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# Exploration of the optimized parameters for bioactive prodigiosin mass production and its biomedical applications *in vitro* as well as *in silico*

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**Running title**

Prodigiosin pigment unveils antimicrobial, anti-viral, anti-neurodegenerative and anticancer activities

**Key words:** *Serratia marcescens*, *Aedes aegypti*, FT-IR, GC-MS, c-RAF and Aβ

## Highlights

- Prodigiosin pigment acts against various pathogens *in vitro*
- Prodigiosin confers larvicidal activity against *Aedes aegypti* larvae
- *In silico* analysis reveals that the prodigiosin have a wide range of biomedical activities

## Abstract

Prodigiosin from *Serratia marcescens* is considered as an important secondary metabolite for various significant applications. This study explores the optimized conditions for the maximal prodigiosin production, characterization of prodigiosin, biomedical applications against therapeutically important diseases. Prodigiosin was extracted, purified using different solvents and the purified prodigiosin was characterized using FT-IR, UV-Vis Spectrometry and GC-MS analysis. The maximal production of prodigiosin was identified using different optimization methods. UV-Vis spectrophotometry showed the absorbance peak at 530 nm and the FT-IR

result revealed prodigiosin contains most of the functional groups that are corresponding to the literature reports and also GC-MS analysis validated the presence of prodigiosin by showing the peak at 323 m/z. Antibacterial activity showed higher activity against *Staphylococcus* sp. than Gram negative bacteria and showed a very effective activity against *Candida* sp. than other tested fungi. The larvicidal activity of prodigiosin against *Aedes aegypti* clearly indicated that 32 % and 76 % mortality rate was seen at 24 and 48 h respectively. *In silico* analysis of prodigiosin against Aβ peptide 1-42, a well-known breast cancer protein RAF-1 and two viral proteins E1A and TMV-CP revealed that it has a strong interaction with less binding energy. Altogether, it is strongly endorsed that the prodigiosin pigment could pave a way to treat various bacterial infections and disorders in the forthcoming existences.

## 1. Introduction

A large amount of unique species such as molds, yeasts, algae and bacteria generate secondary metabolites such as antibiotics, pigments, etc. Meanwhile, benefit of the microbial system relies on the capacity to produce cost effective desired products by means of fermentation. Moreover, not all the group of microbial communities produces negative impact on the environment. Some microorganisms are beneficial to human beings i directly or indirectly. Secondary metabolites such as antibiotics, pigments, phenolic compounds, etc., purified from the bacterial community have been used in vast applications, and thereby they are being produced on large scale in industries. Enhancement and improvement in solubility, stability and safety has made extensive applications such as pigments in the food and, pharmaceutical industries. In general, the secondary metabolites turn out to be the protective mechanisms against their antagonists (for example, prodigiosin from *Serratia marcescens* could act as an antigenic factor during infection) **(Srinivasan et al., 2016; Srinivasan et al., 2017; Ramanathan et al., 2018; Ravindran et al., 2018)**. In some cases, microbial pigments employed as food coloring agents also acts as preservative with potential antioxidant properties **(Suryavanshi et al., 2014).** Chromogenic bacterial species are frequently isolated from soil, water, plants or insects. Among them, *S. marcescens* is a rod shaped belongs to the family Enterobacteriaceae, facultative and Gram negative bacteria categorized by its capability to produce the red colour pigment prodigiosin **(Khanafari 2006; Arivizhivendhan et al., 2018).** It has been reported earlier that among the characterized ten species of *Serratia*, only 3 species is capable of producing

prodigiosin: *S. rubidaea*, *S. plymuthica* and *S. marcescens* **(Grimont et al., 1977; Wei and Chen 2005; Annapoorani et al., 2012).** Since, *S. marcescens* is a facultative bacterium and the pigment is yielded under both anaerobic and aerobic conditions **(Wei and Chen 2005)**. Pigment production is extremely variable among the same genus and relies on physiological and biochemical factors such as pH, incubation time, nitrogen and carbon sources **(Kim et al., 2008; Pandey et al., 2009).**

Prodigiosin (a red colour pigment) is one of the economically important secondary metabolites produced by *S. marcescens* and few other bacterial species such as *Pseudomonas magnesiorubra* **(Lewis and Corpe 1964),** *Vibrio psychroerythrus* **(D'Aoust and Gerber 1975)**, *Alteromonas rubra, Actinomycetes* such as *Nocardia* spp. **(Khanafari et al., 2006)** and *Streptomyces* spp. Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1Hpyrrole) is an alkaloid compound with a distinctive structure that consists of three pyrrole rings and is a pyrryldipyrrlmethane; two of the rings are directly connected to each other and the third is linked by a methane bridge **(Kobayashi and Ichikawa 1991).** The molecular formula of prodigiosin is $C_{20}H_{25}N_3O$ with the molecular weight of 323.432 Da **(Casullo de Araújo et al., 2010).** It forms lustrous, square pyramidal crystals that are dark red with a green reflex and it is found to be light sensitive, insoluble in water, moderately soluble in alcohol and ether, soluble in chloroform, methanol, acetonitrile and DMSO. For the past few decades, these pigments were frequently studied by the researchers throughout the world and emerging as a novel group of compound having distinct biological significance such as antibacterial, antifungal, larvicidal, immuno-modulating, antitumor and nuclease activities.

Prodigiosin is as a strong therapeutic molecule represented specially for its immunosuppressive properties and anticancer properties **(Khanafari et al., 2006).** This prodigiosin pigment has a significant role in antimicrobial activity against Gram positive bacteria such as *Bacillus* sp. and *Staphylococcus* sp. **(Cang et al., 2000);** Gram negative bacteria such as *Escherichia coli* and *Pseudomonas* sp. It is found less active against fungal pathogens like *Fusarium oxysporum*, *Aspergillus flavus* and higher antifungal activity against *Penicillium notatum* **(Suryavanshi et al., 2014).** There is only less accessible information on total antioxidant activity of prodigiosin in *in vitro* conditions employed by phosphomolybdate method which requires cost-effective reagents **(Gulani et al., 2012).** Since, prodigiosin is known for its larvicidal activity, a recent study by **Patil et al., 2011** revealed that at higher concentration (500

ppm), mortality of *Aedes aegypti* larvae starts within 6 h of exposure and more than 50 % mortality occur within first 24 h **(Patil et al., 2011).** In this study, we have analyzed the larvicidal activity of prodigiosin against *Aedes aegypti* larvae and their survival against the treatment at minimal concentrations.

Generally, *in silico* studies provide the preliminary knowledge about the binding efficiency of a compound towards its target protein molecule. RAF proto-oncogene is a serine/threonine-protein kinase which is also known as c-RAF/Raf-1 is an enzyme that is encoded by the *raf-1* gene. A recent report by **Ma 2017** suggested that *Raf-1* still exists as a pharmaceutical target to proliferate radiotherapy or chemotherapy sensitivity on cancer cells. So, we have chosen c-RAF as a target molecule to test the prodigiosin's binding efficiency. Also, we have selected E1a protein as a target molecule to check the binding energy of prodigiosin if any. In general, Adenovirus early region 1A (E1a or E1A) protein is expressed during replication of Adenovirus to synthesis a variety of E1A family proteins for their cell cycle **(Routes et al., 2000).**It has been also considered as an oncogene and also can act as a tumor suppressor gene. Thus, the binding efficiency of the prodigiosin compound against the abovementioned proteins was elucidated using molecular docking study. As far as we know, this is the first study to provide the molecular interaction of prodigiosin against these potential drug targets using *in silico* analysis.

Amyloid beta (A-beta or Aβ) designates the series of peptides ranging from 36–43 amino acids that significantly plays an important role in Alzheimer's disease. Recent research study suggests that on the formation of amyloid plaques in brain **(Hamley 2012),** the soluble oligomeric forms of Aβ (1-42) peptide turns into a crucial causative agent for Alzheimer's disease. It is generally believed that Aβ oligomer deposition in brain is the most toxic condition than the differential regulation of upstream protein players **(Prelli 1988; Shankar 2008).** A novel exploration on the molecular interaction of prodigiosin with Aβ peptide has been carried out in the present study. Altogether, the current study has been focused on the optimization of enhanced prodigiosin production using various factors including sugars, salt concentrations, solvents, nitrate substrates, carbon substrates, etc., Also, the important parameters such as antimicrobial activity, *in silico* studies has also been carried out to explore the multifaceted applications of prodigiosin. It is renowned that the studies upon potential secondary metabolites and their enhanced production obviously make a way to explore their activity thereby aids in

treating various infectious diseases. Undisputedly, the current study creates an impact among the researchers to explore the multi-dimensional activities of secondary metabolites from various sources.

## 2. Materials and methods

### 2.1. Isolation of red colour pigment producing *Serratia marcescens*

Soil contains large different groups of pigment producing bacteria. Out of this, only red colour pigment producing bacteria was isolated using serial dilution method (Nutrient agar medium and Trypticase Soy Agar Medium). The isolated organism was subjected to morphological identification and biochemical identification for the confirmation of *Serratia marcescens.* Cultures were grown in nutrient broth medium for optimal prodigiosin production. The prodigiosin production was estimated at both stationary and agitated phases to investigate the effect of aeration production. After 24 and 48 h, the quantity of prodigiosin was estimated in those conditions using standard calorimetric method.

