For publication

Sulfate as a pivotal factor in regulation of *Serratia* sp. strain S2B pigment biosynthesis

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Abstract

In the present work, we investigated the prodiginine family as secondary metabolite members. Bacterial strain S2B, with the ability to produce red pigment, was isolated from the Sarcheshmeh copper mine in Iran. 16S rDNA gene sequencing revealed that the strain was placed in the *Serratia* genus. Pigment production was optimized using low-cost culture medium and the effects of various physicochemical factors were studied via statistical approaches. Purification of the produced pigment by silica gel column chromatography showed a strong red pigment fraction and a weaker orange band. Mass spectrometry, FT-IR spectroscopy and ¹H NMR analysis revealed that the red pigment was prodigiosin and the orange band was a prodigiosin-like analogue, with molecular weights of 323 and 317 Da, respectively. Genotoxicity and cytotoxicity studies confirmed their membership in the prodiginine family. Analysis of the production pattern of the pigments in the presence of different concentrations of ammonium salts revealed the role of sulfate as an important factor in regulation of the pigment

1 biosynthesis pathway. Overall, the data showed that regulation of the pigment biosynthesis
2 pathway in *Serratia* sp. strain S2B was affected by inorganic micronutrients, particularly the
3 sulfate ions.

4
5 **Keywords:** Prodiginine biosynthesis; Prodigiosin production optimization; *Serratia marcescens*;
6 Sulfate effect.

11 **1. Introduction**

13 *Serratia* family members are Gram-negative, rod shaped bacteria known by their secondary
14 metabolites, red pigment, 2-methyl-3-pentyl-6-methoxyprodiginine, prodigiosin althiomycin,
15 serratamolide/serrawettin and a β -lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid,
16 carbapenem [1-4]. Among these secondary metabolites, the prodiginine family is considered an
17 antimicrobial, antimalarial, immunosuppressive, proapoptotic and anticancer agent [5-8]. A
18 pyrrolyldipyrrolylmethene (PPM) skeletal core with different alkyl substituents, ranging from
19 linear to cyclic carbon chains, constitutes the prodiginine family members, including prodigiosin,
20 undecylprodigiosin, cycloprodigiosin, nonylprodigiosin, butyl-meta-cycloheptylprodigiosin and
21 cyclononylprodigiosin [2, 9-12]. A few genera like *Serratia*, *Pseudomonas*, *Hahella*, *Vibrio* and
22 *Alteromonas*, Gram-negative bacteria *Streptomyces* and *Streptoverticillium*, like Gram-positive
23 ones, have been reported to produce this family of pigments [2]. *Serratia marcescens*, *S.*

plymuthica, *Hahella chejuensis*, *Pseudomonas magnesorubra* and *Vibrio psychroerythreus* are the main producers of prodigiosin, and *Streptomyces longisporus ruber*, *Streptoverticillium rubrireticuli*, *Actinomadura madurae*, *Streptomyces coelicolor* are undecylprodigiosin producers [9, 13, 14]. Among these, *S. marcescens* and *Streptomyces coelicolor* are the most well-known species and are characterized by reddish colonies when cultured at low temperatures [15, 16].

Since prodiginine pigment analogues are bioactive compounds and have potential use in biopharmaceutical fields, optimization of their production and understanding of the mechanisms of biosynthesis regulation are of great concern. Complex media consisting of fatty-acid- and saccharide-derived carbons, along with various nitrogen sources, have been recommended to optimize the growth and secondary metabolite production of *Serratia* species [17-19]. Optimization of secondary metabolite production in bacterial strains mainly depends on variation in growth conditions, and their regulatory systems. Several regulatory agents, including physicochemical factors like nutrient starvation, temperature alteration, oxygen, pH, light, ionic strength, salt concentration and phosphate availability, along with quorum sensing (AHL and AI-2) [20], transcriptional regulators (CRP, HexS, RssAB, SpnR, PigP, CarR, PigQ, PigR, Rap, PigS, PigV and PigX) and two-component systems (PigQW), have also generated the complex regulatory network of secondary metabolite production in *Serratia* species [2, 5-8, 21-23]. Although the effects of individual factors have been characterized, correlations between environmental factors and gene cluster products remain a mystery. The effect of some inorganic factors, such as nitrogen, in regulation of antibiotic biosynthesis has been shown [24]; in the case of sulfate, its direct role in synthesis of sulfur amino acids such as methionine has been reported [25]. Since the role of methionine in increasing prodigiosin production by *S. marcescens* has been elucidated [26], an indirect effect of sulfate in the prodigiosin biosynthesis

