).

Preliminary Screening of Isolates for Prodigiosin Production

Prodigiosin production by the *Serratia marcescens* isolates was first screened for the potential to release large volumes of pigment following the method described by Kanika *et al*. (2023), and the highest pigment producer was used for further analysis and production. For the preliminary screening, a nutrient broth medium supplemented with glucose was used as the fermentation medium. A loopful of 24-hr-old culture of the isolates was inoculated into 50 ml of the medium in a 100 ml Erlenmeyer flask and incubated at 25°C under 150 rpm agitation for 72 hours. Prodigiosin yield was determined by measuring the absorbance (optical density) using UV-VIS spectrophotometry at 550 nm. The experiment was performed in triplicate, and the obtained values were recorded as mean ± SD.

Optimization Of Prodigiosin Production Conditions

The one-factor-at-a-time method of optimization was employed to identify the optimum conditions of growth and production in the selected isolate, as described by Rakh *et al*. (2017). The tested production conditions encompass fermentation medium, incubation duration, temperature, and agitation velocity.

The various fermentation media tested included nutrient broth supplemented with different sugars (glucose, sucrose, and mannitol) and peptone glycerol broth. While keeping all factors from the preliminary screening constant, we tested the various media and selected the one that produced the highest yield of prodigiosin for further analysis. Prodigiosin yield was determined by measuring the optical density of the fermentation medium as earlier described (Kanika *et al*., 2023).

The optimum incubation period for production by the isolate was determined using the best fermentation medium while keeping all other factors constant; the production yield was tested at 24-hour intervals over a total of 72 hours. Yield was determined by measuring the optical density of the fermentation medium as earlier described (Kanika *et al*., 2023).

The best temperature and agitation speed for achieving optimum prodigiosin yield by the isolate were identified by incubating the fermentation medium at various

temperatures (25-40°C) and agitation speeds (150-200 rpm), while keeping all other factors constant. Yield was determined by measuring the optical density of the fermentation medium as earlier described (Kanika *et al*., 2023).

Production And Extraction of Prodigiosin

Optimum conditions of growth and prodigiosin production as identified in earlier analyses were applied. A loopful of 24-hr-old culture of the isolate was inoculated into 50 ml of peptone glycerol broth in a 100 ml Erlenmeyer flask and incubated at 30°C under 150 rpm agitation. After 72 hours of incubation, pigments were extracted by first centrifugating at 8000 × g and 4°C for 15 minutes to separate the whole cells and residuals (Kanika *et al.*, 2023). The supernatant was collected using a separating funnel and then mixed with 96% ethanol acidified with 0.1 N HCl at 1:1 v/v and subsequently neutralized with a few drops of Tris buffer, resulting in the formation of reddish prodigiosin (a pigment). The mixture was centrifuged at 8000 × gravity for 10 minutes to separate the pigment, which was collected in a pre-weighed beaker. The pigment contained in the beaker was dried in a hot air oven, and the weight (dry mass in g) was measured by subtracting the initial weight of the beaker from the final weight.

Determination Of Bioactive Components of The Prodigiosin

The bioactive composition of prodigiosin was analyzed using gas chromatography-flame ionization detector (GC-FID). The crude pigment was mixed with 20 ml of a chloroform and ethanol solution (1:2 v/v) and allowed to sit for 30 minutes before being vortexed and centrifuged at 5000 × gravity for 10 minutes. The organic layer was gathered, dried, and treated with pyridine and TMCS (99:1 v/v) at 100°C for 1 hour. The GC-FID analysis was performed using an Agilent Technologies GC system (GC-7890A/FID-5975C) with an HP-5FID column. The relative quantity of bioactive compounds was expressed as a percentage based on peak area in the chromatogram (Cocito and Delfino, 1994).

