Original Research

Production and toxicological evaluation of Prodigiosin from Serratia marcescens UCP/WFCC1549 on Mannitol Solid Medium

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Summary. Prodigiosins, a family of natural red pigments, were produced by a new strain of Serratia marcescens UCP/WFCC1549 using peptone-glycerol and mannitol solid media. Prodigiosin is a secondary metabolite alkaloid (5((3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl)-2-methyl-3-pentyl-1H-pyrrole) with a unique tripyrrole chemical structure, and has antimicrobial, antimalarial, antifungal, immunomodulating, antitumor and anti-proliferative properties. The mannitol medium produced a high amount of biomass, and the red pigment was extracted by methanol and scanned at 200-700nm. The raw pigment was purified by exclusion chromatography by Sephadex LH-20, resulting in 96 fractions. The isolated red pigment was analyzed by electrospray ionization mass spectrometry (GC-MS), its molecular weight was 323.4Da, and it was identified as Prodigiosin. Cytotoxic activity using Artemia salina showed LC50 = 78.33 µg/mL.

Industrial relevance. Prodigiosin produced by Serratia marcescens is a promising drug owing to its reported characteristics of having antifungal, anti-microbial, anti-malarial, anti-cancer and immunosuppressive properties. Of these, its immunosuppressive and anti-cancer activities have received greatest attention because they have clinical promise. From the point of view of industrial production, we obtained a suitable medium so as simultaneously to enhance the growth of Serratia marcescens and production of the pigment. The red purified fraction was identified as Prodigiosin and is a promising molecule owing to its low toxicity and potential for therapeutic application in the future.

Keywords. cytotoxicity; mannitol medium; mass spectrometry; phytotoxicity; Serratia marcescens; Prodigiosin.

INTRODUCTION

Various bacteria possess a huge ability to produce biopigments that can be synthesized to produce medicinally important products. Red pigment produced as a secondary metabolite alkaloid with a unique tripyrrole chemical structure has been isolated from a few species such as Serratia, Pseudomonas and Streptomyces [Bennett and Bentley, 2000; Giri et al., 2004; Song et al. 2006].

Serratia marcescens, Prodigiosin is characterized as (5(3(methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl)-2-methyl-3-pentyl-1H-pyrrole) as an alkaloid with a unique tripyrrole chemical structure [Isaka et al., 2002; Furstner, 2003; Kim et al., 2008a; Lee et al., 2011; Ryazantseva, Andreyeva, 2014]. However, Prodigiosin appears only in the later stages of bacterial growth, while other microorganisms such as Zooshikella rubidus, Vibrio sp., Streptomyces griseoviridis, and Hahella chejuensis are described as Prodigiosin producers [Kawasaki et al. 2008; Kim et al. 2008b; Borić et al.2011; Lee et al. 2011].

Prodigiosin belongs to the family of prodiginines that is characterized by having a tripyrrolic linear structure, and called Prodigiosinide, which is common to all the members of this family such as (? apagar Prodigiosin porque já é citado – 1a. palavra desta sentença,) CycloProdigiosin, MetacycloProdigiosin, Dipyrrrolidipirromethan, all of which have a common pyrrolydipyrrolmethene skeleton. The chemical structure of Prodigiosin comprises three rings which form a pyrrolylpyrromethene skeleton with a C-4 methoxy group, with the molecular formula C20H25N3O [Harris et al., 2004; Song et al., 2006; Williamson et al., 2006a; 2006b] (Figure 1). Macrocyclic prodiginines appear to be derived from decyclprodiginine by oxidative cyclisation [Azuma et al., 2000; Cerdeño et al., 2001; Azambuja, Garcia, 2004; Fineran et al., 2005; Baldino et al., 2006; Song et al., 2006]. Chromogenic biotypes from the natural environment have rarely resulted
in infections and the clinical isolates are rarely pigmented. The function of this red pigment remains unclear and no role has been defined for it in the physiology of the producer strains [Han et al., 2001; Giri et al.,2004; Gulani et al., 2012].

