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EFFECT OF MINERAL SUPPLEMENTATION AND TYPE OF STARCH ON THE PRODUCTION OF PRODIGIOSIN FROM A CULTURE OF *Serratia marcescens* BS303

EFECTO DE LA SUPLEMENTACIÓN MINERAL Y EL TIPO DE ALMIDÓN EN LA PRODUCCIÓN DE PRODIGIOSINA A PARTIR DE UN CULTIVO DE *Serratia marcescens* BS303

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Abstract
Prodigiosin is a secondary metabolite that has become attractive for industrial microbiology by the large number of potential applications, but limitations like high price related to production, recovery and purification are responsible of the low industrial application. The production of the red pigment prodigiosin in *Serratia marcescens* is a regulated mechanism that depends on several factors, such as the presence of specific metal ions and carbon sources, for that reason the effect of four minerals and three types of starch as carbon sources on the cultivation behavior of *Serratia marcescens* BS303 was studied. The role of minerals on prodigiosin content resulted in 1.8-fold increase on the metabolite production using a combination of copper sulfate and ammonium ferric citrate at 0.05 g/L and it is related with the ability of the strain to regulate metal toxicity. The use of hydroxypropylated modified starch as a carbon source caused an increase of 8.1-fold on prodigiosin content and a growth rate of 0.06 h^{-1} showing pigment adsorption and probably scaffold properties for bacterial growth, enhancing prodigiosin production by 8.1-fold. Prodigiosin content raise 570 mg/L with mineral supplementation and polartex instant addition.
Keywords: prodigiosin, *Serratia marcescens*, 2^k factorial method, culture media, mineral supplementation.

Resumen
La prodigiosina es un metabolito secundario que se ha vuelto atractivo para la microbiología industrial por el gran número de aplicaciones demostradas, pero los altos precios relacionados con la producción, recuperación y purificación son responsables de limitar la aplicación industrial. La producción de prodigiosina a partir de *Serratia marcescens* es un mecanismo regulado que depende de varios factores, tales como la presencia de iones metálicos y la fuente de carbono, por esa razón se estudió el efecto de cuatro minerales y tres tipos de almidón como fuente de carbono en la producción de prodigiosina. El papel de los minerales en el contenido de prodigiosina resultó en un aumento de 1.8 veces, usando una combinación de sulfato de cobre y citrato de amonio férrico en una concentración de 0.05 g / L y se relacionó con la capacidad de la cepa para regular la toxicidad de metales. El uso de almidón hidroxipropilado como fuente de carbono causó un incremento de 8.1 veces el contenido de prodigiosina y una tasa de crecimiento de 0.06 h^{-1}, originado por una adsorción del pigmento y generación de un andamio para el crecimiento bacteriano, obteniendo hasta 570 mg / L de prodigiosina.
Palabras clave: prodigiosina, *Serratia marcescens*, diseño 2^k, medio mineral.

1 Introduction

Prodigiosin is a red pigment produced by some strains of *Serratia sp*., also by other species like *Hahella chejeuensis* (Huh *et al*., 2007) , *Vibrio psychroerythrus* (Aoust & Gerber, 1974) and *Streptomyces griseoviridis* (Kawasaki *et al*., 2008). This secondary metabolite, typically produced in the stationary phase has a pyrrolyldipyrrolylmethene structure with a molecular mass of 324 Da (Chang *et al*., 2011), and has shown antimalarial/antiprotozoal (Siva *et al*., 2012), antimicrobial (Gulani *et al*., 2012), algicidal (Park *et al*., 2013), insecticide (Wang *et al*., 2012), immunosuppressive (Fürstner, 2003), antineoplastic (Montaner *et al*., 2000) and other related cytotoxic activities. Applications according to above activities are broad and cover areas like health, water management and pest control, but also there have been reports to common uses as a dye for textiles (Alihosseini *et al*., 2008; Gulani *et al*., 2012; Siva

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et al., 2012). The actual uses of prodigiosin is for analytical or research purposes, its price rise to up 100 USD per 500 μg (Santa Cruz Biotechnology), limiting its potential applicability on pharmaceutic or textile industry.