### 2.2. Estimation of prodigiosin

To quantify the prodigiosin produced by *S. marcescens*, broth suspensions were subjected to centrifugation at 10,000 rpm for 10 min. 15 ml of 1 % acidified ethanol was added to the cell pellet and centrifuged at 10,000 rpm for 10 min. 1 % acidified ethanol was used as a blank. The absorbance of prodigiosin at was measured at 530 nm and additionally bacterial cell absorbance in nutrient broth was measured at 620 nm. Since, solvent extract of prodigiosin showed maximum absorbance at 530 nm, extracted prodigiosin was estimated using the following equation **(Rakh 2017).**

$$\text{Prodigiosin unit/cell} = \frac{[OD530 - (1.381 \times OD620)] \times 1000}{OD620}$$

OD – Optical Density OD620 – Bacterial cell absorbance

1.381 – Constant OD530 – Pigment absorbance

**2.3. Use of different media for the maximal production of prodigiosin**

In order to determine the media supporting the maximal production of prodigiosin, the culture was grown in different media such as Nutrient broth, Peptone glycerol broth, Gelatin broth, LB broth, Tryptone soy broth, Glycerol beef extract broth in agitated phase (120 rpm) at room temperature for 48 h. After the incubation period, the broth cultures were centrifuged at 10,000 rpm for 10 min, prodigiosin was solvent extracted, estimated separately and plotted **(Modified from Casullo de Araújo et al., 2010).**

**2.4. Influence of different temperatures for the maximal prodigiosin production**

Bacterial isolate was inoculated into 100 ml of nutrient broth and incubated at different temperatures such as 25 °C, 37 °C, 45 °C, 55 °C and 4 °C for 48 h with agitation. The prodigiosin unit/cell was estimated after incubation described above. The temperature at which maximum production of prodigiosin observed was chosen for the further studies **(Modified from Suryawanshi et al., 2014).**

**2.5. Influence of different pH on the enhanced prodigiosin production**

Bacterial isolate was inoculated in nutrient broth with various pH 3, 5, 7, 9 and 11 and were incubated at room temperature for 48 h with gentle agitation. The prodigiosin production was estimated after incubation. The pH at which maximum production of prodigiosin observed was chosen for the subsequent studies **(Modified from Casullo de Araújo et al., 2010).**

**2.6. Effect of different salt concentration on maximal prodigiosin production**

The bacterial isolate was inoculated into nutrient broth containing different concentrations (w/v) of NaCl 0.4 %, 0.5 %, 0.6 %, 0.7 %, 0.8 % and 0.9 %. The inoculated broth cultures were incubated at room temperature for 48 h with gentle agitation. After 48 h of incubation, the level of prodigiosin production was estimated using abovementioned standard formula **(Modified from Suryawanshi et al., 2014).**

185

186 **2.7. Influence of different nitrogen sources on enhanced prodigiosin production**

187 In order to study the effect of different nitrogen sources such as ammonium oxalate,
188 ammonium nitrate, ammonium sulphate, ammonium phosphate, urea, tryptone an+d peptone in a
189 concentration of 0.5 % (w/v), the bacterial isolate was inoculated in nutrient broth and incubated
190 at room temperature for 48 h with gentle shaking. After incubation, the amount of prodigiosin
191 production was estimated **(Modified from Suryawanshi et al., 2014).**

192

193 **2.8. Effect of different carbon sources on maximal prodigiosin production**

194 The bacterial isolate was inoculated in 100 ml of nutrient broth containing different
195 carbon sources (1 % w/v of Glucose, lactose, mannitol, maltose, sucrose and fructose) and was
196 incubated at room temperature for 48 h with gentle shaking. The prodigiosin was estimated after
197 incubation using standard formula and graph was plotted **(Modified from Suryawanshi et al.,**
198 **2014).**

199

200 **2.9. Effect of different solvents for the maximal extraction of prodigiosin**

201 In order to extract the maximal prodigiosin content from the cell lysate, the culture was
202 grown in nutrient broth media and incubated at room temperature for 48 h with gentle shaking.
203 After incubation, the bacterial broth was centrifuged at 10,000 rpm for 10 min and the
204 prodigiosin was extracted with various solvents such as ethanol (99.9 %), acidified ethanol (1 %
205 of 1 N HCl in 99 ml ethanol), acetone, methanol (95 %), chloroform and diethyl ether separately.
206 The amount of prodigiosin was estimated using the standard formula and the solvent which have
207 higher extraction levels has been used in further studies.

208

209 **2.10. Solvent extraction of prodigiosin**

210 *Serratia marcescens* was inoculated into nutrient broth medium and incubated for 48 h in
211 agitated phase with the optimized parameters for the maximal production of prodigiosin. The
212 cells were harvested by centrifugation at 10,000 rpm for 10 min. The supernatant was discarded
213 and the pellet was resuspended in 1 % acidified ethanol. The mixture was vortexed and the
214 suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to the
215 fresh vial which will be containing prodigiosin. About 10 ml of ethanol extracted sample was put

in a separating funnel and double the volume of petroleum ether was added. The separating funnel was shaken vigorously and kept undisturbed for 10-15 min allowing the two liquid phases to separate. Prodigiosin was extracted in the petroleum ether layer which was removed carefully from the separating funnel. This petroleum ether layer was poured in a petridish and kept at 50 °C to 70 °C in order to evaporate the solvent completely. The powdered crude sample was used for thin layer chromatography, FT-IR, UV-Vis spectrophotometry, GC-MS, column chromatography and antimicrobial studies **(Modified from Suryawanshi et al., 2014).**

### 2.11. Presumptive test for prodigiosin

The suspended pellet from broth culture was centrifuged at 10,000 rpm for 10 min. Debris was removed and the supernatant was taken in two test tubes. The content of one of the test tubes was acidified with a drop of concentrated hydrochloric acid (HCl) and the other tube was alkalinized with a drop of concentrated sodium hydroxide (NaOH) solution. An indication of pink or red colour in the acidified solution and a tan or yellow colour in the alkaline solution concludes that a positive presumptive test for the presence of prodigiosin **(Rakh 2017).**

### 2.12. Identification and purification of prodigiosin

The crude extract of prodigiosin was dissolved in acidified ethanol and the absorption spectrum was measured using SpectraMax® M3 Multi-Mode Microplate Reader instrument over the range from 300 nm to 750 nm **(Nakashima et al. 2005).** The crude extract was further characterized using Fourier transform infrared (FT-IR) spectrophotometer (Thermo nicolet avatar 380 FT-IR). Crude extract was assorted with KBr (Potassium Bromide) powder and hard-pressed into pellet for the functional group analysis using FT-IR spectroscopy with a frequency range of 4,000–400 $cm^{-1}$ **(Suryavanshi et al., 2014).**

### 2.13. Separation of prodigiosin using Chromatographic techniques

Followed by the solvent extraction of prodigiosin, the crude product was dissolved in 20 ml of methanol and the solution was passed through a hexane-balanced silica gel column to trap the target product within the column. The loaded column was eluted with 10 M ethyl acetate to liberate the adsorbed product and dried at 50 °C – 60 °C. Powdered sample was then dissolved in methanol and used for antimicrobial activity analysis **(Modified protocol from Casullo de**

**Araújo et al., 2010).** And also, the extract was dissolved in methanol and subjected for TLC (Thin layer chromatography) by employing the solvent system containing methanol, ethyl acetate and chloroform in the ratio 6: 3: 1 (v/v) **(Casullo de Araújo et al., 2010).** 50 µl of methanolic extract (in duplicates) was loaded onto the silica gel slides and run against the solvent till the solvent front reaches 2/3$^{rd}$ of the slide. After the development of the chromatograms, slides were removed and dried. The retardation factor ($R_F$) values of the chromatogram were calculated.

**2.14. Gas Chromatography-Mass Spectrometry analysis**

The purified prodigiosin was subjected to GC-MS analysis (Agilent GC 7890A/MS5975C; Capillary DB5MS – 30 m/ 0.25 mm internal dia./0.25 micron film thickness) in order to confirm the presence of prodigiosin. The flow rate was set to 1 ml/min in splitless mode and the oven temperature was 50 °C for 1 min then 10 °C/min to 300 °C for 2 min and the run time was 28 min. The MS ion source temperature was 230 °C and the MS quadrupole temperature was 150 °C. **(Fei et al., 2013; Pore et al., 2016).**

**2.14. Antimicrobial activity**

The antimicrobial activity of the purified prodigiosin was assessed by Muller-Hinton agar well plate method. The bacterial (*Staphylococcus aureus, Escherichia coli, Pseudomonas* sp. *Bacillus* sp*, and Salmonella* sp.) and fungal (*Penicillium* sp. *Aspergillus* sp. *Fusarium* sp. *Mucor* sp. *Candida* sp.) broth cultures were grown in nutrient and Potato Dextrose Broth at 37 ˚C and 27 °C, respectively. From the 24 h grown broth cultures of each bacteria and fungi, swabbing method was employed to spread them on the surface of Muller-Hinton agar in Petri plates **(López-Oviedo et al. 2006).** Agar deep wells were made with sterile micro tips (1 ml) and the extracts of 100 µl each was incorporated into the each wells aseptically. The plates were retained at 2-4 ˚C for 1-2 h to permit prediffusion and then the plates were incubated for 24 h at 37 ˚C for bacteria and 27 ˚C for 48 h for fungi. Zone of inhibition was measured in millimeter from the perimeter of the wells to the circumference of the inhibition zone in each set-up and plotted. The antimicrobial assays were done with experimental duplicates with solvent serving as controls **(Suryawanshi et al., 2014).**