However, interest in it has been reported in various areas of knowledge, given its great potential for clinical and environmental applications owing to its reported characteristics of having antibacterial, antifungal, antimalarial and antiprotozoal properties. In addition, Prodigiosin is reported as inducing apoptosis and antitumoral activities [Carbonell et al., 2000; Díaz-Ruíz et al., 2001; Campás et al., 2003; Williamson et al. 2005; Costa et al., 2010].

The Prodigiosin-like pigment, Prodigiosin, is known to have antitumor activities and potential as an anticancer drug, and is reported to have had a positive effect on the increased chances of survival of mice undergoing heart transplants [Montaner et al., 2000; Campás et al., 2003; Montaner et al., 2005; Williamson et al. 2007; Elahian et al., 2013].

In recent years, SSF (Solid-state surface fermentation) has shown much promise when developing bioprocesses and products. More recently, it has gained importance in the production of microbial enzymes due to its having several economic advantages over conventional submerged fermentation [Sathya et al., 2009].

This paper sets out to find a medium that may support the growth of bacteria and at the same time prove efficient at triggering the accumulation of high levels of Prodigiosin pigment. Therefore, this study explores the effects of the nutrients of the solid media mannitol and glycerol-peptone on Prodigiosin production by a new strain of *Serratia marcescens* UCP/WFCC1549. The isolate biopigment was characterized and its toxicity evaluated using the microcrustacean *Artemia salina*.

**MATERIALS AND METHODS**

**Microorganisms.** A strain of *Serratia marcescens* UCP/WFCC1549 to be used as a Prodigiosin producer was isolated from the semi-arid soil of Pernambuco, Brazil, and identified by Araujo et al. (2010). The strain belongs to the Culture Collection of UCP (Universidade Católica de Pernambuco), registered in the World Federation for Culture Collection (WFCC), and is maintained on Nutrient Agar at 5°C.

*Artemia salina* was obtained from the Nucleus of Research in Environmental Sciences and Biotechnology-NPCIAMB, Catholic University of Pernambuco (Recife, PE, Brazil).

**Substrates, media, chemicals, organic solvents and reagents.** The components peptone, glycerol, mannitol and agar were obtained from Difco. Chemicals, organic solvents and reagents, and analytical chromatograph were obtained from Merck, and Sephadex LH-20 from Sigma-Aldrich.

**Biomass production and red pigment.** *S. marcescens* grown in 250 mL Erlenmeyer flasks containing 100 mL of Nutrient Broth [Peptone 10g; NaCl 5g; Yeast extract 3 g/L], was incubated at 28°C, at 150 rpm, until turbidity of OD\textsubscript{600}=2 (corresponding to 10^9 cells/mL), and was used as inoculum. This inoculum (10%) was transferred to forty 500 mL Erlenmeyer flasks containing 300 mL of solid media Peptone-Glycerol -PG [peptone 10g; glycerol 100mL; added agar 17g/L], and Mannitol Agar medium -MM [peptone 5g; yeast extract 2g; mannitol 20g; added 17g of Agar/L], and pH 7, as per Giri et al. (2004). The flasks were incubated at 28°C, for 48 hours under solid state fermentation (SSF).

**Estimate of Biomass.** After this period the biomass was removed by scraping it gently off the surface and transferred to Falcon tubes using sterile saline solution, washed three times with deionized water, and then freeze dried until constant weight, when the biomass was weighed and expressed in g/L.

**Isolating and quantifying the pigment.** The red pigment was extracted from the biomass obtained from solid state fermentation (SSF) for which organic solvent systems were used: chloroform:methanol (1:1; 2:1 and 1:2 v/v), which was followed by the extraction using pure methanol. The extracts were pooled and concentrated by rotating on an evaporator at 45°C, and raw Prodigiosin was obtained as per the literature [Nakashima et al., 2005; Nakashima et al., 2006].