1 pathway could be emphasized. On the other hand, PigS is a putative ArsR/SmtB family
2 transcriptional regulator that represses two related operons, *blhA-orfY* and *pmpABC* [27]. PigS
3 indirectly influenced prodigiosin biosynthesis, *pigA-O*, via transcription balance of these two
4 operons. Previous studies reported the effect of an S-containing compound (z), generated by
5 OrfY and BlhA, on prodigiosin production pathways [27]. In the model proposed by Gristwood
6 and her colleagues, PmpC, as an anion transporter, imports an unknown sulfur-containing
7 compound (4-toluene sulfonate, sulfate or sulfite) into the cell; then, enzymes OrfY and BlhA
8 produce an S-containing product (z) which influences prodiginine biosynthesis reactions.
9 Alterations in the z compound concentration may indirectly inhibit or stimulate *pigA-O*
10 transcription and modify prodigiosin output levels. Moreover, under rare conditions, changing
11 the medium components could influence the production pattern and produce several pigment-
12 related analogs. Although there exist a few reports on the effect of growth conditions on
13 production pattern alteration, and only one report on Gram-positive actinomycetes like
14 *Streptomyces lydicus* [28], our findings revealed that the pigment pattern and its concentration
15 ratio were changed, even by variations in *Serratia* growth medium ingredients.

16 In the present study, the effect of sulfate as a new player in regulation of prodiginine
17 biosynthesis has been reported for the first time, and a possible mechanism for the corresponding
18 process has been discussed. Furthermore, a new low-cost medium for pigment production was
19 introduced and optimized using response surface methodology (RSM). Following the
20 optimization procedure, two secondary metabolites were isolated, and alterations in production
21 ratios were characterized and discussed. Genotoxicity and cytotoxicity of both related pigments
22 were also evaluated. Finally, inhibitory effects of excess sulfate on prodigiosin production,
23 probably as an effective factor in the z compound concentration, as well as its regulatory effect,

were elucidated.

2. Materials and methods

2.1. Microorganism culture conditions and phylogenetic classification

The red-pigment-producing bacterium, strain S2B, was isolated from a soil sample collected in the Sarcheshmeh Copper Mine in the Kerman province, Iran. The organism was precultured on nutrient agar plates (Difco) at 30 °C and pH 7.0. After colonies were formed, plates were kept at 7 °C for seven days and the colony with highest red pigment production was selected for further study. Prodigiosin production was monitored in 250 ml Erlenmeyer flasks at 50 ml culture medium using a matrix ingredient of 1% (v/v) carbon sources including sugar beet molasses, sunflower oil or olive oil with 0.5% (w/v), as well as various nitrogen sources including yeast extract, peptone, NH_4NO_3 or $(\text{NH}_4)_2\text{SO}_4$. A four- day old nutrient broth (NB) seed culture was inoculated (2% v/v) into pigment production medium and incubated at 27 °C with 180 rpm for 72 h.

For phylogenetic classification of strain S2B, chromosomal DNA was extracted according to a procedure described by Sambrook et al. [29]. The 16S rDNA nucleotide sequence was amplified with primers: HRK1 5'-AGAGTTGATCCTGGCTCAG-3' and HRK2: 5'-AAGGAGGTGATCCAGCCGCA-3' using a thermal cycler (Biometra, USA) with the following program: initial denaturation at 95 °C for 5 min, 35 cycles denaturation at 93 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 90 s, and a final synthesis at 72 °C for 10

min. The 1,012-bp-amplified DNA fragment was isolated from agarose gel, sequenced and submitted to the GenBank database under accession number KC164755. Sequence alignment and phylogenetic tree were performed by MEGA 6.0 software [30]. Strain S2B was deposited in the Persian Type Culture Collection under accession number PTCC 1877.

2.2. Pigment production optimization (statistical design)

The effect of five independent parameters, including two different physical factors (incubation temperature and agitation rate) and three chemical factors (sugar beet molasses, $(\text{NH}_4)_2\text{SO}_4$ and peptone) upon pigment production was monitored using 2^{5-1} fractional factorial design using Design-Expert 8.0.7.1 software [31]. Concentrations were designed at minimal (-1), maximal (+1) and central point (0) ranges. After evaluation of the responses, the most significant factors affecting pigment production were selected and used for further spherical central composite design (CCD) experiments using five levels (-1.73, -1, 0, +1, +1.73).