**2.15. Larvicidal activity**

Test larvae for assessing the larvicidal activity of prodigiosin, third-instar larvae of *Aedes aegypti* were obtained from Field Station, Mettupalayam (A field branch of National Centre for Disease Control, Coonoor, Tamil Nadu). The *A. aegypti* larvae were stored in plastic trays filled with dechlorinated tap water and the larvae were maintained at 25±2 ˚C. Larvae were fed with a diet of dog biscuits and finely ground brewer's yeast in the ratio (3:1). 100 mg of dried solvent extracted crude prodigiosin was dissolved in 10 ml of acidified ethanol (serves as stock solution). Acidified ethanol was considered as vehicle control. In order to check the larvicidal activity of prodigiosin against *Aedes aegypti* larvicidal forms, the powdered prodigiosin was taken in the concentration of 100 ppm and added to the dechlorinated water containing 50 numbers third instar stage larvae. The numbers of dead larvae were counted after 12 h, 24 h and 48 h of exposure, and the percentage mortality was recorded for two replicates **(Patil et al., 2011).**

### 2.16. *In silico* analysis of prodigiosin for antiviral, anticancer and anti-neurodegenerative activities

The crystallographic 3D structure of Aβ (PDB ID code: 1IYT), RAF-1 (PDB ID code: 3OMV), E1A (PDB ID code: 2KJE) and TMV-CP (PDB ID code: 1EI7) were obtained from the Protein Data Bank (PDB). Water molecules, Natural ligands and other hetero atoms were excluded from the protein molecules using Autodock MGL tools version 1.2.6. The molecular docking comprises a conformational analysis of the target compound which consist a target binding site with the aim of identifying the best matching posture along with the active site to perform docking. The default parameters were used to do the molecular docking for all the target proteins. The docked models which had the highest docking score were selected to scrutinize the mode of binding. The docking scores (fitness), binding energy, molecular bonding and Van der Waals interactions were visualized by Maestro (Schrodinger) software version 10.0.

## 3. Results and discussion

### 3.1. Isolation of pigment producing organism and estimation of prodigiosin

Five different soil samples were subjected for serial dilution and the red colored colony was sub-cultured and checked with biochemical tests for the confirmation of *S. marcescens*. Moreover, the isolate was taken for IMViC test in which Indole test was found to be negative,

Methyl red test was found to be negative, Voges Proskauer test was found to be positive, and citrate test was found to be positive. Subsequently, with the use of Vitek automated bacterial typing instrument, the sugar fermentation details were analyzed which revealed the tested isolate was *Serratia marcescens*. The colony formation of *S. marcescens* in nutrient agar, tryptone soy agar and nutrient broth has been showed in the **Fig. 1a and Fig. 1b.** Once then the confirmation of *S. marcescens*, the prodigiosin was estimated using the formula given in materials and methods. *S. marcescens* was inoculated into nutrient broth and the prodigiosin production was appeared as red color in the culture flask **(Fig 1c)**. Many reports suggest that the secondary metabolites found to be produced higher at room temperature not with other temperatures **(Giubergia, S., et al., 2016; De Carvalho, C.C. and Fernandes, P., 2010).** In the present study also, prodigiosin production in nutrient broth with shaking after 24 h was found to be insufficient and sufficient (enhanced) prodigiosin was observed after 48 h in nutrient broth with gentle agitation. Hence, aeration resulted in enhanced levels of prodigiosin and the result indicated that the maximum amount of prodigiosin is produced in nutrient broth with shaking in comparison with normal room temperature (27℃) without shaking **(Fig. 2a)**.

### 3.2. Nutrient broth favors the maximal production of prodigiosin

*S. marcescens* was inoculated in different media such as Nutrient broth, Peptone glycerol broth, Tryptone soy broth, Luria Bertani broth, Gelatin broth and Glycerol beef broth medium then it was incubated for 48 h with gentle agitation. Among these different types of media, the maximum production of prodigiosin was seen in nutrient broth **(Fig. 2b)**. Hence, the nutrient broth was used for further studies. In general, the enriched nutrient broth medium favors the higher growth of the organism but not with the secondary metabolites until it gets the proper environment. But in the present study, we found that the nutrient broth itself produced prodigiosin production in higher levels **(Giri, A.V et al., 2004).** Nutrient Broth is a basic media composed of peptone, NaCl, beef and yeast extracts as major components. Peptone delivers the organic nitrogen (in the form of amino acids and long-chained fatty acids). Meanwhile, beef and yeast extract provides additional vitamins, carbohydrates, salts and other organic nitrogen compounds. In the presence of optimal temperature and the nutrients provided in the medium allows the *S. marcescens* to produce more prodigiosin.

### 3.3. Incubation at room temperature delivers the maximal prodigiosin production

Inoculated bacterial cultures were employed for solvent extraction of prodigiosin obtained at various temperatures such as 4 °C, 27 °C, 37 °C, 45 °C and 55 °C. The maximum prodigiosin production occurred over a relatively narrow range of temperatures 25 °C and 37 °C. In the present study, a completely block in the prodigiosin production was observed when *S. marcescens* was incubated at 4 °C. The organism was found to produce more prodigiosin at 27 °C **(Fig. 2c)** and the rate of prodigiosin production was reduced as the temperature increases. The main reasons for the restriction of temperature were relatively studied well. The limitation for temperature is imposed by the varied rates of denaturation/suppression of key cellular components as temperature varies. Mostly, the higher temperature is not preferable for a given bacterial species due to degree of adaptation in terms of modifications of key structures such as cell membranes and involvement of HSPs (Heat Shock Proteins) which protect cellular processes **(Nedwell, D.B., 1999).** Thus, the optimal temperature for individual bacterial type is required to obtaine the maximum growth of the bacteria.

### 3.4. Neutral pH improves the enhanced prodigiosin production

The bacterial culture inoculated to nutrient broth medium contains various pH ranging from 3 to 11 was checked to identify the maximum prodigiosin production. Less prodigiosin production was seen in the acidic pH 3.0 and alkaline pH (9.0 & 11.0). Maximum pigment production was seen in neutral pH 7 **(Fig. 2d)**. The soil contains various pH based on the location, still bacteria either adopts the environment or it will die due to the surrounding conditions. Usually, many bacteria and plants prefer 5.5-7.0 pH. In our study, we found that neutral pH was found to be optimal for the growth of *S. marcescens* which facilitated the maximum production of prodigiosin. Thus, pH at which maximum production of prodigiosin was observed was chosen and used in the following studies.

### 3.5. Maximal prodigiosin production requires the minimal salt concentration

*S. marcescens* was inoculated in nutrient broth containing different concentration of NaCl ranging 0.4 %, 0.5 %, 0.6 %, 0.7 %, 0.8 % and 0.9 % respectively. The quantity of prodigiosin starts gradually increasing from 0.4 % NaCl concentration to 0.7 % and decreases after 0.8 %. Among the different concentrations, the maximum prodigiosin production was seen in 0.7 %

concentration **(Fig. 3a)**. Generally, the presence of NaCl in nutrient broth maintains required salt concentration in the medium that is similar to the cytosol of a microorganism. 0.5 % NaCl concentration in nutrient broth facilitates the prodigiosin production but not much more. Thus, the optimized condition of salt concentration for prodigiosin production was determined and was followed for the further studies.

### 3.6. Peptone triggers the enhanced prodigiosin production

Different nitrogen sources like peptone, tryptone, ammonium oxalate, ammonium phosphate, ammonium nitrate and urea were incorporated in the nutrient broth medium. The enhanced prodigiosin production was seen in the presence of peptone than other sources **(Fig. 3b).** Whereas, pigmentation was delayed in media amended with urea and ammonium nitrate. The moderate level of prodigiosin production was seen in tryptone and ammonium phosphate supplemented media.

### 3.7. Lactose and maltose provides the maximal prodigiosin production

Different sugars were added to the nutrient broth medium and the maximum amount of pigment production was observed in the presence of lactose and maltose followed by relatively moderate level of pigment production in medium amended with fructose, mannitol and the least being in the medium amended with sucrose **(Fig. 3c)**. Our study revealed that the sucrose when incorporated in the media resulted in decreasing the pigment production and the maximum production of prodigiosin was seen in lactose.

### 3.8. Effect of different solvents for the maximal extraction of prodigiosin

*S. marcescens* was inoculated into optimized nutrient broth medium and incubated at room temperature with gentle agitation. After 48 h incubation, for the effective extraction of prodigiosin various solvents were used (ethanol, acidified ethanol, acetone, methanol, chloroform and diethyl ether). In this present study, acidified ethanol extraction was found to be effective one **(Fig. 3d)**. So that, acidified ethanol extraction was carried out further for the purification and identification of prodigiosin pigment.

### 3.9. Purification of prodigiosin compound

The extracted prodigiosin crude extract was mixed with 20 ml acidified ethanol and passed through hexane balanced silica gel column package **(Fig 5b)**. With the help of ethyl acetate, the prodigiosin was eluted out and then powdered. The powdered sample was further used for thin layer chromatography and for other applications. The prodigiosin was analyzed with silica gel plate **(Fig. 5a)** and the solvent system used was methanol: ethyl acetate: chloroform at ratio (6: 3: 1) in which the prodigiosin was observed as pink color spot. The $R_F$ value fraction was found to be 0.92 which suits well with the literature reports **(Fig. 5b)**.