Prodigiosin biosynthesis in *Serratia marcescens*, a small rod shape, gram-negative, motile aerobic bacteria, is affected by typical environmental factors as pH (Fender *et al*., 2012), temperature (Williams *et al*., 1971), type/concentration of carbon source (Cang *et al*., 2000; Giri *et al*., 2004), inoculum size (Fineran *et al*., 2005), inorganic phosphate (Slater *et al*., 2003), illumination (Ryazantseva *et al*., 2012), and salts (Chen *et al*., 2013; Silverman & Munoz, 1973). The high heterogeneity observed in different strains may be due to different prodigiosin biosynthesis regulation (Harris *et al*., 2004), Nima strain enhance prodigiosin synthesis with iron (Silverman & Munoz, 1973), O8 strain enhance synthesis with sodium dodecyl sulfate (Feng *et al*., 1982), UCP1549 strain enhance synthesis with cassava based medium (Araújo *et al*., 2010), SMΔR enhance synthesis with oil based medium (Wei & Chen, 2005), and so on. The genetic variability observed between strains could be explained by its successful adaptation to different environments, for example UCP1549 strain was isolated from arid soil and whereas BS303 strain was isolated from pond water. Given these differences in the strain origins, it is necessary to determine which factors affect the biosynthesis of prodigiosin in order to establish an optimal biosynthetic production process. To have a better understanding of the effect of the different nutrients on the production of prodigiosin from *S. marcescens* BS303, different culture conditions were studied and optimized for prodigiosin production, taking as variables the carbon source and mineral supplementation. This study narrows the most important factors and develops an optimal production medium that enhances prodigiosin biosynthesis in a strain with little studies related to prodigiosin production.

2 Materials and methods

2.1 Materials

Casein peptone, iron (II) sulfate heptahydrated, ammonium iron(III) citrate, α-amylase (E.C. 3.2.1.1), methanol Chromasolv® for HPLC, and triton X - 114 were purchased from Sigma-Aldrich (St. Louis, MO, USA),phosphoric acid, glycerol, ethyl acetate, magnesium sulfate heptahydrated, sodium chloride, calcium chloride, anhydrous copper sulfate, n-hexane, acetonitrile and soluble starch were purchased from DEQ (Monterrey, Mexico), manganese sulfate heptahydrated from Productos Químicos Monterrey S.A. (Monterrey, Mexico), triethanolamine from Fischer Chemicals (Zurich, Switzerland), nutrient broth from BD Bioxon (Franklin Lakes, NJ, USA) Polartex® Instant 12640 and Stabitex® Instant from Cargill (MN, USA) Water HPLC grade from Honeywell (Morristown, NJ, USA) and Dextrozyme (E.C. 3.2.1.3) from Novozyme (Bagsvaerd, Denmark).

2.2 Microorganism and culture conditions

S. marcescens BS 303 (ATCC® 13880^{TM}) was acquired from ATCC bacteriology collection and was activated in nutritive broth for 24 h at 28ºC and 250 rpm, further subcultures of the strain were done in a mineral glycerol peptone medium (MGP), 10.5 g/L casein peptone, 16 ml/L glycerol, 0.125 g/L $FeSO_4 \cdot 7H_2O$, 0.72 g/L $MnSO_4 \cdot H_2O$, 0.62 g/L $MgSO_4 \cdot 7H_2O$ (adapted from Harris *et al*., 2004) at pH 7 in 24 h batch cultures. To obtain a pigmented inoculum, the culture broth was centrifuged 10 min at 8000 x g in 50 mL tubes using an Thermo-scientific centrifuge SL16R (Thermo Fisher Scientific Inc, Waltham, MA, USA), the supernatant was discarded and the pigmented pellet of bacteria was suspended in a specific volume of distilled water to reach a concentration of 4.5 mg/ml. The inoculum was maintained in refrigeration at 4ºC until its use.

Inoculum size and initial pH were studied to find the best culture conditions for both cases measuring prodigiosin as response, four different inoculum sizes were studied (3%, 3.5%, 4.3% and 5% v/v) and five initial pH: 7, 7.5, 8 and 9 (Harris *et al*., 2004; Silverman & Munoz, 1973; Williams, 1973) in mineral supplemented starch-glycerol-peptone medium (MSSGP) obtained from factorial design (2^4) 50 ml of MSSGP medium were incubated at 250 rpm in 250 ml Erlenmeyer flask at 28ºC for 18 h in MAXQ6000 incubator (Thermo Fisher Scientific Inc, Waltham, MA, USA), experiments were performed with replicates (n = 2). Statistical analysis (ANOVA) was performed using Minitab® 17.