Bacterial biomass and total pigment production were quantified as the response of the experiments. Each production medium was divided into two 25-ml samples to assess the total biomass and dried pigment weights. Acetone-1 N HCl 1N (24:1) solution was added to the cell mass of equal volume culture broth followed by 30 min incubation at room temperature. In the next step, centrifugation at 8,000 g was applied to remove pigment-free cell pellets, and then, pigment-containing solvent was concentrated by a rotary evaporator (Buchi, Flawil, Switzerland). Finally, the dried cell pellet weight (DCW) and dried pigment weight (DPW) were measured. All experiments and measurements were performed in triplicate and statistical analysis was performed using SPSS 22.0 [32].

2.3. Pigment purification and molecular characterization

Extracted pigments from a cell pellet of 5 L S2B culture medium were further purified by the method described by Wei et al. with some modifications [15]. Crude extracts were washed with chloroform and passed through petroleum ether balanced silica gel columns (28×200 mm). Various pigments were harvested with petroleum ether/ethyl acetate gradient, concentrated with a rotary evaporator and dried in a vacuum drier at 50 °C to obtain the purified products. The purified pigments were analyzed using silica gel thin-layer chromatography (TLC) to assess their purity and chemical nature. The purity of the pigments was confirmed by HPLC (KNAUER, Germany) using methanol-water (30:70) as a mobile phase at a flow rate of 0.7 ml/min (5 mm x 150 mm, C₁₈ column).

Prodigiosin concentrations were determined according to the previous report by a UV–Vis spectrophotometer in 95% EtOH–HCl solution ($\epsilon_{535}=112000 \text{ M}^{-1} \text{ cm}^{-1}$) [33] and the prodigiosin-like analogue extinction coefficient was determined using serial concentrations of the pigment and the Beer-lambert equation ($\epsilon_{538}=99000 \text{ M}^{-1} \text{ cm}^{-1}$). Mass spectra were measured with a QP 1000EX mass spectrometer (Shimadzu, Tokyo). The UV and IR spectra were recorded on a T90⁺ U-1800 spectrophotometer (PG Instrument, England) and an FTIR RX1 spectrometer (PerkinElmer, Germany) in KBr disc, respectively. The ¹H NMR spectra were recorded on a spectrometer (Bruker Avance DPX) operating at 250 MHz. Methanol was used as a solvent in UV-Vis, mass spectrometry and FT-IR analysis; D-chloroform (CDCl₃) was used for ¹H NMR analysis of the pigments.

2.4. Effect of inorganic salts on the pigment production ratio and pattern alteration

In order to investigate the effect of inorganic salts on the pigment production ratio, a constant concentration (15.2 mM) of five different ammonium salts was tested, including ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium acetate, ferric ammonium citrate in optimized production conditions containing 1.0% sugar beet molasses and 0.1% peptone (MP medium) at 27 °C for 72 h. Dried pigment and total biomass weights were measured using the method described above. Production ratios were calculated using the following equation:

$$\text{Pigment production ratio} = \frac{\text{Dried pigment weight (DPW)}}{\text{Total dried cell weight (TDCW) - DPW}}$$

To study the effect of sulfur-containing compounds on the pigment production ratio, cultures consisting of MP medium supplemented by various concentrations of ammonium sulfate ranging from 0.05 to 1.2 % and ammonium sulfite ranging from 0.02 to 0.2% were prepared. The effect of sulfur ions alone on pigment production and its ratio were assessed using ammonium chloride as the reference.

2.5. DNA cleavage studies

DNA cleavage analysis was carried out by incubation of an 8 µL total sample volume containing pBlu2KSM DNA (500 ng) and different concentrations of the pigments. Supercoiled plasmid DNA was treated over time (10, 20, 30, 45, 60 and 90 min) with prodigiosin and prodigiosin-like pigments (- in the presence of -30 µM CuCl₂) and the mixtures were incubated

in a dark environment for 60 min at 37 °C. The samples were subjected to 1% agarose gel electrophoresis (TAE buffer containing 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH~ 8.2) for 1 h at 50 V. Finally, the gel was stained with 0.5 µg/mL ethidium bromide and visualized by UV-light and photographed for analysis.

2.6. MTT assay

An MTT assay was carried out as described previously [34]. The pigments in the concentration range of 0.5–50 µM, dissolved in methanol, were added to the 96 wells 24 h after seeding of 1×10^4 and 2.5×10^4 cells per well of MCF-7 and Jurkat cell lines, respectively, in 100 µl of fresh culture medium. Methanol was used as the vehicle control. After 24 h incubation, 10 µl of MTT solution (5 mg ml⁻¹ in phosphate-buffered saline) were added to each well and plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The purple formazan product was dissolved by addition of 100 µl of solubilization solution containing 40% (v/v) DMF and 16% (w/v) SDS, at pH~ 4.7, to each well [35]. Then, absorbance was monitored at 590 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Biotek, Hercules, CA, USA). The stock solutions of the pigments were prepared in methanol in such a way that the percentage of methanol was maintained in the range of 0.1–1% in all experiments. Methanol by itself was found to be non-toxic to cells up to a concentration of 1% concentration. Data were collected for three replicates each and were used to calculate the mean value. The percent inhibition was calculated from these data using the following formula:

$$= \frac{\text{Mean OD of untreated cells (control)} - \text{mean OD of treated cells}}{\text{Mean OD of untreated cells (control)}} \times 100$$

IC₅₀ values were calculated by the GraphPad prism 5.04 program using three independent data.