### 3.10. Identification of prodigiosin

The crude extract of prodigiosin was red in color (shown in the **Fig. 4a)** and the presumptive test for prodigiosin revealed that pink color was observed in the acidic reaction and yellow color was observed in the basic reaction thereby it confirms the presence of prodigiosin **(Fig. 4b)**. UV-Vis spectrophotometry revealed that the maximum absorbance was seen at 530 nm **(Fig. 4d)**. FT-IR spectroscopy **(Fig. 7)** of prodigiosin showed broad and strong wavelength absorption at 3,374.67$cm^{-1}$ (O–H or N-H stretch), 2,927.38 $cm^{-1}$ (Stretch of aliphatic C-H group), 1,621.85 $cm^{-1}$ (aromatic ring C=C and C-N). Furthermore, absorption at 1,198.42 $cm^{-1}$ showed C–N C–O (carboxylic) stretch, whereas 1,030.11 $cm^{-1}$, 863 $cm^{-1}$ and 706 $cm^{-1}$ indicate C–H phenyl ring bend at fingerprint region **(Fig. 7).** The functional groups that were matched to literature confirm the presence of prodigiosin **(Suryawanshi et al. 2014).** Subsequently, the molecular mass of the purified compound was found to be 323 Da which corresponds to prodigiosin using GC-MS analysis.

### 3.11. Antimicrobial activity of prodigiosin

From the antimicrobial results, it is clear that prodigiosin showed higher activity against Gram positive (*Staphylococcus* sp. and *Bacillus* sp.) than Gram negative organisms (*Pseudomonas* sp. and *Salmonella* sp.) with zone of inhibition in the diameter range of 6-15 mm at 100 μg $ml^{-1}$ **(Fig. 7a)**. The antimicrobial property of the prodigiosin molecule could be possibly due to the presence of protonated methoxy group in the prodigiosin molecule **(Arivizhivendhan et al., 2018)**. Also, the Gram positive bacteria was found to be more sensitive than Gram negative which could be possibly due to Gram positive cell membrane is relatively simple whereas the Gram negative bacteria contain outer membrane which could protect the

attachment and penetration of the compound. Followed by, the prodigiosin showed very effective activity against the fungal pathogen *Candida* sp. and slightly less activity against the other fungal pathogens such as *Penicillium* sp. *Mucor* sp. *Fusarium* sp. and *Aspergillus* sp., with zone of inhibition in the diameter range of 4-12 mm **(Fig. 7b)**. The antimicrobial activities are the preliminary aspects of a compound to identify them as a potential drug molecule. From the results, it is clear that prodigiosin can act as a bioactive molecule against various bacterial and fungal diseases.

### 3.12. Prodigiosin has Larvicidal activity against *Aedes aegypti*

The larvicidal activity was performed against *Aedes aegypti* with 100 ppm concentration. The prodigiosin was found to be effective against developing larval third instar stage of *Aedes aegypti* mosquito based on the larvicidal activity result. Within 24 h of incubation, the prodigiosin pigment showed 32 % mortality rate and 76 % mortality was seen after 48 h **(Fig. 8)**. After 48 h, the survived larvae of third and fourth instar stages either pupated or emerged as adults before they deceased. Previous report by **Patil et al., 2011** revealed that the prodigiosin has larvicidal activities at higher concentration **(Patil et al., 2011).** In this study, we have used less concentration of prodigiosin and found that it has strong mortality efficiency towards the larvae.

### 3.13. Molecular docking analysis of prodigiosin against potential protein targets

Molecular docking analysis of prodigiosin against a well-known Neurodegenerative (Aβ peptide 1-42) protein suggested that the binding energy is -4.3 and the 3$^{rd}$ position of Glutamate aminoacid is directly interacting with the prodigiosin **(Fig. 9)**. A breast cancer (oncogene) related protein RAF-1 revealed the binding energy of -5.0 against prodigiosin and it is also predicted that openly interacts with Glutamate 348$^{th}$ aminoacid residue **(Fig. 10)**. *In silico* antiviral activity of prodigiosin against the 2 critical viral proteins (Adenoviral E1A and TMV-CP) predicted that the binding energy is -4.1 and -4.7 correspondingly **(Fig. 11a & b)**. The molecular docking analysis is considered to be a preliminary analysis to predict the binding efficiency of any compounds towards their target proteins. In this study, we have found that prodigiosin binds to most of the proteins with less binding energy at their respective aminoacid residues. To validate the *in silico* analyses, the prodigiosin can be subjected for *in vivo* toxicity analysis and

characterization studies using the generously available well-known invertebrate genetic model systems such as *Caenorhabditis elegans* **(Marudhupandiyan et al., 2017; Muthamil et al., 2018a; Vigneshwari et al., 2018; Gowrishankar et al., 2018; Muthamil et al., 2018b; Kannappan et al., 2019; Balasubramaniam et al., 2019a; Balasubramaniam et al., 2019b Shanmuganathan et al., 2019)**, *Drosophila melanogaster* **(Nehme et al., 2007)**, etc., followed by vertebrate model systems such as *Mus musculus* **(Pope et al., 1961; Bridges et al., 2018)**, *Rattus norvegicus* **(Jemilehin et al., 2016)**, etc.

## 4. Conclusion

Various secondary metabolites secreted by microorganisms are found to be present in soil and the environment. The need for identifying therapeutic molecules is enormously high to treat various diseases and disorders. Prodigiosin obtained from *S. marcescens* is considered to be an important secondary metabolite which can be used for various applications based on the literature. From the obtained results in the present study, it is suggested that prodigiosin can be produced in large scale level by employing the optimized protocol explained which can be used for therapeutic applications against various infections and disorders in near future Moreover, there were no reports about the *in silico* activities of prodigiosin against the tested oncogenes and neurodgenreative genes. In particular, the cumulative analysis of prodigiosin in all aspects such as optimization, purification, characterization, *in vitro* antimicrobial activities along with the *in silico* activities gives more worth to this study. In this context, the present study opens up a new platform to study the importance of prodigiosin in near future by using model system based studies.

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in the FT-IR analysis.

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**Figure legends**

**Fig. 1. a.** Cultural characteristics of *Serratia marcescens* in nutrient agar medium. **b.** Trypticase soy agar. **c.** nutrient broth.

**Fig. 2. a.** Analysis of effect of room temperature along with agitation on maximal prodigiosin production revealed room temperature along with agitation resulted in enhanced prodigiosin production. **b.** Analysis of effect of different media on the maximal prodigiosin production revealed nutrient broth is the media in which maximal prodigiosin production was seen. **c.** Analysis of effect of different temperature for the maximal production of prodigiosin showed room temperature is the suitable temperature for the maximal prodigiosin production. **d**. Analysis of effect of different pH revealed that pH 7 is the apt pH for maximal prodigiosin production.

**Fig. 3. a.** Analysis of effect of different salt concentrations on maximal prodigiosin production revealed 0.7 % is the optimal salt concentration. **b.** Analyzing the different nitrate substrates on the maximal prodigiosin production revealed peptone is enhancing the prodigiosin production. **c.** Analyzing the effects of different sugar substrates for the maximal production of prodigiosin showed lactose is the suitable sugar for the enhanced prodigiosin production. **d**. Analysis of different solvents for the maximal extraction of prodigiosin revealed acidified ethanol is the apt solvent for the maximal extraction.

**Fig. 4. a.** Solvent extracted Prodigiosin. **b.** Presumptive identification of prodigiosin. **c.** separation of prodigiosin using ethyl acetate. **d.** UV-Vis spectrophotometry of prodigiosin showed that lambda max at 530 nm acquired from Spectramax M3 equipment.

**Fig. 5. a.** Thin layer chromatographic separation of prodigiosin showed the $R_F$ value of 0.92. **b.** Column chromatographic separation of prodigiosin.

**Fig. 6. a.** FT-IR analysis of prodigiosin showing various functional groups. **b.** GC-MS analysis of the pigment isolate shows the prodigiosin peak at 323 Da (Arrow head).

**Fig. 7. a.** Antibacterial activity of Prodigiosin reveals that *Staphylococcus* sp. is more susceptible; *Salmonella* sp., *E. coli*, *Bacillus* sp. and *Pseudomonas* sp. is less susceptible against prodigiosin. **b**. Antifungal activity suggests that the prodigiosin is highly active against *Candida* sp.

**Fig. 8.** Larvicidal activity of prodigiosin showed that 76 % mortality was observed at 48 h of treatment in the third and fourth instar stage larvae.

**Fig. 9. a.** *In silico* Anti-neurodegenerative (Aβ peptide 1-42) activity of prodigiosin revealed that the binding energy of -4.3. **b.** molecular interaction model for the anti-neurodegenerative activity of prodigiosin

**Fig. 10. a.** Molecular docking analysis of prodigiosin against a well-known breast cancer protein RAF-1 revealed the binding energy of -5.0. **b.** Molecular interaction of prodigiosin against RAF-1 protein.

**Fig. 11. a & b.** *In silico* antiviral activity of prodigiosin against the two crucial viral proteins (E1A and TMV-CP) revealed that the binding energy of -4.1 and -4.7 respectively. **b & d**. Molecular interactions of prodigiosin against antiviral proteins respectively.