The volume of the raw Prodigiosin was estimated using the following equation [Mekhael and Yousif, 2009].

$$\text{Prodigiosin unit/cell} = (\text{OD}_{499} - (1.381 \times \text{OD}_{620})) \times 1000/ \text{OD}_{620}$$

**Chematography methods.** The crude extract was solubilized in 3 mL of methanol, submitted to exclusion chromatography (column 22 x 1 cm), filled with Sephadex LH-20, as adsorbent. The elution process was carried out using the solvent system: chloroform: methanol: ethyl acetate (5:30: 65 v/v), as per Nakashima et al. (2005; 2006). The solvent system was modified to chloroform: methanol: acetone (4:2:3 v/v), and 96 fractions were collected amounting to 10mL on separating the pigment. The purified pigments were separated using Thin layer Chromatography (TLC). The purified red and blue pigments were separated using silica gel coated on TLC aluminum foil. The developing solvent system methanol: chloroform: acetone (2:3:4 v/v) was standardized and poured into the chromatography tank that was saturated using a filter paper soaked in the mobile phase as modified by the literature [Williams et al.,1956; Lynch et al. 1968].
After running this system, the Rf value of chromatogram on the TLC plates was noted, and the similar fractions corresponding to the same Rf were pooled.

**Determining the absorption spectra.** Spectral analysis was conducted on dried pigment extracted by the above method by dissolving it in 10 ml of absolute ethanol. Acidic conditions for spectral analysis were obtained by adding 1 ml of 1 N Hydrochloric acid, to 10 ml of the ethanol extract [Araújo et al., 2010; Gulani et al.2012]. Spectral analysis was carried out on a Systronics UV-visible spectrometer model 2203.

**Characterizing the red pigment.** The purified red pigment was characterized by spectrophotometry scan in a wavelength of 200-700 nm [Pyrece and Terry, 2006], using chloroform as solvent. The molecular weight of the pigment was determined by using mass spectrometry, GCMS, so as to compare Prodigiosin and its derivatives, as per the literature. A computerized search was carried out to match library m/z values, and their respective compounds were registered. The coincidence of mass spectra was used to highlight the likely structure of the Prodigiosin compound produced by *S. marcescens* [Khanafari et al., 2006].

**Evaluating toxicity.** The test of toxicity on *Artemia salina* was carried out in three stages: incubation, exposure to the substance and counting the number of living and dead nauplius cells, after 48 hours of exposure to the compound. Initially, the eggs of *A. salina* were transferred to a container containing 100 mL of navy (blue RX dye) and incubated at 30°C. After this period where of the outbreak occurred larval (nauplius). After ten days, *A. salina* nauplii were transferred to volume 5 mL vials containing a solution of Prodigiosin in concentrations of 100, 50, 20, 5 and 2.5 µg/mL, and were incubated at 30°C for 48 hours. Thereafter, the number of live and dead nauplii was counted. The lethal concentration LC50 was calculated using a PROBIT statistical programme.

**RESULTS**

**Effect of different media on Prodigiosin production by *S. marcescens***

*S. marcescens* was isolated from the semi-arid soil of Pernambuco, Brazil and found to grow as a red-pigmented colony on nutrient agar. Biomass was obtained from MM and PGA media (Figure 2). Figure 2 shows the biopigment accumulation by *S. marcescens* UCP/WFCC 1549 in nutrient agar medium (A), and the biomass and organic extracts in both MM and PG media. On comparing the results, it can be confirmed that a stronger red color was formed in the MM medium than in the PG one.

In this study the new isolate of *S. marcescens* produced a high amount of biomass on solid media (48.50 g/L) on Mannitol (MM) at 48 h of cultivation. However, only 17.50 g/L of biomass on Peptone-Glycerol media (PG) was produced. The maximum raw pigment extracted from the Mannitol Agar (MM) medium corresponded to a 5.82g/total biomass (12.00%), while a maximum of 1.75g/total biomass (10.00%) was obtained from the PG medium (Table 1).

The results with the MM medium showed the red fraction of the the isolate of the red pigment was 3.20g, corresponding to 54.98%. However, in the same fraction, we isolated a blue fraction corresponding to 0.87g, and 14.94% of that pigment. The results show that the raw red fraction from PG medium (1.75g) was a purified fraction of 0.647g, corresponding to 36.97%.