Safety Evaluation of The Prodigiosin

The safety of prodigiosin produced for consumption was evaluated in a rat model (Suvarna *et al*., 2018). Before the use of laboratory rat, ethical approval for the use of laboratory animal was procured from the Nnamdi Azikiwe University Animal Research Ethics Committee (NAU/AREC/2025/0089). A total of Twelve healthy, eight weeks old male Wistar rats were used. The animals were kept in a spacious and well-ventilated cages at room temperature, Standard food (Vita feeds for growers) and drinking water were provided *'ad libitum*', the animals were left for Seven (7) days to acclimatize and grouped into 3 sets comprising 4 rats each. Group 1 was labeled "control," which was fed with sterile distilled water; group 2 was labeled "prodigiosin" and was fed with 10% of the prodigiosin, while group 3, labeled "synthetic colorant," was fed with 10% Tartrazine E102. The substances were administered to the rats orally every 6 hours for 3 days. At the end of the period. The rats were sacrificed by placing into a desiccator containing cotton wool soaked in chloroform, the internal organ (liver) of the rats were collected, processed, and stained with H&E (Hematoxylin and Eosin) for histopathological examination under an Olympus microscope.

Statistical Analysis of Data

Data was collected in triplicate and presented in tables as mean±SD. The mean and standard deviation were calculated using Microsoft Excel 365. Minitab software version 20 was used to carry out the analysis

of the variance (ANOVA), p-value < 0.05 was regarded as statistically significant.

Results And Discussion

Bio-pigment production from bacteria continues to gain interest in research owing to the rapid growth of bacteria, and the rapidity and simplicity of production and pigment extraction (Chavan *et al*., 2025). In this study, a total of five bacterial isolates were recovered from the grey water samples using caprylate thallous agar for the production of prodigiosin and were identified as *Serratia marcescens* based on cultural and morphological characteristics (Table 1). The isolates exhibited convex elevation, smooth edges, and lobate margins, with a circular shape, smooth surface, opaque opacity, and 1 mm size. Biochemical characterization revealed that *S. marcescens* was a Gram-negative rod, motile, catalase-positive, citrate-utilization positive, and coagulase- and lactose fermentation-negative. The successful isolation of *Serratia marcescens* from grey water using the selective medium, caprylate thallous agar, highlights the ubiquity and adaptability of microorganisms in diverse environments and corroborates the report of Anaukwu *et al*. (2024), who sampled different environmental samples and isolated *Serratia marcescens* from grey water. Moreover, sourcing *Serratia marcescens* for bio-pigment production from grey water is a cost-effective process that aligns with circular bioeconomy principles (Mesa *et al*., 2024) and a promotion of waste maximization.

The biochemical profile of the isolates further confirms the isolates' identities as *S. marcescens,* as they are consistent with standard biochemical descriptions and distinguished from other closely related Enterobacteriaceae (Garrity, 2025).

Table 1: Morphology and Biochemical Characteristics of *Serratia marcescens*

Parameter	Observation
Form	Circular
Edge	Smooth
Elevation	Convex
Margin	Lobate
Gram reaction	-ve rods
Catalase production	+
Coagulase production	-
Citrate Utilization	+
Indole production	-
Oxidase production	-
Methyl red	+
Voges-Proskauer	+
Motility	+
Glucose fermentation	+
Lactose fermentation	-
Sucrose fermentation	+
Maltose fermentation	+
Mannitol fermentation	+
Organism name	*Serratia marcescens*

Key: + = Positive; - = Negative

The *S. marcescens* isolated from the grey water were coded S1 to S5 and screened for prodigiosin production. The optical density (OD), which determined the level of prodigiosin released into the fermentation medium, ranged from 0.019 ± 0.0011 to 1.057 ± 2.329 (Table 2). The isolate with the highest yield (S1) was selected for further production. The variation in pigment yield among the isolates is indicative of strain-specific differences in metabolic capacity and pigment biosynthetic efficiency. Such variability occurred in a study during which twelve strains of *Serratia marcescens* isolated from a hospital environment in Cairo produced varying levels of prodigiosin (Elkenawy *et al*., 2017). The highest prodigiosin-producing ability of S1 may be attributed to efficient nutrient utilization during its growth; consequently, its selection for further optimization is justified, as screening for high-yielding strains is a critical preliminary step in sustainable bio-pigment development (Lyu *et al*., 2022