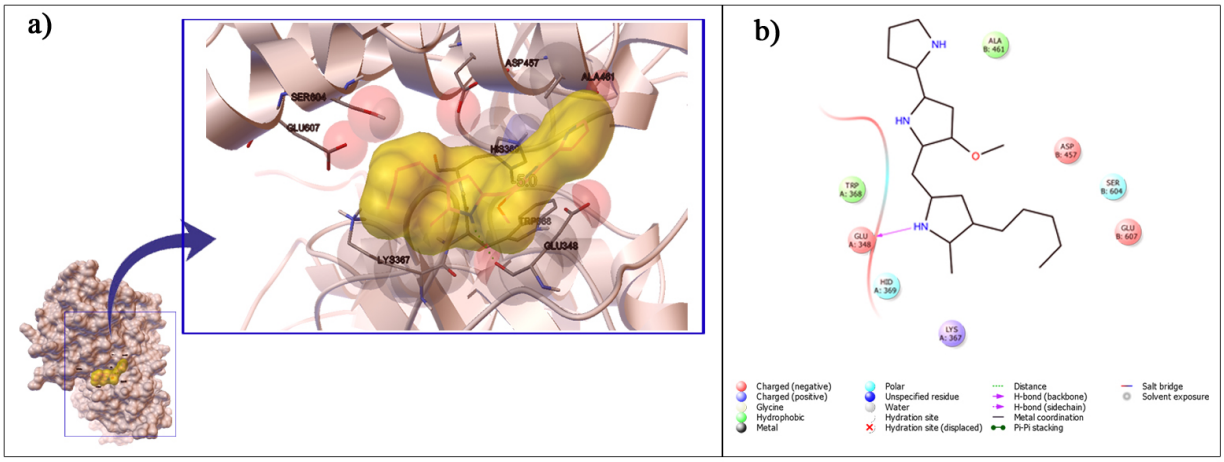
**Tab. 1.** Biochemical characteristics of the isolate. The sugar fermentation revealed that the

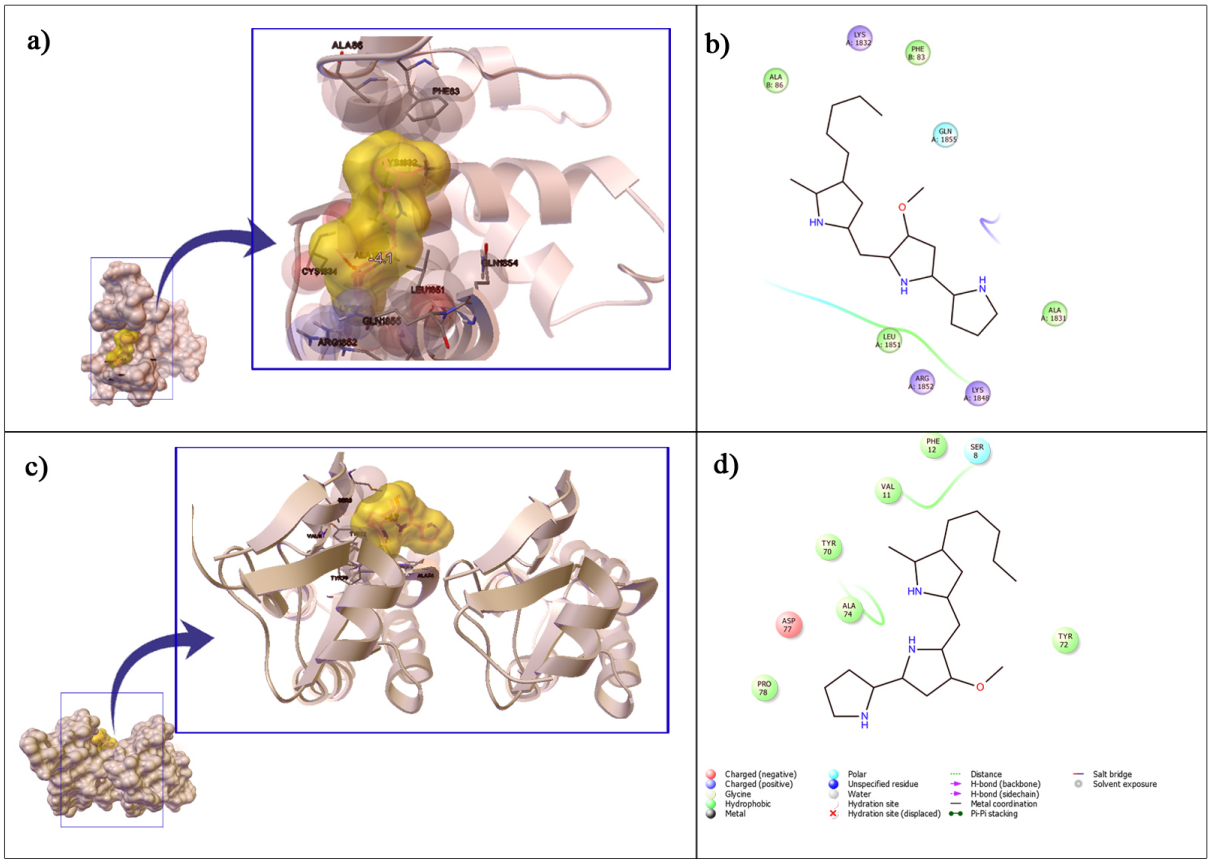
712 isolate corresponds to *Serratia marcescens*.

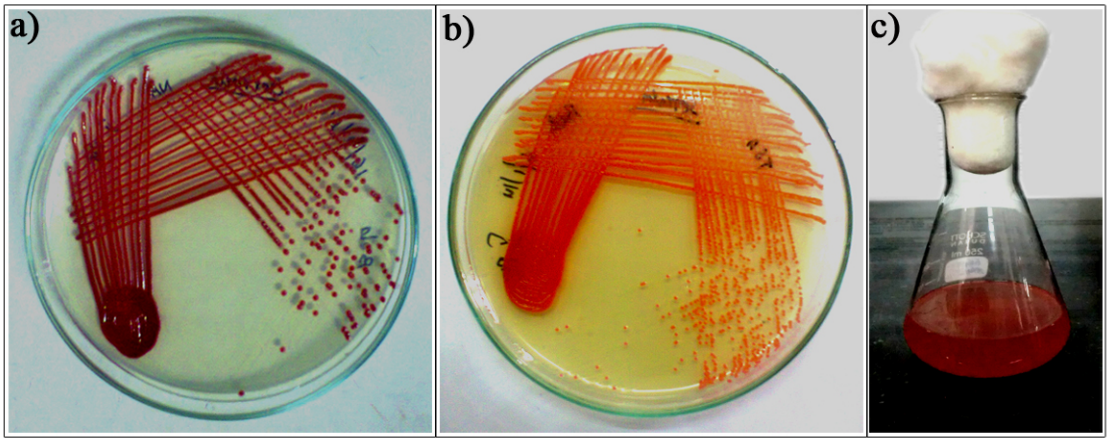
Table 1. Biochemical characteristics of the isolate