Table 1. Variables and levels considered for 2^k factorial design for the optimization of the culture media (n= 2).

Factor	Variable (abbreviate)	Level	Concentration (g/L)
a	Sodium	-1	0
	Chloride	1	5.0
b	Calcium	-1	0
	Chloride	1	8.0
c	Ammonium Iron	-1	0
	(III) citrate	1	0.05
d	Copper	-1	0
	Sulfate	1	0.05

2.3 *Effect of salts on prodigiosin production by 2^4 factorial designs*

A factorial design (2^k) was performed to find a relationship between four different salts and prodigiosin production, sodium chloride, calcium chloride, ammonium iron (III) citrate and anhydrous copper sulfate were added to MGP medium with the addition of 10 g/L of soluble starch as an additional carbon source.

Selection on the minerals was based on previous reports on the production of prodigiosin from different strains of *S. marcescens* by several authors (Harris *et al*., 2004; Silverman & Munoz, 1973), levels and concentrations of salts are presented in Table 1. All experiments were performed during 18 h of culture, pH 7.0 and aerated by hosepipes in 50 ml tubes, with replicates (n=2). Inoculum size was set up at 4% v/v, and aeration was maintained by an air pump ELITE 802 (Rolf C. Hagen Inc, Ontario, Canada) divided with pipes into eight 50 mL PYREX tubes.

2.4 *Type of starch effect on cell growth and prodigiosin production*

The two different pregelatinized and chemically modified starches used in this study: hydroxypropylated distarch phosphate Polartex Instant 12640 and acetylated distarch phosphate Stabitex Instant (Cargill) were kindly provided by Dr. Esther Perez Carrillo. Modified starches were used to replace the soluble starch in MSSGP medium formulation and the effect of the type of starches was tested separately. 55 ml of MSSGP medium were cultivated for 95 h at 28ºC and 250 rpm as a control. Specific growth rate was calculated by the formula described by Pirt, (1975):

$$\mu = \frac{\ln(X) - \ln(X_0)}{T - T_0} \quad (1)$$

X_0 and X represent the biomass in g/L in earlier and late exponential points of the growth and the T_0 and T correspond to the time of the previous biomass points respectively.

2.5 *Determination of prodigiosin and cell growth*

Bacterial cell growth was monitored by dry weight measurement. To quantify the real cell weight a starch digestion method was developed and implemented to eliminate the weight of insoluble not consumed starch. An aliquot of the culture broth (1 ml) was centrifuged at 8000 x g by 10 min, the supernatant was discarded and the pellet was resuspended in two milliliters of an enzyme solution in 0.1M potassium phosphate buffer at pH 6. The enzyme solution consists of 3.6 U/ml of α-amylase and 0.7 U/ml of glucoamylase. The reaction was incubated at 30ºC for 30 minutes. The mixture was centrifuged at 8000 x g by 10 min and the supernatant was discarded, the pellet was placed in a drier at 60ºC for one hour and the dry cell weight was determined.

Prodigiosin from the production media was determined based on the method reported by Ryazantseva *et al*. (2012). Briefly, an aliquot of 1 ml of the medium was diluted in 9 ml of acidified ethanol (1:10 of 1N HCl in ethanol), under constant agitation until the pellet was discolored (c.a. 10 min). The spectrophotometric determination of the prodigiosin content was made by measuring the absorbance of the supernatant at 535 nm in a Genesys 10S UV-VIS spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) and correlated to a standard curve of a purified prodigiosin standard.