**Purification of prodigiosin by TLC chromatography.** After the purification was determined by thin-layer chromatography (TLC), two bands appeared from Mannitol-purified extract which had the following reference values: the Rf for prodigiosin (red fraction) was 0.59, and the blue fraction was 0.22. However, the purified extract from PG medium showed only one similar corresponding Rf band (0.59) (Figure 3).
Evaluation of Prodigiosin from *Serratia marcescens*

**Isolation and characterization of Prodigiosin.** Therefore, the methodology used here for purification was Sephadex LH-20, as the adsorbent eluted using chloroform: methanol ethyl acetate (5:30:65 v/v). However, we modified the solvent-system to chloroform: methanol: acetone (4:2:3 v/v) and 96 fractions were collected which were characterized by scan spectrophotometry (200-700 nm), where the maximum absorption of the blue fraction was 272 nm, and the red fraction 534 nm, which correspond to a Prodigiosin (Figure 4).

**Toxicity assessment.** The Prodigiosin was evaluated as to its toxicity on *A. salina* and the results exceeded LC$_{50}$ = 78.33 µg/mL, with level (0.05), which gives the data obtained 95% significance.
Figure 5. Mass spectrometry (GC-MS) of purified red fraction (prodigiosin) produced by Serratia marcescens UCP/WFCC 1549 in Mannitol Agar (MM) medium

DISCUSSION

In this study S. marcescens UCP/WFCC1549 was found to produce a significant yield of Prodigiosin in MM solid medium (Table 1, Figure 2) when compared with the yields reported by Nakashima et al., (2005; 2006), Sundaramoorthy et al.,(2009) and Patil et al., (2011).

In this paper, Prodigiosin production followed the methodology described for conventional Mannitol and Peptone glycerol media. Carbon and nitrogen sources were used as necessary to multiply the micro-organisms. The maximum production of biomass was obtained after 48 hours, and the results are as given by Azambuja et al. (2004).

However, Sundaramoorthy et al.(2009) found Serratia marcescens to produce more Prodigiosin in medium containing maltose. However, when the microorganism was allowed to grow in various media, the organism was found to produce more Prodigiosin in nutrient broth. (?? Galba, é isto mesmo?: even although glucose and lactose did not influence the production of Prodigiosin. The authors described that the organism produced 1610 units/cell and 1616 units/cell of Prodigiosin using glucose and lactose in medium respectively. (Galba, para mim, esta sentença contradiz a anterior.)

Baldino et al., (2006) showed that the maximum production of Prodigiosin occurs in the presence of light, but this reduces the biomass concentration. Moreover, the liquid media currently being described for Prodigiosin biosynthesis are nutrient broth [Lee et al.2011], peptone glycerol broth [Patil et al.2011], and M9 medium (0.4% mannose, 0.01% Methionine, 0.003% cysteine and 0.1% ammonium chloride) [Wei et al.2005].

The results obtained for producing Prodigiosin by S. marcescens UCP/WFCC1549 were enhanced using mannitol in the medium, as described in Table 1, and are supported by the literature [Montaner et al., 2005; Nakashima et al., 2006; Leeper and Salmond, 2006; Lee et al. 2011].

Anuradha et al.(2004) reported that the production of Prodigiosin was greater in nutrient broth (0.52 mg/mL) than in peptone glycerol broth (0.302 mg/mL). In our results (Table 1, Figure 2) the MM medium differs from the PG medium since the former uses yeast and meat extracts. Taking into consideration the basic role of carbon source in enhancing pigment production, two justifications can be made. The first point is that in nutrient broth, which is basically devoid of carbon source, the addition of maltose is associated with yeast extract sources. The yeast and meat extracts contain rich tissues (yeast, beef muscle, liver, brain, heart, etc.), which are extracted by boiling and followed by concentration to a paste or dried powder. In addition, these extracts are frequently used by fastidious organisms as a source of amino acids, vitamins and coenzymes, as growth factors. On the other hand, peptone-glycerol is in PG, and glycerol was the only carbon source. Therefore, the important substrates present in the MM medium, especially the carbon and nitrogen sources favorably influence the production of red biopigment by S. marcescens UCP/WFCC 1549.