Table 2: Preliminary Screening of Isolates for Prodigiosin Production

Isolate	Yield (OD at 550 nm)
S1	1.057 ± 2.339
S2	0.058 ± 0.005
S3	0.329 ± 0.008
S4	0.174 ± 0.062
S5	0.019 ± 0.001

Results of the one-factor-at-a-time optimization of production conditions show that prodigiosin yield by the selected *Serratia marcescens* strain varied in different fermentation media tested. While there was production in nutrient broth supplemented with conventional sugars, the significantly highest yield (OD: 1.290 ± 0.109) was observed in peptone glycerol broth (Figure 1). This study suggests that the combination of peptone as a nitrogen source and glycerol as a carbon source provides optimal nutritional conditions for prodigiosin biosynthesis. Peptone supports robust cell growth and metabolic activities (Davami *et al*., 2015), while glycerol serves as an efficient carbon source that promotes secondary metabolite formation rather than only biomass accumulation (Kamasaka *et al*., 2025; Kumokita *et al*., 2023; Darshan and Manonmani, 2015). Previous studies have reported peptone glycerol medium as a suitable medium for prodigiosin synthesis (Anaukwu *et al*., 2024; Islan *et al*., 2022; Hu *et al*., 2016).

Among the incubation periods tested, prodigiosin yield peaked at 72 hours (OD: 1.307 ± 0.003) (Figure 2). This time point corresponds to the early stationary phase of the bacterial growth during which secondary metabolites such as prodigiosin are usually synthesized. According to Haddix *et al*. (2008), prodigiosin production generally occurs as microbial growth dynamically transitions from the exponential phase to the stationary phase, during which growth rates slow due to nutrient limitation or environmental stress. A similar 72-hour incubation period was reported by Bhagwat and Padalia (2020) in their study on optimization of prodigiosin biosynthesis by *S. marcescens* using waste and unconventional substrates. The study by Tejada *et al*. (2024) also reported that the highest prodigiosin yield was observed at 72 hours of incubation using tilapia scale hydrolysates as a substrate.

The influence of temperature on prodigiosin production showed that 30°C was best suited for optimal yield by the isolate (Figure 3). Earlier works have reported that

S. marcescens produces maximum pigment at a mesophilic temperature range of 25 to 30°C (Anaukwu *et al*., 2024; Rakh *et al*., 2017). At higher temperatures, enzyme denaturation occurs, resulting in suppression of pigment formation (Suryawanshi *et al*., 2015). Thus, it is essential to maintain the incubation temperature around 30°C for sustained pigment yield. Furthermore, a decreasing prodigiosin yield was observed in the study at increasing agitation speed (Figure 4), with the highest yield recorded at 150 rpm speed. Identifying the suitable agitation speed for pigment synthesis is critical, as excessive agitation can disrupt cell membranes and cause shear stress, while lower agitation can create anaerobiosis in the medium and impede pigment synthesis (Singh *et al*., 2019). The moderate agitation speed of 150 rpm, which yielded optimal results for the isolate in this study, suitably promoted nutrient and oxygen dispersion in the fermentation medium. This corroborates the report of previous studies. For example, Shaba *et al*. (2016) reported an agitation speed of 150 rpm as the best for pigment and biomass yield in *Serratia marcescens* UCP 1549.

Applying the optimal production conditions—peptone glycerol broth, 30°C incubation temperature for 72 hours, and 150 rpm agitation speed—the selected *Serratia marcescens* strain yielded 0.91 g of crude prodigiosin. This yield demonstrates the isolate's biosynthetic potential; thus, its application in sustainable pigment production is highly recommended. Moreover, this study successfully extracted nearly one gram of pigment under laboratory conditions, demonstrating that scalable fermentation could yield commercially relevant quantities with minimal environmental impact.