2.6 *Prodigiosin isolation and purification*

To obtain a purified standard 1 L of MSSGP medium at pH 7.0 was inoculated with 5% v/v and an extractive phase adapted from Ferrer *et al*, (1996) consisting of 30 g/L of Triton X-114 was added. The flask was incubated at 28ºC for 24 hours with vigorous aeration of 3000 cm^3/min using an air pump ELITE 802 (Rolf C. Hagen Inc.). After incubation period, culture was allowed to stand to obtain two phases consisted of a surfactant-rich phase and a micellar phase. Surfactant-rich phase (around 100 ml) was recovered and placed in 1L beaker; 100 ml of distilled water was added

and mixed to wash cell debris. The mixture was then heated to 80ºC by 30 min to separate phases and water was removed. Surfactant-rich phase obtained was adjusted to pH 9 with triethanolamine (Fischer Chemicals, Zurich, Switzerland), 400 mL of n-hexane were poured into the beaker and stirred at 600 rpm. Then water was added to obtain reverse micelles, the reaction was recognized by the high viscosity change. Mixing was maintained during 30 minutes, the reverse micelles were broken with addition of 50 to 80 ml of acetonitrile. Hexane-rich phase was recovered and dried in an oven at 60ºC (Shen *et al*, 2014). Purification of the pigment was performed in a HPLC Agilent 1100 Series (Agilent Technologies, Santa Clara, USA), using a C18 preparative column Prodigy (250 x 21.2 mm, 5μm). Detection for prodigiosin was at 535 nm and 223 nm for Triton X-114 with a photo diode array detector (Rodríguez-Sánchez *et al*., 2013).

Mobile phases consisted of acidified (pH 3) water (Phase A) and methanol (Phase B) mixture as mobile phase with a elution program of 0 min - 0% , 1 min - 10%, 3 min - 30%, 8 min - 50%, 15 min - 60%, 20 min - 70%, 30 min - 100%, 40 min - 0% Phase B. Eluted fractions were neutralized to pH 6.5 with 1M NaOH and dried in a rotary evaporator R210 (Buchi, Flawil, Switzerland) at 35ºC. The dry powder was dissolved in ethyl acetate to remove salt crystals formed by neutralization and ethyl acetate was dried in an oven at 60ºC. Purified prodigiosin was compared with a standard of prodigiosin (Axxora, NY, USA) using the absorbance profile in a spectrophotometer and the reported by Hardjito *et al*. (2002). Purity and yield were calculated using the model according to Belter *et al*. (1988) with the following equations using HPLC chromatogram:

$$\text{Yield} = \frac{1}{2}\left[1 + \text{erf}\left(\frac{t}{t_0} - \frac{1}{\sqrt{2}}\sigma\right)\right] \quad (2)$$

$$\text{Purity} = \frac{y_0(i)Yield(i)}{\Sigma_j y_0(j)Yield(j))} \quad (3)$$

3 Results

3.1 Effect of salts on prodigiosin production by 2^k factorial designs

The effect of four minerals was evaluated using the Minitab 17 statistical software by a 2^k factorial design and related with prodigiosin content; the results obtained from experimental design are summarized in Figure 2, but the effects of mineral salts and interactions can be seen in Figures 3 and 4 obtained from the Minitab software. It can be observed that a specific combination of minerals yields different amounts of prodigiosin. The statistical analysis performed in Minitab 17 demonstrates that all the salts have significance to response (p-value < 0.05), but not all of them benefits prodigiosin biosynthesis as it can be seen in Figure 3. Both sodium chloride and calcium chloride decreased prodigiosin biosynthesis; from Figure 4 it can be observed that interactions of the four mineral salts led to different response in prodigiosin content. Copper sulfate in combination with ammonium iron citrate was shown the best interaction by the highest prodigiosin content reached. The addition of sodium chloride decreased 20% of prodigiosin production in the concentration range studied. Nevertheless copper sulfate and ammonium iron (III) citrate in combination increased 1.8-fold prodigiosin production in comparison to control. The MSSGP obtained was used for further studies.

3.2 Prodigiosin isolation and purification

The recovery of prodigiosin using the cloud point extraction method (CPE) achieved a high recovery of the metabolite, in this case the 91.5% of prodigiosin was in the down phase called coacervate phase or surfactant rich phase and this represents a novel extraction system for prodigiosin *in situ* product removal by the high recovery. The back-extraction of the pigment from the surfactant-rich phase by silica gel column clean-up (Ferrer *et al*, 1996) was discarded since this is time-consuming and intensive method, besides his difficulty to be applied in a continuous industrial process. The reverse micelle extraction method used here was optimized from a proportion of 4:1 hexane/coacervate phase resulting in a final surfactant clearance extract with a prodigiosin concentration around 1 mg/mL. An efficient back-extraction of the pigment is explained by the increase in the interaction between n-hexane and prodigiosin on the out-layer surface of the micelles due to higher