3. Results

3.1. Microorganism characterization and pigment production optimization

Among several strains isolated from the Sarcheshmeh copper mine, strain S2B produced a significant amount of red pigment and was selected for further optimization. It was a Gram-negative bacterium isolate with excellent growth ability in nutrient broth at 27 °C, pH 7 and 180 rpm. The 1012-bp 16S rDNA gene sequence of the present strain was deposited in the GenBank database under accession number KC164755. The constructed phylogenetic tree, based on the neighbor-joining algorithm, indicated that the isolated S2B was part of the cluster within the genus *Serratia*, with 99 % sequence similarity to *S. marcescens* DSM 30121, indicating that strain S2B is a variant of *S. marcescens* (Fig. S1).

Firstly, a matrix of different carbon and nitrogen sources in single factor experiments was done. Primary results showed that, while the growth rate and pigment production were increased using sugar beet molasses (as carbon source) and ammonium sulfate (as nitrogen source), olive oil, sun flower oil and yeast extract did not show any significant effect on prodigiosin production (Table S1). Based on these initial data, five physico-chemical factors were chosen and their interactions on pigment production were analyzed using a fractional factorial design. As presented in Table 1, sugar beet molasses, peptone and agitation rate were the most significant effectors; however, temperature and $(\text{NH}_4)_2\text{SO}_4$ concentration had the lowest effect on DPW values (mg/L). Pigment production showed a range of 610 to 1,440 mg/L, with the best

response at the highest tested concentration of peptone and sugar beet molasses (at least 1,232 mg/L; Run 4, 8, 10 and 16). Furthermore, in designed culture conditions containing maximal molasses (1%) and minimal peptone (0.05 %) concentrations, the maximum agitation rate (180 rpm) had a significant effect on increasing pigment production (Run 7, 12, 14 & 15). For more optimization of pigment production, sugar beet molasses, peptone and the agitation rate as significant factors were selected, and the spherical central composite design (CCD) at five different levels was conducted, are denoted by -1.73 , -1 , 0 , $+1$, $+1.73$ (See Table 2 and Table S2). A 14 factorial design and six axial points ($\alpha = 1.73$) with six replicates at the central point, for yielding a total of 20 experiments, were designed. Based on secondary-order fitted variance analysis, X_1 , X_2 , X_3 , X_{23} and X_3^2 were highly significant (“probe > F” less than 0.1), while X_1^2 , X_2^2 and other interactions were not. The second-order polynomial equation for pigment production (Y), molasses concentration (X_1), peptone concentration (X_2) and agitation rate (X_3) in terms of coded factors were expressed as follows:

$$Y = 328.25 X_1 + 245.80 X_2 + 134.72 X_3 + 112.93 X_2 X_3 - 77.64 X_3^2 + 1147.5$$

In original variables, the above equation can be rewritten as:

$$Y = 1312.99 V_1 - 12605.33 V_2 + 19.15 V_3 + 149.58 V_2 V_3 - 0.09 V_3^2 - 1506.92$$

The response observed by various tested levels with an R^2 value of 0.90 revealed flat curves with the best condition in maximal points of the selected levels for all significant variables (1% molasses, 0.1 % peptone and 180 rpm of shaking condition) with a total yield of 2,007 mg/L (see Fig. S3). Regarding the predicted model, a composition of 1% molasses, 0.1% peptone, 0.2% $(\text{NH}_4)_2\text{SO}_4$, at 27 °C and 180 rpm constituted the optimal condition for pigment production (Run 5, Table 2). Comparative analysis of three effective factors revealed that positive effects of 1% molasses in pigment production were stronger than 0.1% peptone and a 180 rpm agitation rate.

Comparison of runs 2, 8 and 7 with 992, 690 and 692.5 mg/L pigment production, respectively, confirmed the point. In addition, increasing molasses and peptone concentrations to 1.18% and 0.118 %, enhanced pigment production up to 1,645 (run 9) and 1,517 mg/L (run 12) compared to 1,147 mg/L (run15-20) in the central point, but a maximized agitation rate, 202 rpm, reduced pigment production to 882 mg/L (run 13).

