

Optimization of physicochemical parameters influencing the production of prodigiosin from *Serratia nematodiphila* RL2 and exploring its antibacterial activity

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Received: 6 July 2017 / Accepted: 13 September 2017 / Published online: 20 September 2017
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Abstract In the present study, role of various physicochemical parameters influencing the production of antimicrobial pigment prodigiosin from *Serratia nematodiphila* RL2 was determined and optimized. The pigment-producing strain was isolated and based on molecular characterization (16S rRNA sequencing), was identified as *S. nematodiphila* RL2. The pigment produced by *S. nematodiphila* RL2 was characterized by thin layer chromatography (R_f 0.94), spectrophotometrically (λ_{\max} 535 nm) and identified as prodigiosin. Optimization of production parameters of prodigiosin revealed, nutrient broth medium supplemented with lactose and yeast extract at 1% concentration each, have a positive effect on the bacterial growth (10.25–4.6 mg/ml DCW) as well as pigment production (0.46–0.6 mg/ml). Prodigiosin production (0.64 mg/ml) increases optimally after 46–48 h of incubation, at 35 °C at pH between 6 and 7 with addition of metal ions such as Uranyl acetate. An increase of 65% in prodigiosin production (0.46–0.76 mg/ml) was observed after optimizing the various production parameters than unoptimized conditions. Antimicrobial activity of the prodigiosin was also evaluated and found to be effective antimicrobial agent against bacterial pathogens including *Listeria* sp., *Pseudomonas* sp., *Yersinia* sp. and *Shigella* sp. Present study indicate that *S. nematodiphila* RL2 is a potent source of pigment prodigiosin which can be further explored for production of prodigiosin.

Keywords Antimicrobial activity · Optimization · Prodigiosin · *Serratia nematodiphila* RL2 · Thin layer chromatography

Introduction

Pigments are the colouring agents that can be produced either by living organisms or chemical reagents. Natural pigments are mainly obtained from plants and microorganisms and represent one of the potential sources of chemicals as well as medicines (Isaka et al. 2002). At physiological temperature, *Serratia* spp. may express a pink/red pigmentation, known as prodigiosin, a kind of secondary metabolite produced by other bacteria also (Lewis et al. 2000). Prodigiosin is a secondary metabolite alkaloid with a unique tripyrrole chemical structure (Grimont et al. 1977; Khanafari et al. 2006) and has been shown to be associated with extracellular vesicles or present in intracellular granules (Matsuyama et al. 1986; Kobayashi and Ichikawa 1991). Prodigiosin has no defined primary role in the growth and physiology of producing strains, but has been reported to have antimicrobial (Ibrahim et al. 2014), antimalarial (Isaka et al. 2002), immunomodulating (Giri et al. 2004) and anticancer activities (Montaner et al. 2000).

Keeping the importance of this pigment in mind, the present study was performed to explore the sources of prodigiosin, to optimize various physicochemical parameters for the production of prodigiosin from *S. nematodiphila* RL2 and exploring its potential as a potent antimicrobial agent.

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Materials and methods

Isolation and identification of *S. nematodiphila* RL2

The pigment-producing organism was isolated from the soil sample collected from the Lahul region of Lahul and Spiti district of Himachal Pradesh, India. *Listeria* sp., *Pseudomonas* sp., *Yersinia* sp. and *Shigella* sp. were obtained from Indira Gandhi Medical College (IGMC), Shimla, India. Various biochemical tests and 16S rRNA sequencing followed by comparative analysis of sequence was performed for identification of strain.

Optimization of growth conditions for pigment production by *S. nematodiphila* RL2

Selection of the medium

Four different media, i.e. Nutrient broth, Luria–Bertani broth, Tomato juice broth and Yeast potato dextrose broth were tested for the growth of *S. nematodiphila* RL2 and prodigiosin production. The initial pH for all media was adjusted to 7.0 and incubation was at 35 °C for 50 h.

Carbon source

Effect of carbon source was studied on the growth and pigment production. For this, organic and inorganic carbon sources (sucrose, galactose, glucose, maleic acid, ammonium acetate, citric acid, glycerol, sodium oxalate and lactose) were used at concentration of 1% (w/v) in the production medium (pH 7.0).