The method used to extract the red pigment described from these media was modified considering that pigment extractions with basic or acidic solutions do not eliminate any impurities present, even after purification by chromatography, according to Kim et al.(2008) and Sundaramoorthy et al.(2009).

Samrot et al. (2011) extracted the pigment and subjected it to TLC and the Rf value of the fraction was 0.87. However, we have a different Rf value for prodigiosin (Figure 3). The isolate blue fraction obtained from the red pigment appeared only in MM medium when the extract of Prodigiosin was being purified. This was first described by Williams et al.(1956) while Patil et al., (2011) isolated the blue component and studied its properties.

However, the presence of the blue component associated with red pigment had previously been reported in the literature [Benenett, Bentley, 2000; Pryce and Terry, 2006], since they had observed a slow moving purple band in their chromatograms of Prodigiosin. It is possible that this purple band was the same blue component reported here, but admixed
with red fractions. The literature suggests that blue fraction is produced in parallel biosynthetic routes that form the monopirrol MAP and the BMC, both of bipyrrol Prodigiosin biosynthesis route which converge to form the pigment tripyrrole rings [Williams et al., 1956; Araújo et al., 2010].

Our results are supported by Song et al.(2006) who extracted the red pigment directly from the internal adsorbent using acidified methanol and phase separation. The maximum UV absorbance of the isolated Prodigiosin was observed at 536 nm (Figure 4), corresponding to Prodigiosin. The results here described are in agreement with prodigiosin purified from Serratia sp KH – 95 [Song et al., 2005; Nakashima et al. 2005; 2006; Araújo et al., 2010].

Prodigiosin has been known to be a natural compound showing a broad range of cytotoxic, antifungal, antibacterial, algicidal, antiprotozoal, antimarial, immunosuppressive, anticancer and antiproliferative properties as described by Cerdeno et al. (2001) and Furstner et al. (2003). Prodigiosin is normally produced in nutrient broth and peptone glycerol broth [Siva et al., 2012]. However, our results obtained in the toxicity test of Prodigiosin produced on MM medium was performed using the micro crustacean A. salina as a bioindicator which is very sensitive to any environmental toxic compounds (Krishnarajana et al., 2005). The results showed a lower value to LC50 at 48 hrs observed for Prodigiosin obtained from S. marcescens UCP/WFCC 1549 suggesting its potential as an antitumoral effect, according the literature [Montaner et al., 2005; Montaner et al., 2005; Patil et al., 2011]. The mass spectrometry date was compared with the literature data, and the purified pigment corresponding to a Prodigiosin, as per the literature [Kim et al., 2008; Sundaramoorthy et al., 2009; Siva et al., 2012], and revealed a molecular weight of 323.4 Da for the purified pigment (Figure 5), in agreement with results reported by Song et al. (2006) and for prodigiosin.

CONCLUSION

The final conclusion based on the experimental results of producing Prodigiosin by S. marcescens UCP/WFCC1549 gave a maximum yield in MM medium with Peptone 5 g, Yeast extract 2 g; Mannitol 20 g; added 17 g of Agar/L, pH 7 at 28°C under SSF for 48 hrs. Yeast extract and Mannitol together resulted in better Prodigiosin production and was found to exhibit a synergistic carbon source effect. The MM medium induced stronger pigmentation than did the PG medium. Extracting Prodigiosin from MM medium showed blue and red absorbance peaks. Chloroform was found to be a more effective solvent for Prodigiosin extraction. The best condition for purifying the molecule of prodigiosin was by Sephadex LH-20 column using the solvent-system chloroform: methanol: acetone (4: 2: 3 v/v). The structure of Prodigiosin was eluted using electrospry ionization mass spectrometry (GC-MS), and the molecular weight was 323.4 Da. In this study, LC50= 78.33 µg/mL to purified Prodigiosin for microcrustaceous Artemia salina was found. The results indicated its biotechnological potential and further appropriate innovations using SSF to produce Prodigiosin.

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