3.2. Pigment analogue characterization

Silica gel column chromatography revealed two significant red compounds that were separated into various ratios of petroleum ether and ethyl acetate. As shown in Fig. 1a, UV-visible spectroscopy (200–700 nm) of the purified pigments showed different spectra with a maximum absorption peak at 544 nm for an orange pigment and 538 nm for the red one. The UV-visible spectra of both orange and red pigments showed the existence of two related analogues of prodigiosin.

As shown in Fig. 1b, Fourier transform infrared spectroscopy (FT-IR) analysis revealed several functional groups in the pigments. The presence of a pyrrole ring, secondary amines and methyl groups in both pigments was confirmed by these spectra. The molecular masses of both orange and red pigments dissolved in methanol indicated a major peak at 317 Da for the orange pigment and at 323 Da for the red one (Fig. 2S,a-b). Concerning this, only the molecular weight of the red pigment was correlated with the molecular weight (MW) of the known pigment, prodigiosin; the orange pigment showed about a 7 Da decrease in its MW. ¹H NMR analysis of the red pigment showed several peaks at 7.19 ppm, 6.69 ppm, 6.61 ppm, 6.30 ppm and 6.29 ppm, associated with the PPM skeleton, and a strong peak belonged to the methyl group at δ 3.94 ppm,

confirming the chemical structure of prodigiosin [36] (Fig. 2S,c).

3.3. Pigment production regulation in *Serratia sp. strain S2B*

To study the effect of inorganic salts on regulation of pigment production in strain S2B, the introduced pigment production ratio (PPR) was monitored by adding a constant concentration of various ammonium salts under optimized practical conditions. Pigment production ratios PPRs showed 1.89-, 1.37- and 1.33-fold increase in molasses+peptone (MP) supplemented by equal concentration of ammonium salts, including sulfate, nitrate and chloride salt, respectively, compared to the basal medium, MP (Table 3). The data represent the positive effect of these components on the pigment production ratio of *Serratia sp. S2B*. Moreover, TLC analysis of the normalized pigment concentration of these culture extracts confirmed that the presence of sulfate in the culture medium not only increased the PPR value, but also induced production of prodigiosin; however, inorganic salts such as chloride and nitrate also acted as prodigiosin-like pigment production stimulators (Fig. 2). There is evidence that secondary metabolite production is primarily repressed when an excess effector is used and remains in the culture medium until the stationary phase [37]. Consequently, according to current experiments, an extra concentration of a sulfur-containing compound might have a negative effect on strain S2B pigment production. To investigate whether or not sulfate, sulfite or 4-toluenesulfonate could act as a repressor, increasing concentrations of ammonium sulfate, ammonium sulfite and ammonium chloride (as the control) were added to the basal medium, MP. As shown in Fig. 3, using concentrations up to 0.2, 0.1 and 0.15% of sulfate, sulfite and chloride led to a positive effect on PPR, respectively. The extremities of sulfate, sulfite and chloride were 0.2%, 0.1% and

0.15%, and extra values inhibited pigment production significantly. In addition, excess concentrations of ammonium sulfate significantly showed negative effects on pigment production when compared to the ammonium chloride effect. High concentrations of sulfate could completely inhibit pigment biosynthesis in basal production medium MP, without any effect on cell growth. Nevertheless, excess sulfite initially had concomitant effects on pigment production and on cell growth (see Table S3 for more details).

3.4. DNA cleavage and cytotoxicity analysis

The ability of isolated pigments to mediate DNA cleavage was assessed using pBlueScript the II KS (+) plasmid and 1% agarose gel electrophoresis. As shown in Fig. 4, double-strand DNA cleavage was observed when using 100 μM of purified pigments in the presence of Cu^{+2} ions at 15 μM (physiological concentration of cancerous cells), and no effects was seen at lower concentrations. To facilitate DNA cleavage activity of the pigments similar to that of a previous study by Melvin [38], the copper ion concentration was increased to 30 μM and the results showed that DNA cleavage was observed in a time-dependent manner at lower concentration of the pigments (30 μM) (See Fig. S3). In this experiment, DNA cleavage activity started at 10 min and prodigiosin was slightly faster than prodigiosin-like pigment at cleaving the DNA double helix. Moreover, cell toxicity activities of prodigiosin and prodigiosin-like pigments toward human MCF-7 and Jurkat cancer cell lines were examined using the MTT assay. According to Table 4, the IC_{50} of both pigments for the Jurkat cell line were 5.64 and 5.29 μM after 24 h contact, respectively. In the case of the MCF-7 cell line, almost similar IC_{50} were obtained for two related pigments and were 2.82 and 2.13 μM , respectively.