Nitrogen source

To analyse the effect of nitrogen source on the growth of the organism and pigment production, organic and inorganic nitrogen sources (yeast extract, peptone, tryptophan, beef extract, glycine and ammonium sulphate) at concentration of 1% (w/v) were used.

Medium pH

To evaluate the optimum pH for the growth of the organism and production of prodigiosin, the medium pH was varied from 3 to 10.

Temperature

Effect of temperature ranging from 20 to 55 °C was studied on the growth of the organism and production of prodigiosin by *S. nematodiphila* RL2.

Incubation time

To find out the optimum incubation time for the growth of the organism and production of pigment by *S. nematodiphila* RL2, culture was incubated up to 50 h and culture samples were withdrawn at an interval of 2 h for assessment of the growth and pigment production.

Metal ions

The effect of metal ions such as UO_2^{+2} , Mo^{+6} , Co^{+2} , Fe^{+3} , Cu^{+2} and Hg^{+2} at 100 mM on the pigment production as well as on the cell growth was studied.

Production of pigment by *S. nematodiphila* RL2

Estimation of cell mass

The cells of *S. nematodiphila* RL2 were harvested from culture broth by centrifugation at $10,000 \times g$ for 10 min and corresponding absorbance of harvested cells was measured at 600 nm in a spectrophotometer. Cell pellet was dried in oven overnight and the amount of cell was measured to calculate the dry cell weight (DCW) and absorbance relationship.

Extraction of pigment

The dry cells of *S. nematodiphila* RL2 were ground using motor pistol, the powder obtained was suspended in acetone and centrifuged at $10,000 \times g$ for 10 min. The supernatant containing pigment was collected and absorbance was measured at 535 nm. The supernatant was allowed to evaporate overnight and the amount of obtained dried pigment was measured. The known dried weight of pigment corresponding to their optical density was recorded after suspending it in solvent used (Picha et al. 2015).

Characterization of pigment produced by *S. nematodiphila* RL2

Thin layer chromatography

The silica gel thin layer chromatography (TLC) was used for effective separation of the impurities from the pigment produced. Totally 10 μl of pigment in acetone was spotted on silica plate. A mixture of dichloromethane, chloroform and acetone (5:5:1%v/v) was used as a mobile phase.

Spectroscopic analysis of pigment

To determine the absorption maxima of the pigment, the pigment was analysed spectrophotometrically in the visible region wave length of 400–700 nm range.

Antibacterial activity of prodigiosin produced by *S. nematodiphila* RL2

Four bacterial cultures, i.e. *Listeria* sp., *Pseudomonas* sp., *Yersinia* sp. and *Shigella* sp. were used to test the antimicrobial activity of the pigment produced by *S. nematodiphila* RL2. A 20 μ l of the pigment (0.70 mg/ml) dissolved in acetone was added in test, against a control (acetone without pigment). The antimicrobial activity was analysed by observing clear zone around the well.

Results

Identification of pigment producing microorganism

Based on the various biochemical tests, morphological characteristics (S1) and 16S rRNA gene sequence (S2), the pigment producing strain was identified as *Serratia nematodiphila* and named as *S. nematodiphila* RL2. Isolated strain exhibited maximum similarity (99.52% sequence similarity) with the 16S rRNA sequence of *Serratia nematodiphila* DSM 21420 as shown in Fig. 1 and sequence has been submitted to the NCBI/Nucleotide databases under accession no. MF417388.

Optimization of parameters for production of pigment prodigiosin from *S. nematodiphila* RL2

Culture medium

To investigate the effect of nutritional media on the growth and pigment production, it was grown in four different media and nutrient broth showed highest pigment (0.46 mg/ml) along with adequate cell mass production (10.25 mg/ml DCW) as shown in Fig. 2.