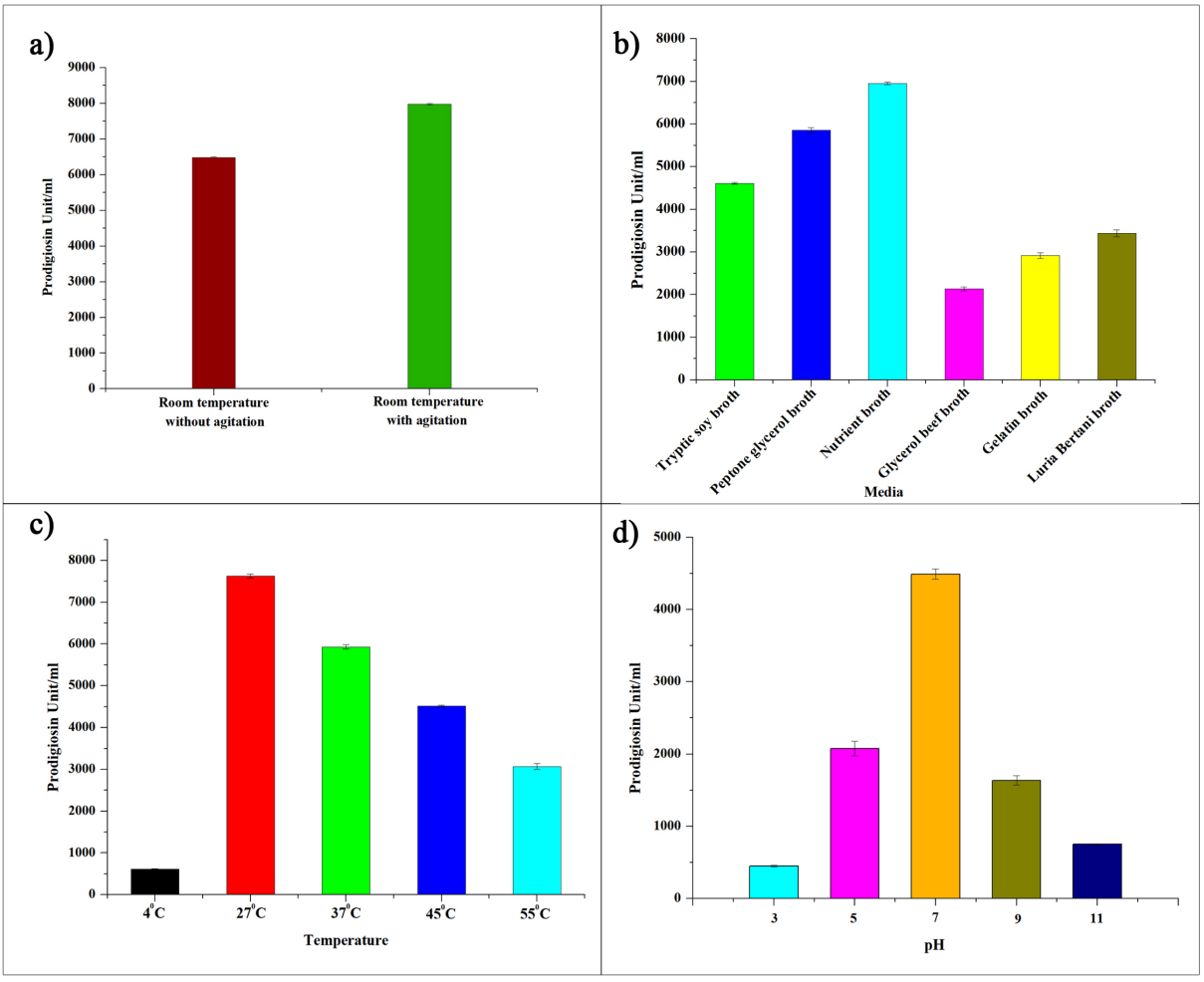
| S. No. | Test substrates | Abbreviation | Result |
|---|---|---|---|
| 1 | Ala-Phe-Pro-Arylamidase | APPA | - |
| 2 | Adonitol | ADO | + |
| 3 | L-Pyrrolydonyl-Arylamidase | PyrA | + |
| 4 | L-Arabitol | IARL | - |
| 5 | D-Cellobiose | dCEL | - |
| 6 | Beta-Galactose | BGAL | - |
| 7 | $H_2$s Production | $H_2S$ | - |
| 8 | Beta-N-Acetyl-Glucosaminidase | BNAG | + |
| 9 | Glutamyl Arylamidase Pna | AGLTp | - |
| 10 | D-Glucose | dGLU | + |
| 11 | Gamma-Glutamyl-Transferse | GGT | - |
| 12 | Fermentation/Glucose | OFF | + |
| 13 | Beta-Glucosidase | BGLU | + |
| 14 | D-Maltose | dMAL | - |
| 15 | D-Mannitol | dMAN | + |
| 16 | D-Mannose | dMNE | + |
| 17 | Beta-Xylosidase | BXYL | - |
| 18 | BETA-Alanine Arylamidase Pna | BAlap | - |
| 19 | L-Proline Arylamidase | ProA | + |
| 20 | Lipase | LIP | - |
| 21 | Palatinose | PLE | - |
| 22 | Tyrosnie ARYLAMIDASE | TyrA | - |
| 23 | Urease | URE | - |
| 24 | D-Sorbitol | dSOR | + |
| 25 | Saccharose/Sucrose | SAC | + |
| 26 | D-Tagatose | dTAG | - |
| 27 | D-Trehalose | dTRE | + |
| 28 | Citrate (Sodium) | CIT | + |
| 29 | Malonate | MNT | - |
| 30 | 5-Keto-D-Gluconate | 5KG | - |
| 31 | L-LACTATE Alkalinization | ILATk | + |
| 32 | Alpha-Glucosidase | AGLU | - |
| 33 | SUCCINATE Alkalinization | SUCT | - |
| 34 | Beta-N-ACETYL-GALACTOSAMINDASE | NAGA | + |
| 35 | Alpha-Galactosaminidase | AGAL | - |
| 36 | Phosphatase | PHOS | + |
| 37 | Glycine ARYLAMIDASE | GlyA | - |

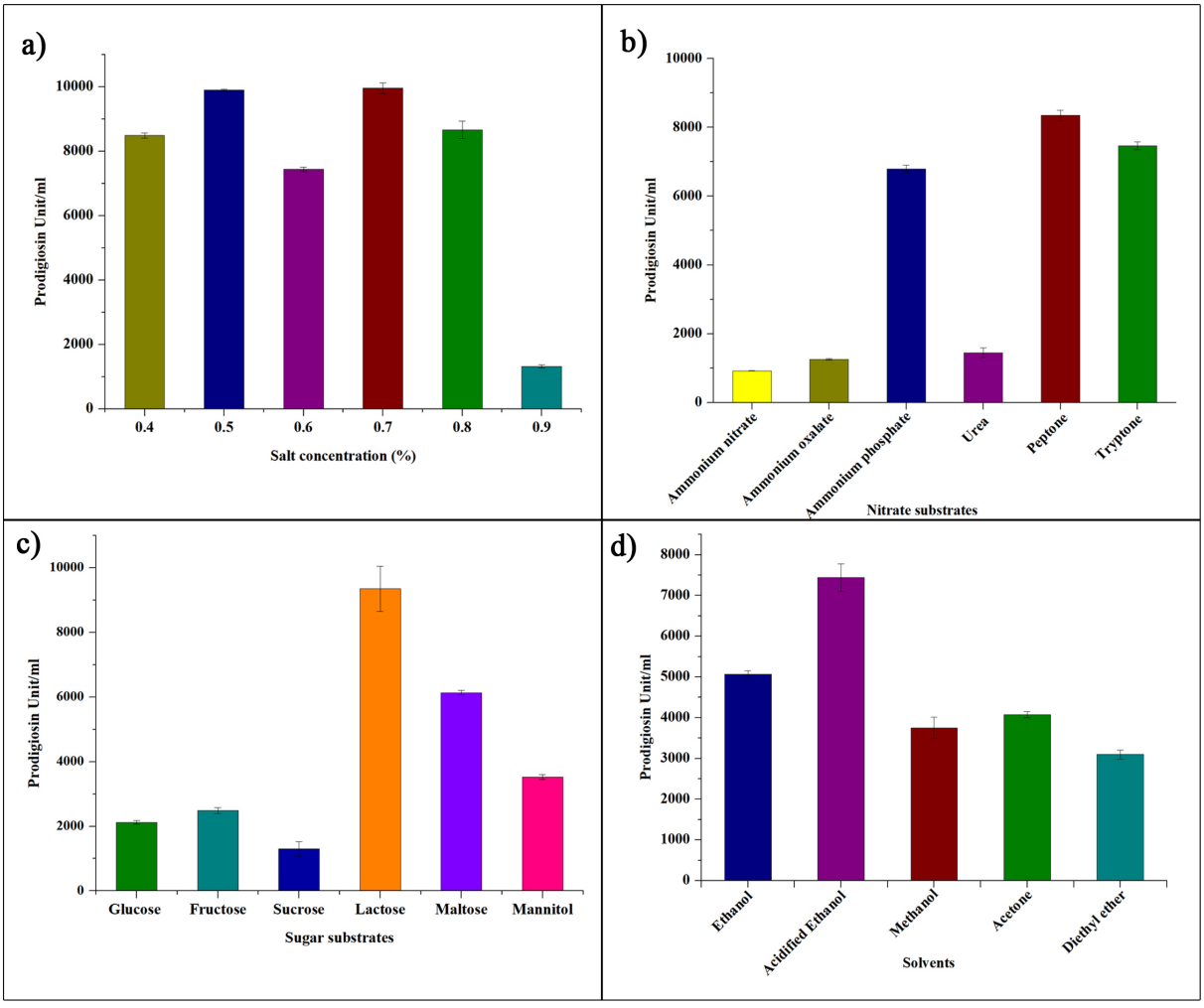
| | | | |
|---|---|---|---|
| 38 | Ornithine Decarboxylase | ODC | + |
| 39 | Lysine Decarboxylase | LDC | + |
| 40 | L-HISTIDINE Assimilation | IHISa | - |
| 41 | Coumarte | CMT | + |
| 42 | Beta-Glucouronidase | BGUR | - |
| 43 | 0/129 RESISTANCE (Comp. Vibrio.) | 0129R | + |
| 44 | Glu-Gly-Arg-Arylamidase | GGAA | + |
| 45 | L-MALATE Assimilation | INLTa | - |
| 46 | Ellman | ELLM | - |
| 47 | L-LACTATE Assimilation | ILATa | - |