4. Discussion

Secondary metabolites are introduced as natural products with valuable medical benefits, including antimicrobial, antiprotozoal, antiparasitic, anti-cancer, immunosuppressant, cholesterol-lowering, anti-HIV and anti-ageing properties [39]. Since most of the secondary metabolites have been successfully used in humans with low side effects, much hope has arisen that they may be applied as future cures for untreatable diseases like Alzheimer, multiple sclerosis, cancer, cystic fibrosis, viral and parasitic diseases [39]. However, the major bottleneck in secondary metabolite production is to guide relevant organisms to act as manufacturers and control their metabolic network for producing our target compound. In this context, understanding of metabolic pathway regulation mechanisms is critical. Prodiginines, the microbial secondary metabolite family, constitute the interesting group of pyrrolic compounds that have attracted considerable recent interest due to their immunosuppressive and selective anti-cancer features [40]. Since 2000, many efforts have focused on finding suitable media for pigment overproduction. Combinations of nutrient components, temperature, pH and agitation rate are common factors that play a crucial role in cell growth and prodigiosin production of *Serratia* strains [17, 41]. Based on the response surface methodology used in this study, a mixture of 0.1% peptone and 0.2% ammonium sulfate along with 1% (V/V) sugar beet molasses showed an excellent cost-effective medium with 2,007 mg/L pigment production (see Table 2). With respect to pigment production optimization, various reports have appeared in the literature. Previous works demonstrated the positive effects of fatty acids and saccharides on *Serratia* sp. pigment production. Fatty acid derivatives like peanuts and sesame seed broth, which are the

most frequently introduced nutrients with sizable amounts of pigment production (38,750 and 16,680 mg/L, respectively), [17] and starch as a cost-effective carbon source with 7,030 mg/L production have been reported [18]. In the present investigation, in contrast to Giri et. al. [17], fatty acids had a slight effect on pigment production that might be due to strain specificity of secondary metabolite production. A medium based on sugar beet molasses was found to be another low-cost medium composition with suitable pigment production that was about 10 times greater than brown sugar with 237 mg/L total prodigiosin production [19]. Sugar beet molasses is mainly composed of sucrose (not less than 48%) and may contain small quantities of nitrogen, sulfur and other minerals, such as raffinose, betaine, calcium, potassium, oxalate and chloride [42]. Regarding the major content of sucrose, molasses was replaced by pure sucrose, but results showed that this substitution could not trigger pigment production to this level in *Serratia* sp. strain S2B (data are not shown). We thus conclude that other ingredients of molasses have an effect on pigment production by strain S2B and, in this regard, a positive effect of inorganic salts is plausible.

Members of the prodiginine family are characterized by a maximum absorption spectra in the wavelength range of 535-545 nm, with a shoulder at around 485-500 nm under acidic conditions [43]. Red and orange pigments with a unique shoulder and maximum absorption at 538 and 544 nm, respectively, show general features of the prodiginine family. Based on mass spectrometry analysis and ^1H NMR, the presence of main functional groups like saturated alkane, secondary amine, methyl and methoxy groups with a total mass of 323 Da represent known prodigiosin pigment production in *Serratia* sp. strain S2B [36]. Moreover, FTIR analysis of the pigments showed that the main functional groups for the red and orange pigments were the same, including pyrrole, methylene, alkane and alkene, which are correlates of the prodiginine family

[44]. DNA cleavage analysis was also in agreement with the fact that prodigiosin-like pigment has copper-mediated DNA cleavage activity similar to that of prodiginine family members. DNA cleavage activity has been linked to therapeutic targets and has been highly correlated with cell toxicity of prodiginine family compounds. This phenomenon is mediated by the bipyrrole moiety, especially pyrrole ring A, that is able to facilitate DNA cleavage in the presence of Cu ions and molecular O₂. The reduction of Cu(II) to Cu(I) is the key step resulting in oxidative stress in cells, and leads to DNA cleavage. Similar to bilirubin- [45] and bleomycin-induced [46] double-strand breaks, formation of a prodigiosin–Cu(II) (π -radical cation) complex upon redox cycling of the metal ion (Cu(II)→Cu(I)) causes production of reactive oxygen species, particularly the hydroxyl radical, which damage double-stranded DNA [47, 48]. The bioactive ability of the pigments, based on the MTT assay, revealed a remarkable IC₅₀ of $\leq 5 \mu\text{M}$, indicating their potent anti-cancer activity. Moreover, both tested cell lines are more sensitive to prodigiosin-like pigment, confirming their slight structural modification (see Table 4).