Carbon source

To determine the effect of carbon source on the production of pigment, *S. nematodiphila* RL2 was grown in 50 ml of culture medium with different carbon sources (1%). It was observed that lactose (0.52 mg/ml pigment; 12.8 mg/ml DCW) was most preferred carbon source for this organism, while relatively lower pigment yield was recorded with other carbon sources as shown in Fig. 3.

Nitrogen source

The effect of different nitrogen sources on the production of pigment and growth of the *S. nematodiphila* RL2 was studied and yeast extract was found to be optimum for the production of pigment (0.6 mg/ml) as well as growth (14.6 mg/ml DCW) of the strain as shown in Fig. 4.

pH

S. nematodiphila RL2 was grown in optimized production medium (nutrient broth) with pH variation from 3 to 10, and it was observed that slight acidic/neutral pH (6–7) was optimum for pigment production (0.6 mg/ml) as well as for cell growth. It was observed that increase in production of pigment occurs up to pH 6 and further increase in pH beyond 7 resulted in suppression of its production as shown in Fig. 5.

Temperature

S. nematodiphila RL2 produced higher amount of pigment as well as cell mass at 35 °C (0.59 mg/ml) and either side variation decrease the production of pigment as shown in Fig. 6.

Time course of pigment production

S. nematodiphila RL2 was grown in nutrient broth and pigment production was assayed after every 2 h up to 50 h. Maximum growth and pigment production was recorded after 46 h of incubation which lasts up to 48 h (0.64 mg/ml pigment) as shown in Fig. 7.

Effect of metal ion

The effect of various metal ions on the production of prodigiosin was studied and it was observed that maximum pigment production was observed with uranyl acetate (0.76 mg/ml) (Fig. 8) while CoCl_2 and HgCl_2 suppressed the growth as well as production of pigment.

Estimation of cell mass

The known dried cell weight corresponding to their optical density was recorded and the results showed that 1 OD corresponds to the 7.8 mg/ml of cells.

Estimation of pigment

The known dried pigment corresponding to their optical density was recorded and the result showed that 1 OD corresponds to the 0.30 mg/ml of pigment.

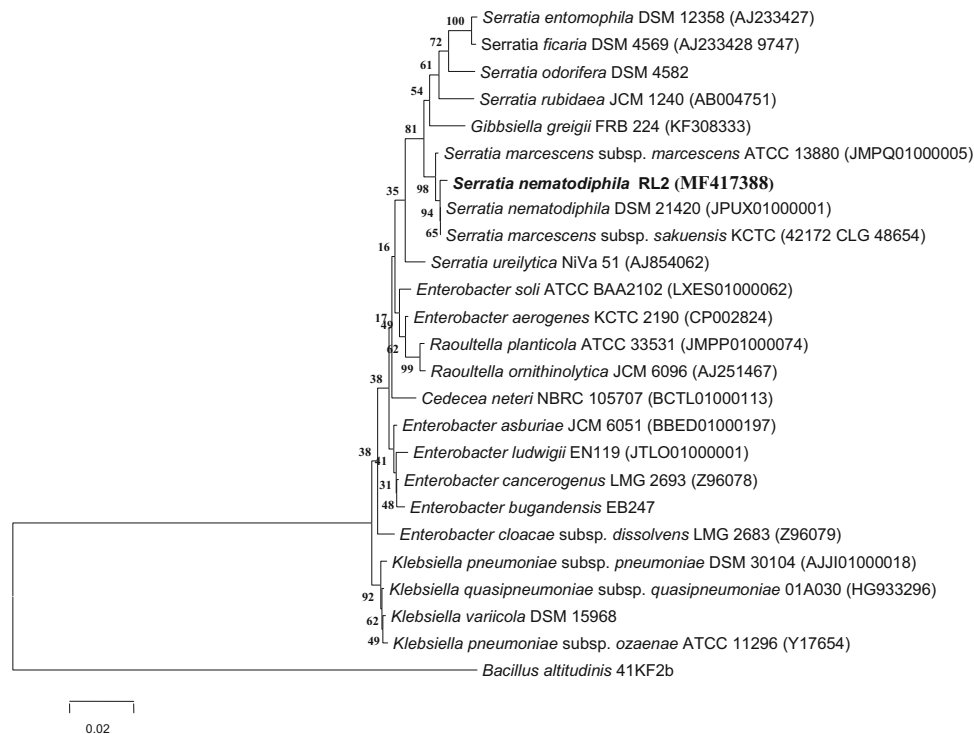
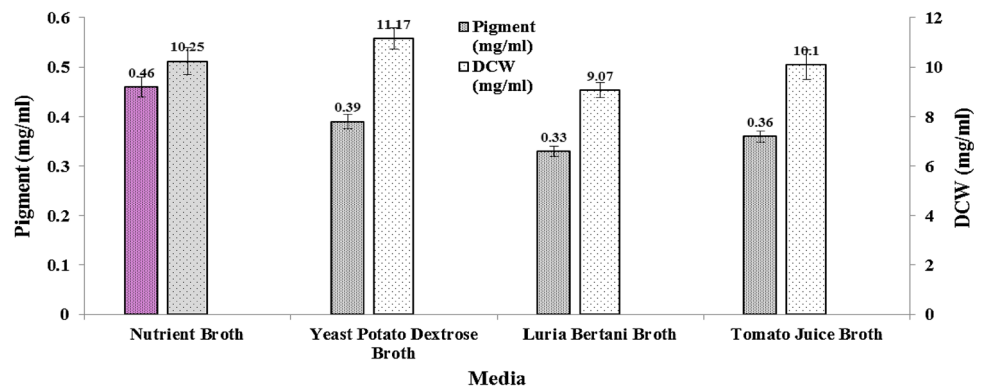