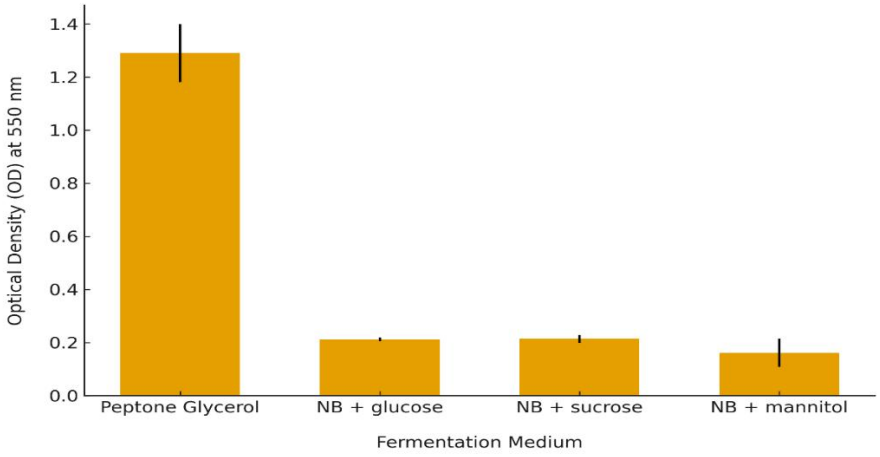


Figure 1: Effects of Fermentation Medium on Prodigiosin Production by *Serratia marcescens* (Key: NB = Nutrient broth)

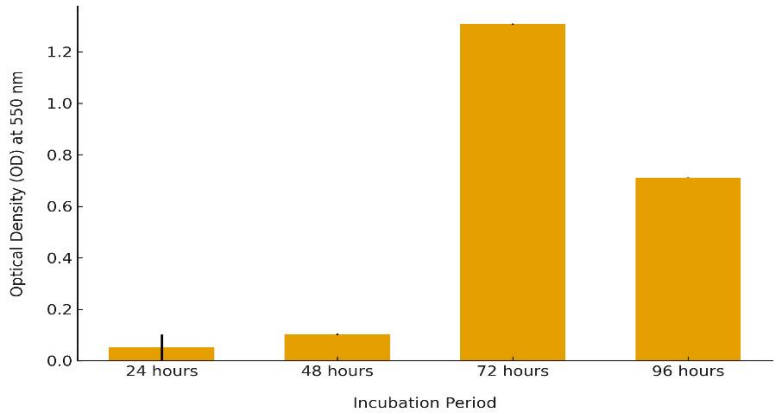


Figure 2: Effects of Incubation Period on Prodigiosin Production by *Serratia marcescens*

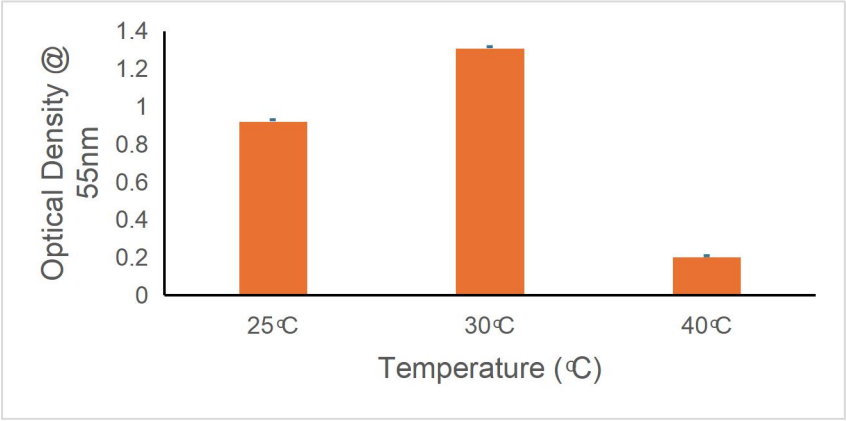


Figure 3: Effects of Temperature on Prodigiosin Production by *Serratia marcescens*