Overall, the red pigment is prodigiosin, while the orange one reveals a structural change in comparison with actual prodigiosin, shows a different absorption peak, molecular weight and also dissimilar mobility on TLC and IC₅₀ against cancerous cells. This phenomenon, which is called “new analogue production”, is rarely seen when the nutrient component is changed [28, 49]. TLC analysis revealed that, compared to basal medium (MP), different anions affected the pattern of pigment biosynthesis in *Serratia* sp. strain S2B (See Fig. 2). Chloride ions changed the production pattern toward prodigiosin-like pigment, and sulfate maximized prodigiosin production alone, while other ammonium salts revealed a median production pattern of these two related pigments. This effect is also seen in production of streptolydigin analogs in *Streptomyces lydicus* [28].

A significant pigment production increase in the presence of ammonium sulfate showed severe effects of ammonium and sulfate ions on the pigment biosynthesis pathway in *Serratia* sp. strain S2B. In order to determine the effect of individual factors, different ammonium sources in equal concentrations were examined. (PPRs show that, in addition to an ammonium effect which increases the PPR, sulfate as an inducer increases prodigiosin production up to 1.89-fold, probably due to a dual effect mechanism: a) a positive effect of sulfate and sulfite (in a concentration range of <0.2% and <0.10%, respectively) on the PPR through methionine biosynthesis induction as a key amino acid in prodigiosin biosynthesis pathway [2, 26]; and b) a negative effect of an excess concentration of sulfate and sulfite ((in a concentration range of more than 0.2% and 0.10%, respectively) on the PPR through acting as a OrfY and BlhA substrate, plus increasing the concentration of the z compound (an unknown sulfur-containing product) in the cell and subsequently reducing *pigA-N* operon transcription and causing a significant decrease in prodigiosin production (See Table S3a-c). Gristwood et al. showed that PmpC, as a permease, uptakes sulfur-containing salts (candidates including: 4-toluene sulfonate, sulfate or sulfite) and enzymes OrfY and BlhA generate an unknown S-containing product (z). The z product may indirectly inhibit *pigA-O* transcription and hence the prodigiosin levels [27]. Moreover, a decrease in cell growth and pigment production in sulfite-containing media is mainly because of the cytotoxic nature of reactive sulfite anion in interacting with disulfide bonds in proteins vital for living organisms [50].

In conclusion, a newly isolated *Serratia* sp. strain S2B, with suitable capacity to produce prodigiosin metabolite in a cost-effective medium--sugar beet molasses--has been introduced. Optimization of production conditions showed that, in addition to previous important factors in prodigiosin production, inorganic ions such as sulfate also played significant roles in regulation

of the prodiginine biosynthesis pathway. To the best of our knowledge, this is the first report on the role of sulfate as an effective factor in regulation of biosynthesis. In the present model, data revealed that sulfate ions had a dual concentration-dependent effect on prodigiosin production by strain S2B. A weak concentration of sulfate ($< 0.2\%$), with a positive effect on synthesis of sulfur amino acids like methionine, increases prodigiosin production and, at higher concentrations ($> 0.2\%$), acts as a substrate of OrfY and BlhA enzymes, and causes an inhibitory effect on expression of prodigiosin operon genes (*Pig A-O*) due to an increase in a sulfur compound concentration called the z product in the cell, subsequently partially decreasing the prodigiosin production ratio.

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends:

Fig. 1. UV-visible (a) and FTIR (b) spectra of pigments extracted from *Serratia* sp. S2B.

Fig. 2. Thin-layer chromatography of pigments produced in different ammonium salts. Line 1: MP; line 2: MP+ ammonium acetate; line 3: MP + ammonium chloride; line 4: MP + ammonium sulfate; line 5: MP + ferric ammonium citrate; line 6: MP + ammonium nitrate. All pigment extracts were prepared in 10 mg/mL.

Fig. 3. PPR in various concentrations of ammonium sulfate (●), ammonium chloride (■) and ammonium sulfite (▲).