Fig. 1 Phylogenetic position of strain *Serratia nematodiphila* RL2 among related bacteria was inferred using the neighbor-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.38090397 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the

phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1322 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013)

Fig. 2 Effect of different media on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2



Characterization of pigment

Thin layer chromatography

Thin layer chromatography of the pigment extracted from the *S. nematodiphila* RL2 was performed and a single coloured band was observed (S3). The R_f value of the pigment was calculated and was found to be 0.94.

Spectroscopic analysis

The spectroscopic analysis revealed a single peak at 535 nm which corresponds to absorption maxima of prodigiosin.

Fig. 3 Effect of different carbon sources on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2

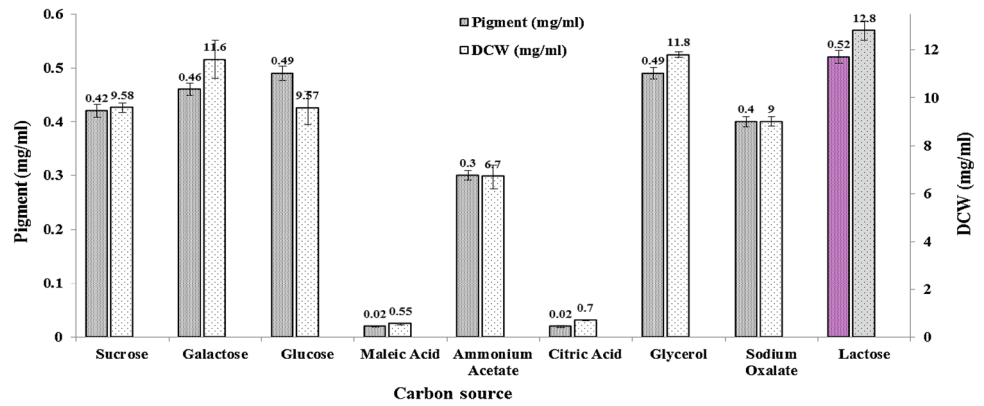


Fig. 4 Effect of different nitrogen sources on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2