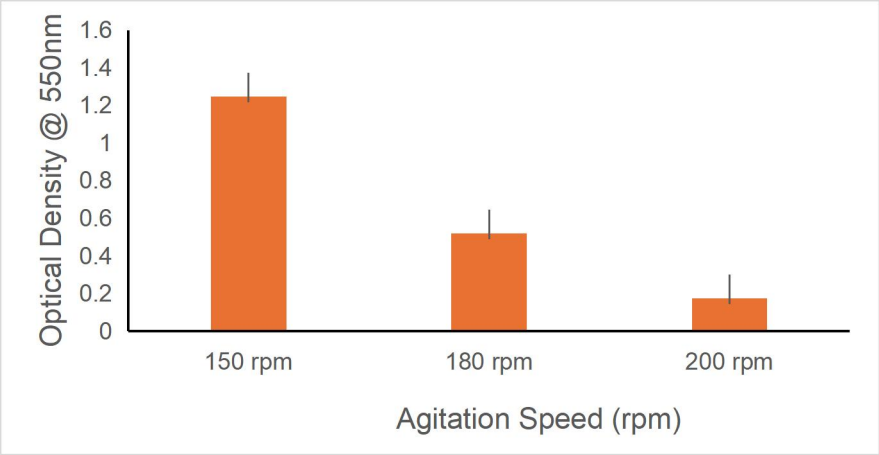


Figure 4: Effects of Agitation Speed on Prodigiosin Production by *Serratia marcescens*

Gas chromatography-flame ionization detection (GC-FID) analysis of the crude extracted prodigiosin revealed the presence of various bioactive constituents (Table 3, Figure 5). The predominant compound was identified as prodigiosin precursor with 56.75% of the total chromatographic profile, followed by methoxy prodigiosin (16.68%), prodigiosin dimer (11.39%), and the main prodigiosin molecule (6.50%). Other minor components included prodigiosin isomer (0.91%) and prodigiosin derivative (0.83%),

while unidentified compounds accounted for 7.02% of the total abundance. The predominance of the prodigiosin precursor suggests that the biosynthetic pathway in the selected *Serratia marcescens* strain was highly active, possibly with the incomplete conversion of intermediates to the fully conjugated prodigiosin molecule. This agrees with the report by Kim *et al.* (2023), who noted that variations in the relative abundance of prodigiosin analogues often reflect differences in enzyme regulation and pathway efficiency within *S. marcescens* strains. The significant presence of methoxy prodigiosin and prodigiosin dimer reinforces the strain's metabolic flexibility, as these derivatives are generally produced via methylation and oxidative dimerization reactions (Darshan and Manonmani, 2015). Multiple prodigiosin analogues identified in the pigment in this study are consistent with earlier findings. For example, Elkenawy *et al*. (2017) opined that changes in carbon source or fermentation parameters are responsible for the diversity of prodigiosin congeners produced. Such chemical diversity is desirable in industrial applications because different analogues possess distinct biological activities, including antimicrobial, antioxidant, and anticancer effects (Suryawanshi *et al*., 2015).

Table 3: Relative Abundance of Bioactive Constituents of Prodigiosin

Bioactive Constituents	Concentration(µg/mL)	Relative Abundance (%)
Prodigiosin	6.09	6.50
Prodigiosin isomer	0.85	0.91
Prodigiosin derivative	0.78	0.83
Unidentified compound	6.58	7.02
Methoxy Prodigiosin	15.63	16.68
Prodigiosin precursor	53.15	56.75
Prodigiosin dimer	10.66	11.39
Total	93.66	