Fig. 4. Prodigiosin and prodigiosin-like pigments induce plasmid DNA cleavage in the presence of Cu(II). Gel electrophoresis diagram of pBlu2KSM DNA in the presence of increasing amounts of (a) prodigiosin and (b) prodigiosin-like pigments (20-100 μ M). Lane 1, HindIII-digested plasmid; lane 2, DNA+ 30 μ M CuCl₂; lane 3, DNA+30 μ M CuCl₂+20 μ M ligand; lane 4, DNA+30 μ M CuCl₂+40 μ M ligand; lane 5, DNA+30 μ M CuCl₂+60 μ M ligand; lane 6, DNA+30 μ M CuCl₂+80 μ M ligand; lane 7, DNA+30 μ M CuCl₂+100 μ M ligand, lane 8; DNA ladder. Linear form (LF) and positive super-coiled (PSC) DNA are labeled, respectively.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Table 1. Fractional factorial design with five independent variables and observed values for prodigiosin production in three independent experiments.

Run	Molasses (%v/v)	(NH ₄) ₂ SO ₄ (g/L)	Peptone (g/L)	Temperature (°C)	Agitation rate (rpm)	PG extracted (mg/L)
1	0.5	2	0.5	27	120	610± 5
2	0.5	5	1	27	120	732.5±37.5
3	0.5	5	1	23	180	792±12.5
4	1	2	1	27	120	1347±57.5
5	0.5	2	1	27	180	975±35
6	0.5	5	0.5	23	120	625±30
7	1	2	0.5	23	120	907.5±7.5
8	1	2	1	23	180	1232.5±62.5
9	0.5	2	1	23	120	695±10
10	1	5	1	23	120	1242.5±22.5
11	0.5	2	0.5	23	180	700±40
12	1	2	0.5	27	180	1175±80
13	0.5	5	0.5	27	180	615±5
14	1	5	0.5	27	120	980±45
15	1	5	0.5	23	180	1247±72.5
16	1	5	1	27	120	1440±45

Table 2. Experimental results observed in RSM study using three independent variables with six center points showing observed values of prodigiosin production (response).

Run	X1	X2	X3	Prodigiosin measured (mg/L)	Prodigiosin Predicted (mg/L)
1	+1	-1	+1	1242.5 ± 7.5	1174.1
2	+1	-1	-1	992.5 ± 67.5	1130.5
3	+1	+1	-1	1442.5 ± 92.5	1396.3
4	-1	-1	-1	575 ± 35	474.0
5	+1	+1	+1	2007 ± 93	1891.6
6	-1	+1	+1	1440 ± 20	1235.1
7	-1	-1	+1	692.5 ± 32.5	517.6
8	-1	+1	-1	690 ± 10	739.8
9	+1.73	0	0	1645 ± 5	1715.4
10	-1.73	0	0	312.5 ± 7.5	579.6
11	0	-1.73	0	815 ± 40	722.3
12	0	+1.73	0	1517 ± 23	1572.7
13	0	0	+1.73	882.5 ± 7.5	948.2
14	0	0	-1.73	742.5 ± 57.5	682.1
15	0	0	0	1175 ± 5	1147.5
16	0	0	0	1120 ± 35	1147.5
17	0	0	0	1140 ± 35	1147.5
18	0	0	0	1135 ± 20	1147.5
19	0	0	0	1122.5 ± 45	1147.5
20	0	0	0	1135 ± 10	1147.5

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3 **Table 3.** Pigment production ratio (PPR) of the media containing 1% molasses, 0.1% peptone (MP)

4 supplemented by different ammonium salts at 15.2 mM concentration.

Medium components	Total biomass (g/L)	PG (g/L)	Total biomass-PG (g/L)	PG/Total biomass-PG (PPR)
MP + ammonium chloride	2.590 ± 0.075	0.945 ± 0.070	1.520 ± 0.014	0.622 ± 0.033
MP + ammonium nitrate	2.570 ± 0.130	1.035 ± 0.035	1.535 ± 0.165	0.674 ± 0.094
MP + ammonium sulfate	3.060 ± 0.215	1.475 ± 0.069	1.585 ± 0.043	0.930 ± 0.094
MP + ammonium acetate	2.855 ± 0.195	0.875 ± 0.055	1.980 ± 0.140	0.442 ± 0.004
MP + ferric ammonium citrate	2.610 ± 0.105	0.925 ± 0.095	1.685 ± 0.010	0.549 ± 0.053
MP	2.670 ± 0.045	0.880 ± 0.025	1.790 ± 0.069	0.492 ± 0.034

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Table 4. The IC₅₀ values of prodigiosin and prodigiosin-like pigments when tested on Jurkat and MCF-7 Cell Lines after 24 hours treatment.

pigments	Jurkat	MCF-7
	24 h	24 h
prodigiosin	5.64 ± 0.59 ^a	2.82± 0.30
prodigiosin-like	5.29 ± 0.31	2.13± 0.47

^a IC₅₀= is the concentration of drug required to inhibit the growth of 50% of the cancerous cells (μM).

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