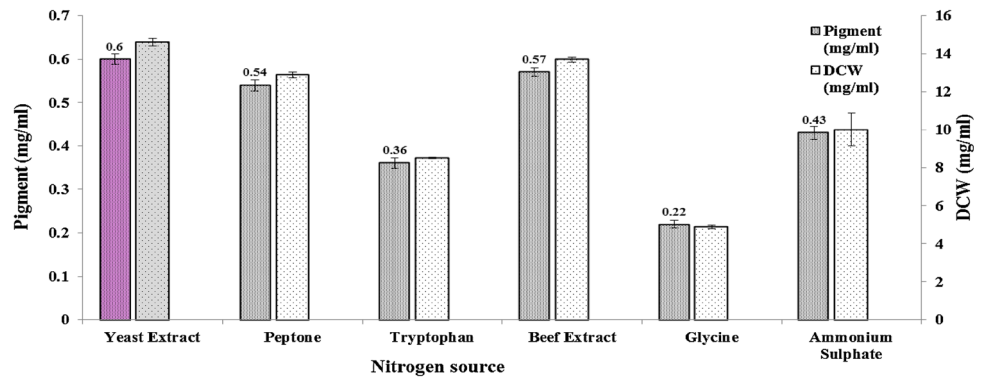


Fig. 5 Effect of different pH on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2

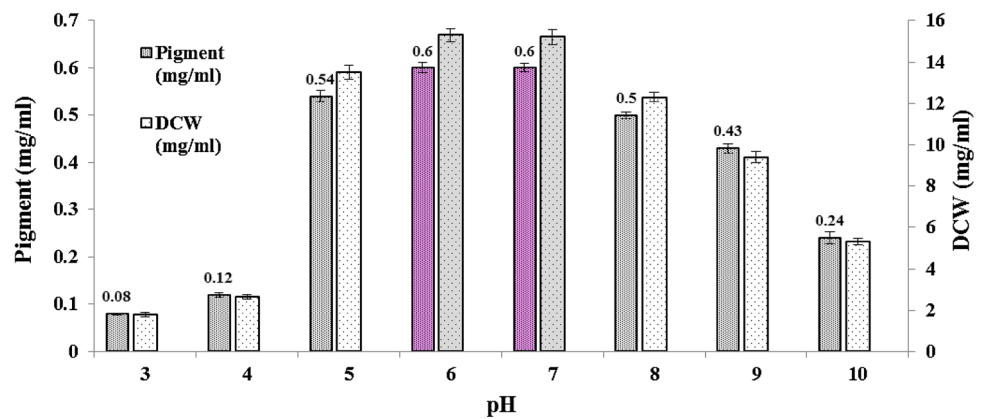


Fig. 6 Effect of different temperatures on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2

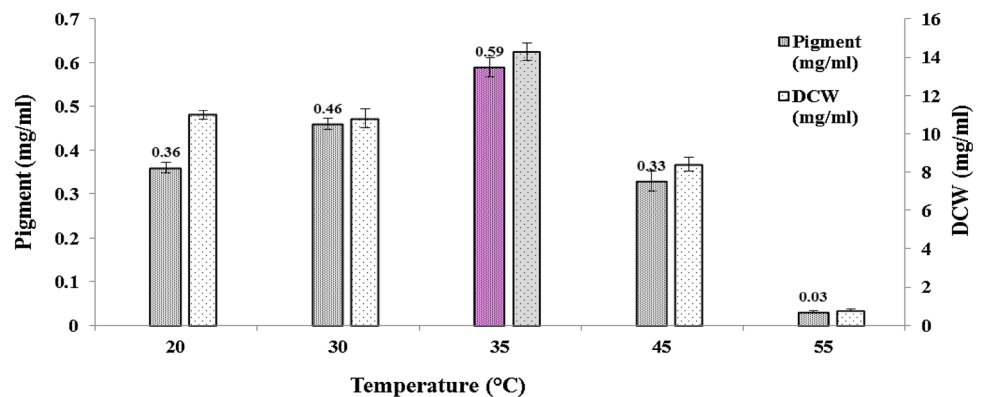


Fig. 7 Effect of incubation time on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2