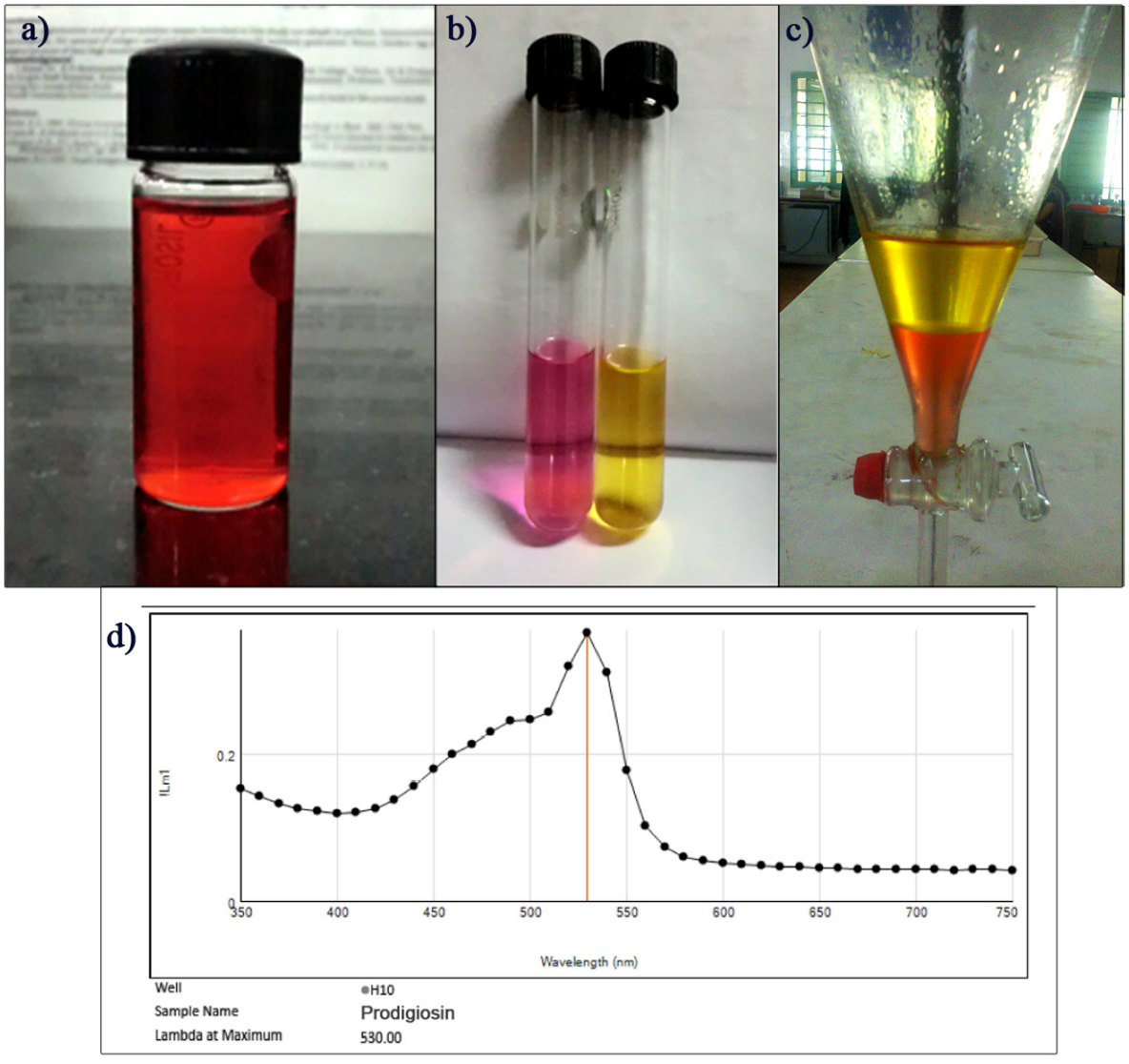


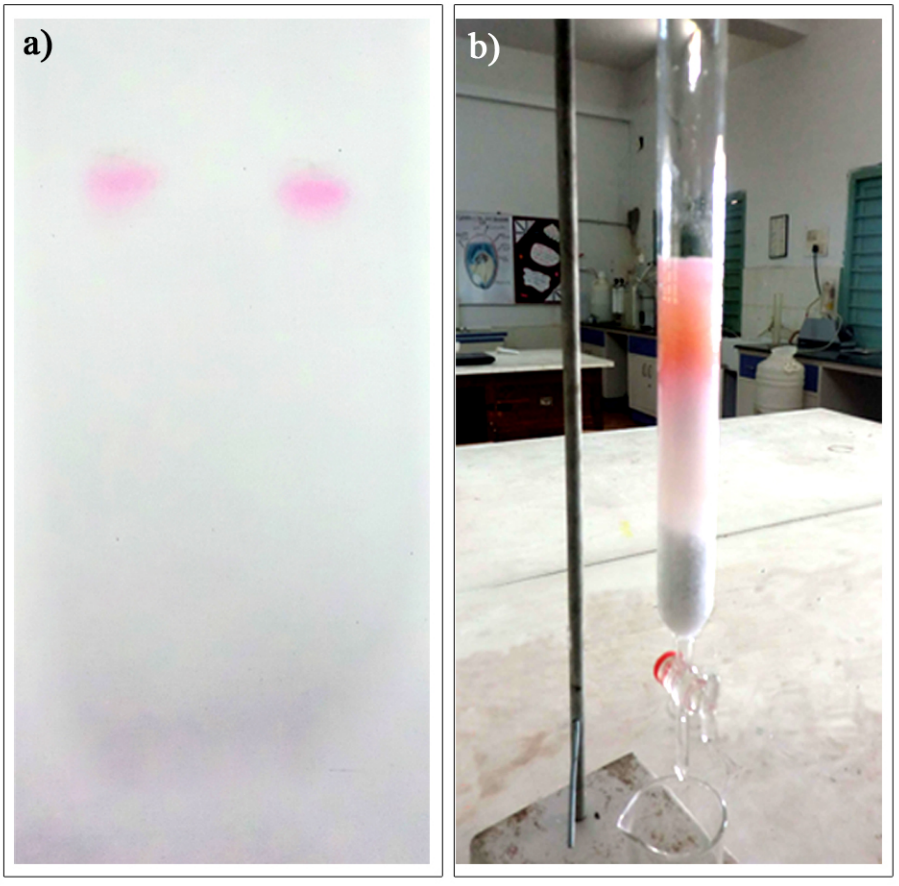

a)
ALA86
PHE83
CYS1834
GLN1854
LEU1851
GLN1855
ARG1852
b)
LYS A: 1832
PHE B: 83
ALA B: 86
GLN A: 1855
HN
O
N H
H N
ALA A: 1831
LEU A: 1851
ARG A: 1852
LYS A: 1849
c)
d)
PHE 12
SER 8
VAL 11
TYR 70
ALA 74
ASP 77
TYR 72
PRO 78
HN
H N
O
N H
Charged (negative)
Charged (positive)
Glycine
Hydrophobic
Metal
Polar
Unspecified residue
Water
Hydration site
Hydration site (displaced)
Distance
H-bond (backbone)
H-bond (sidechain)
Metal coordination
Pi-Pi stacking
Salt bridge
Solvent exposure