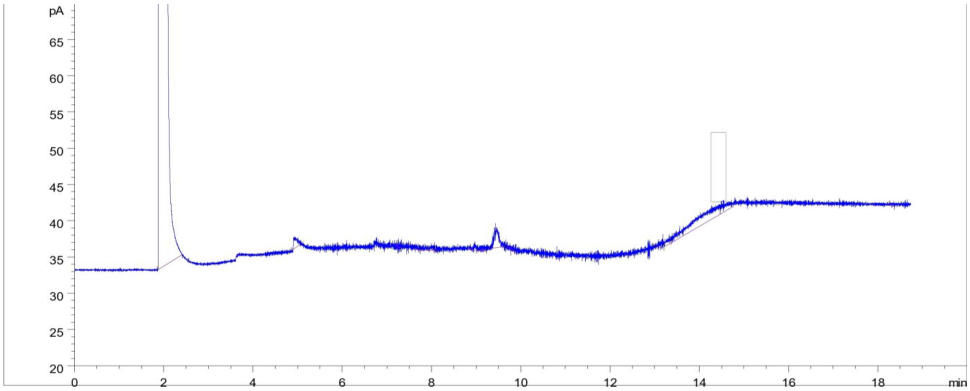


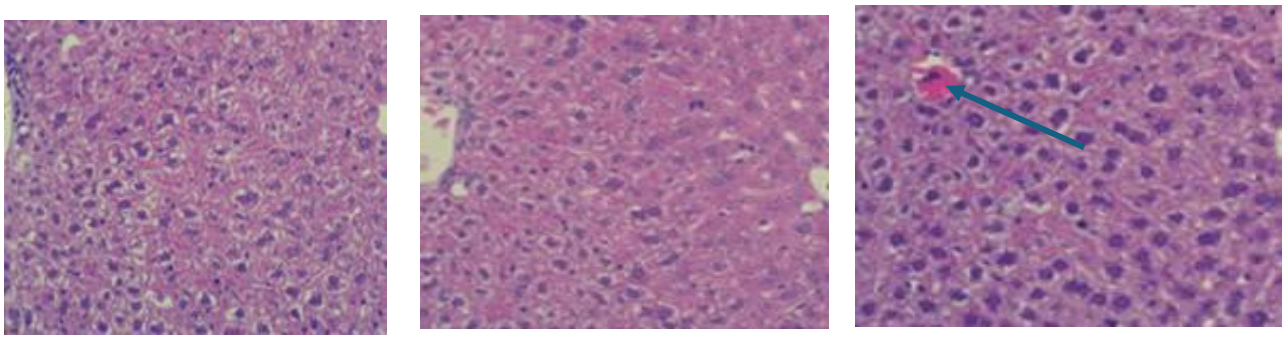
Figure 5: GC-FID Chromatogram of Bioactive Constituents of Prodigiosin

Histopathological evaluation of rat liver tissues following oral exposure to the pigment extracts revealed distinct differences among the treatment groups (Table 4; Plate 1). The control group exhibited normal hepatic architecture, with intact hepatocytes, clearly defined central veins, and no visible inflammatory infiltrates. On the contrary, rats administered with the prodigiosin extract displayed mild changes, such as slight erosions and limited cellular disorganization, while overall tissue integrity remained largely preserved. Conversely, the group exposed to the

synthetic colorant Tartrazine (E102) demonstrated moderate inflammation, focal necrosis, and early fibrosis, indicating significant hepatic injury compared to the natural pigment-treated group. The mild changes observed in the prodigiosin-treated rats can be attributed to transient metabolic stress associated with pigment biotransformation and not obvious toxicity. A similar result was reported by Rahman *et al*. (2019), who reported minimal histological alterations following exposure to microbial-derived natural pigments. Espirito-Santo *et al*. (2022), on the other hand, reported significant hepatic lesions in rats treated with Tartrazine and Sunset Yellow and opined that hepatotoxic effects occur because the chemical colors induce fibrotic changes and lipid peroxidation in the rats. The histopathological results of this study suggest that *Serratia marcescens*-derived prodigiosin has a favorable safety profile, supporting its potential as a natural and sustainable alternative to synthetic colorants in the food industry.

Table 4: Histology of Rats Exposed to the Pigments

Group	Treatment	Histological findings
1 (control)	None	Liver tissue showed normal architecture with no significant histological changes
2	Prodigiosin extract	Liver tissue showed normal architecture with mild erosions (degenerative changes)
3	Tartrazine (E102) Synthetic colorant	Liver tissue showed moderate inflammation, focal necrosis of the tissue and early fibrosis



Group 1: Control Group 2: Prodigiosin extract Group 3: Tartrazine E102

Plate 1: Histological Slides of Liver tissues (H&E X400).

Conclusion

This study demonstrated the production of prodigiosin by *Serratia marcescens* isolated from greywater, with optimal conditions identified as peptone glycerol broth, 30°C

temperature, 72 hours incubation, and 150 rpm agitation. The findings suggest that prodigiosin extract is a safer alternative to synthetic colorants, based on its chemical composition and histopathological evaluation. Prodigiosin show promise as natural food-grade colorant, however further studies are required to optimize and scale up production.

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