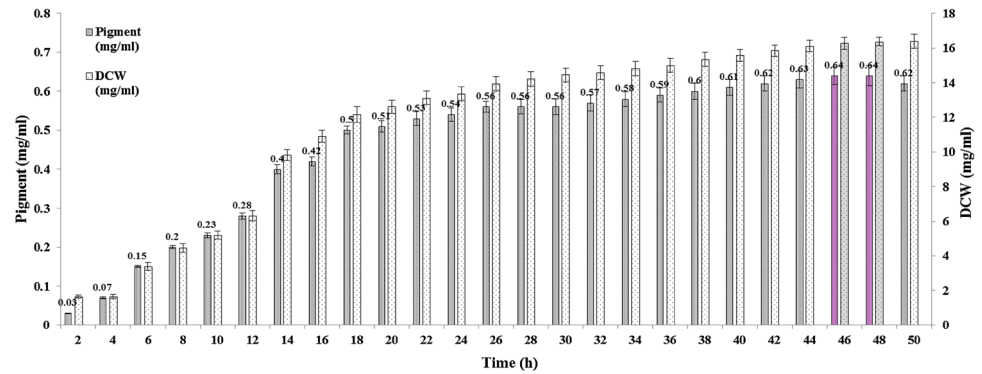
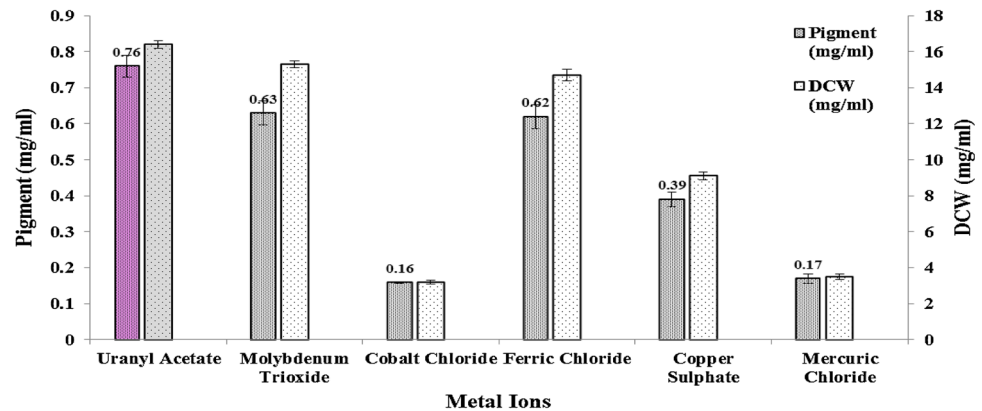


Fig. 8 Effect of different metal ions on growth of *S. nematodiphila* RL2 and pigment production by *S. nematodiphila* RL2



Antibacterial activity of pigment

Four bacterial pathogens (*Listeria* sp., *Pseudomonas* sp., *Yersinia* sp. and *Shigella* sp.) were used to evaluate the antimicrobial activity of the pigment produced by *S. nematodiphila* RL2. Pigment prodigiosin showed potent antibacterial activity as clear zone of inhibition was observed around the well (S4).

Discussion

Prodigiosin, a pigment having pyrrolypyrromethane skeleton was first characterized from *S. marcescens* and reported to be an antifungal, immunosuppressive and anti-proliferative agent (Khanafari et al. 2006). In current study *S. nematodiphila* RL2 was isolated from the cold desert of Himachal Pradesh having the property to produce prodigiosin. Prodigiosin was characterized by spectrophotometric analysis and thin layer chromatography and spectrophotometric analysis of pigment showed spectrum maxima at 535 nm, corresponds to prodigiosin as reported in literature (Song et al. 2006; Samrot et al. 2011; Silva et al. 2012; Kurbanoglu et al. 2015; Darshan and Manmohini 2016). Thin layer chromatography showed R_f value of 0.94, which was also reported by Someya et al. (2004) in

the range of 0.90–0.95, thus concluding that the red pigment as prodigiosin. Davaraj et al. (2009) have also extracted prodigiosin from the standard strain of *S. marcescens* MTCC 97 having R_f value 0.9–0.95 further supported our observation.