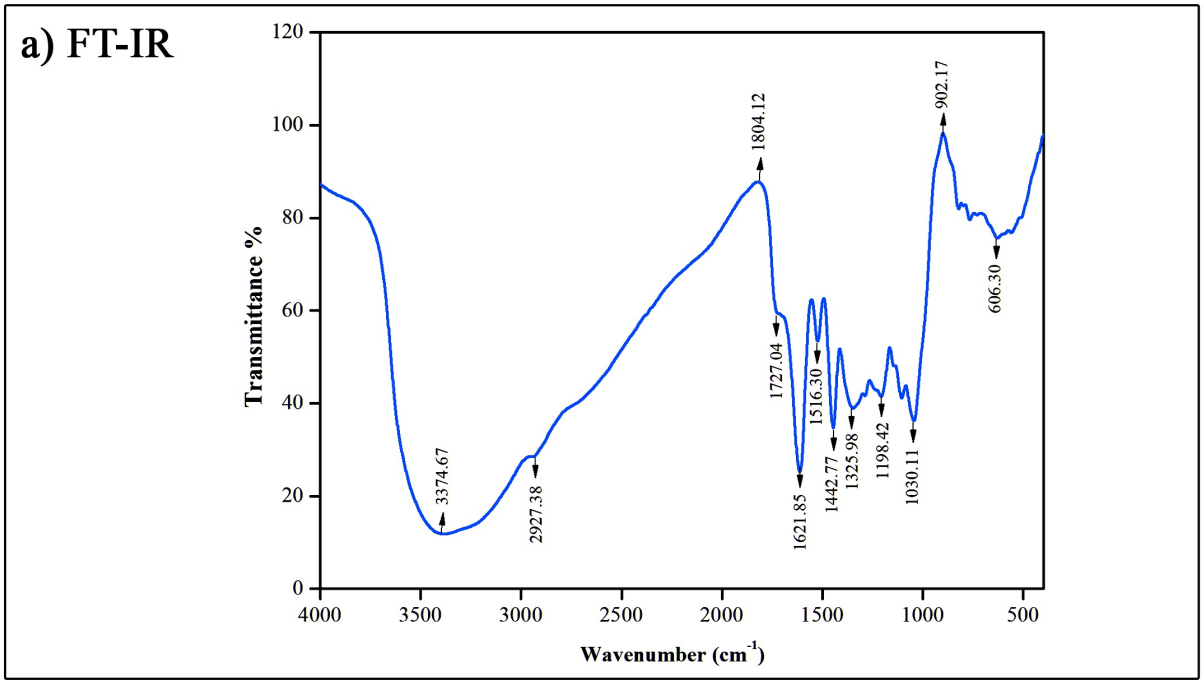


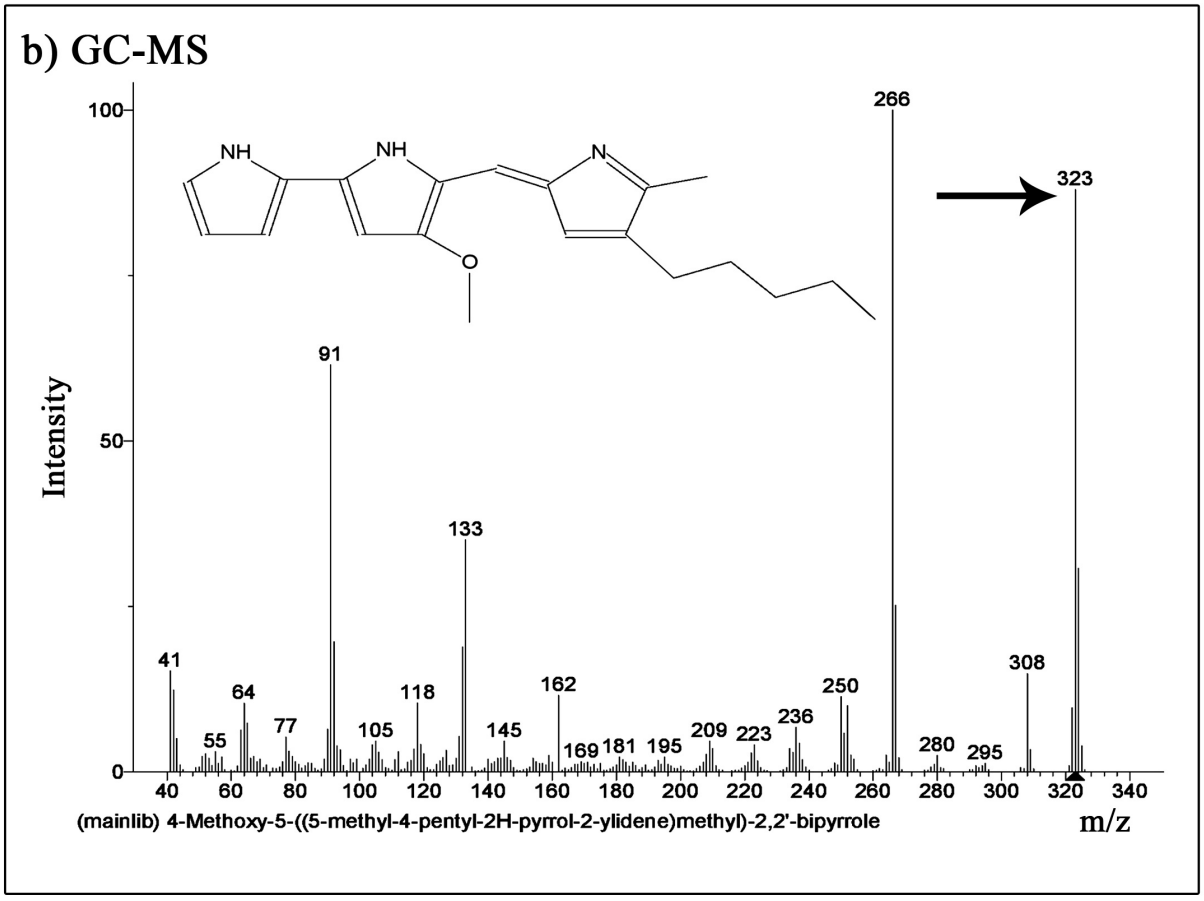

a)
Prodigiosin Unit/ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Room temperature without agitation
Room temperature with agitation
b)
Prodigiosin Unit/ml
8000
7000
6000
5000
4000
3000
2000
1000
0
Tryptic soy broth
Peptone glycerol broth
Nutrient broth
Glycerol beef broth
Gelatin broth
Luria Bertani broth
Media
c)
Prodigiosin Unit/ml
8000
7000
6000
5000
4000
3000
2000
1000
0
4°C
27°C
37°C
45°C
55°C
Temperature
d)
Prodigiosin Unit/ml
5000
4000
3000
2000
1000
0
3
5
7
9
11
pH

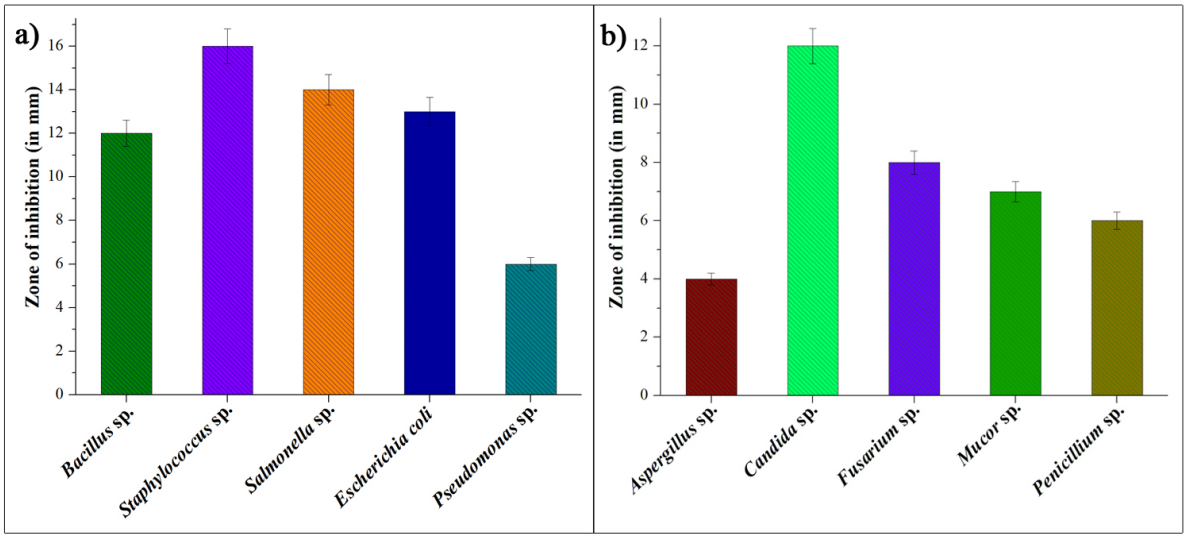

a)
Prodigiosin Unit/ml
10000
8000
6000
4000
2000
0
0.4
0.5
0.6
0.7
0.8
0.9
Salt concentration (%)
b)
Prodigiosin Unit/ml
10000
8000
6000
4000
2000
0
Ammonium nitrate
Ammonium oxalate
Ammonium phosphate
Urea
Peptone
Tryptone
Nitrate substrates
c)
Prodigiosin Unit/ml
10000
8000
6000
4000
2000
0
Glucose
Fructose
Sucrose
Lactose
Maltose
Mannitol
Sugar substrates
d)
Prodigiosin Unit/ml
8000
7000
6000
5000
4000
3000
2000
1000
0
Ethanol
Acidified Ethanol
Methanol
Acetone
Diethyl ether
Solvents

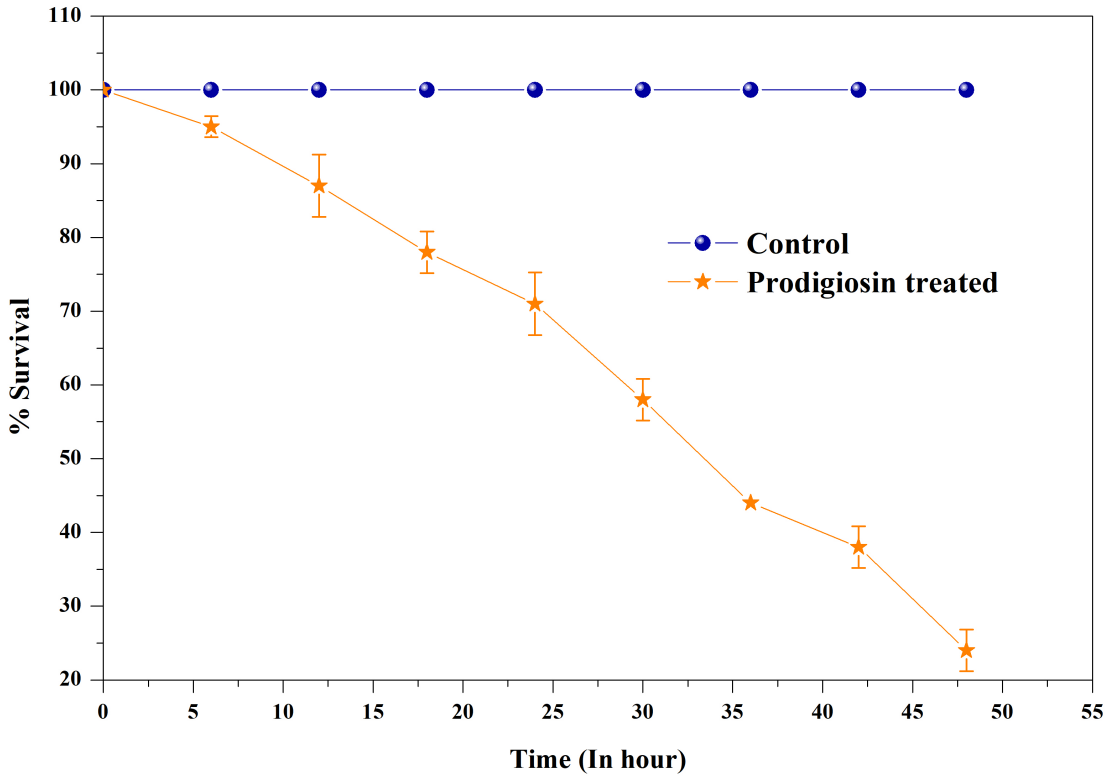

a)
b)
c)
d)
0.2
0
350
400
450
500
550
600
650
700
750
Wavelength (nm)
Well
H10
Sample Name
Prodigiosin
Lambda at Maximum
530.00




a) FT-IR
Transmittance %
Wavenumber (cm-1)
3374.67
2927.38
1804.12
1727.04
1621.85
1516.30
1442.77
1325.98
1198.42
1030.11
902.17
606.30


b) GC-MS
Intensity
m/z
NH
NH
N
O
266
323
91
133
41
64
55
77
105
118
145
162
169
181
195
209
223
236
250
280
295
308
(mainlib) 4-Methoxy-5-((5-methyl-4-pentyl-2H-pyrrol-2-ylidene)methyl)-2,2'-bipyrrole




% Survival
Time (In hour)
Control
Prodigiosin treated
110
100
90
80
70
60
50
40
30
20
0
5
10
15
20
25
30
35
40
45
50
55