Giri et al. (2004) used the powdered sesame seed in water, nutrient broth and peptone glycerol broth as a growth medium for *S. marcescens*. It has been reported that *Serratia* sp. grows well on synthetic media using various compounds as a single carbon source (Furstner 2003) supporting present observations that pigment production was maximum when nutrient broth supplemented with single carbon source, i.e. lactose. Sundaramoorthy et al. (2009) showed that the pigment production was maximum in the presence of maltose, where present study showed that lactose increases pigment production among several carbon sources tested. In the present study, yeast extract as a nitrogen source increases growth as well as pigment production followed by beef extract and peptone. Andrade et al. (2009) reported that the addition of yeast extract as nitrogen source in the medium increased pigment production, such as Corn steep liquor offers a rich source for culture media. Similar results were also shown by Suryawanshi et al. (2014), where yeast extract significantly enhanced the production of prodigiosin, as nitrogen plays

an important role in prodigiosin production (Kurbanoglu et al. 2015).

The pH of medium regulates the pigment production as well as cell growth as there was increase in pigment production from 5 to 7 pH was observed, as Patil-Nilam and Chincholkar (2014) investigated the influence of initial pH on the prodigiosin production by *S. nematodiphila* was highest at 7 pH. Khanafari et al. (2006) reported the reduction of prodigiosin production by *S. marcescens* mediated by glucose and other metabolizable sugars was due to a lowering in pH observed in cell suspensions. Present investigation showed that heavy metal addition also increases pigment production which can be related to the chelating activity of prodigiosin. Morgenstern et al. (2015) also indicated the role of divalent metal ions in prodigiosin production, in which cobalt divalent ions significantly increases the prodigiosin production by inducing sleeping genes. Temperature is an important factor and it was observed that pigment production was maximum at temperature 30 °C as Giri et al. (2004) showed that there is decrease in pigment production above 30 °C but fatty acid medium such as powdered peanut and seed medium can support the prodigiosin production even up to 42 °C. Sundaramoorthy et al. (2009) also showed significant decrease in prodigiosin production by *S. marcescens* at 40 °C and comparable pigment production at 25, 30 and 35 °C. Maximum pigment production was observed at 46–48 h interval of incubation, as the prodigiosin is already reported as a secondary metabolite in literature (Williams 1973). Harned (1954) also reported that no significant prodigiosin production was seen after 48 h incubation and supports present findings.

Lee et al. (2011), Priya et al. (2013) and Ibrahim et al. (2014), showed an antibacterial activity of prodigiosin against a range of pathogens by showing inhibition zone which supports the antimicrobial activity of prodigiosin. Ramina and Samira (2009) concluded that the antibacterial activity of prodigiosin is due to its ability to pass through the membrane and causes inhibition of target enzymes, such as DNA gyrase and topoisomerase IV, which are essential for the cell growth. Darshan and Manmohini (2016) showed that prodigiosin from *S. nematodiphila* can induce programmed cell death which involves DNA fragmentation, ROS generation and expression of caspase like protein in bacterial cells. Arakha et al. (2015) also postulated the antimicrobial activity of prodigiosin may be attributed to the interaction of positively charged prodigiosin with negatively charged bacterial membrane leading to reactive oxygen species production resulting in bacterial cell death. Present investigation also showed that the pigment prodigiosin produced by *S. nematodiphila* RL2 is potent antibacterial agent against several potent pathogenic strains.

Conclusion

The present investigation had led to the isolation of a bacterial source of prodigiosin, i.e. *S. nematodiphila* RL2 with a high potential for prodigiosin production. Optimization of culture conditions, pigment production, pigment characterization and antimicrobial activity of pigment was carried out. Culture conditions were optimized in terms of production media, carbon source, nitrogen source, pH, metal ions, temperature and incubation time. Prodigiosin was found to be in higher yield with heavy metal ions under stress condition. Prodigiosin produces from *S. nematodiphila* RL2 was found to be active against different pathogenic microorganisms showed that it may play important role in microbial competition. These results indicate that *S. nematodiphila* RL2 has emerged as potent source of prodigiosin and can be further explored for its future applications.

Acknowledgements Authors are highly grateful to University Grants Commission (UGC), New Delhi, India for providing financial assistance to Mr. Mohammad Asif.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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