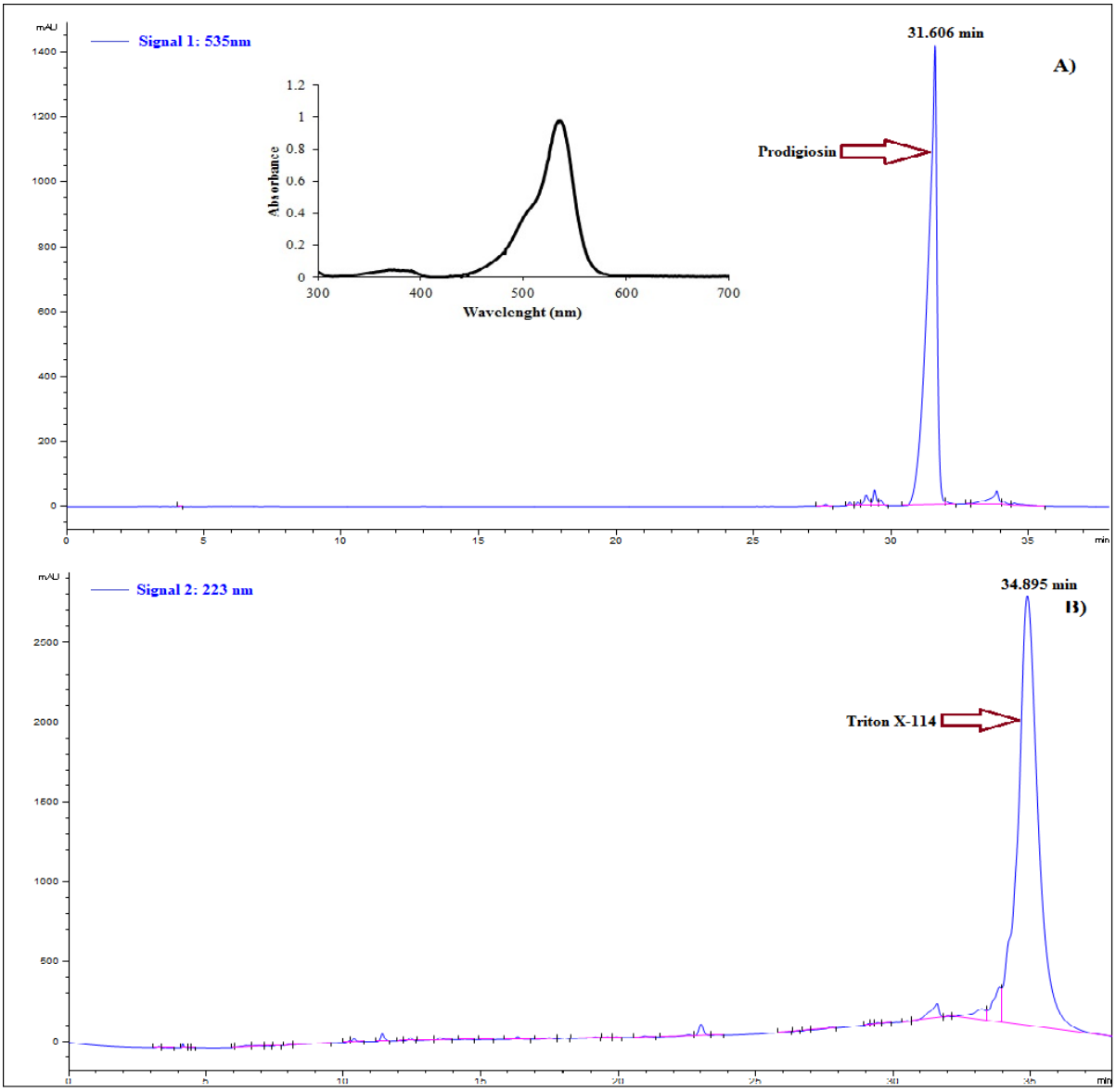

Figure 1. Chromatogram obtained from reverse micelle extraction method. A. The single peak obtained at 535 nm represents the extracted prodigiosin (t_r = 31.6 min), absorbance spectrum of the peak at 31.6 min is presented in the left; B. the mayor peak obtained by detection at 223 nm correspond to Triton X-114 the main contaminant (t_r = 34.89).

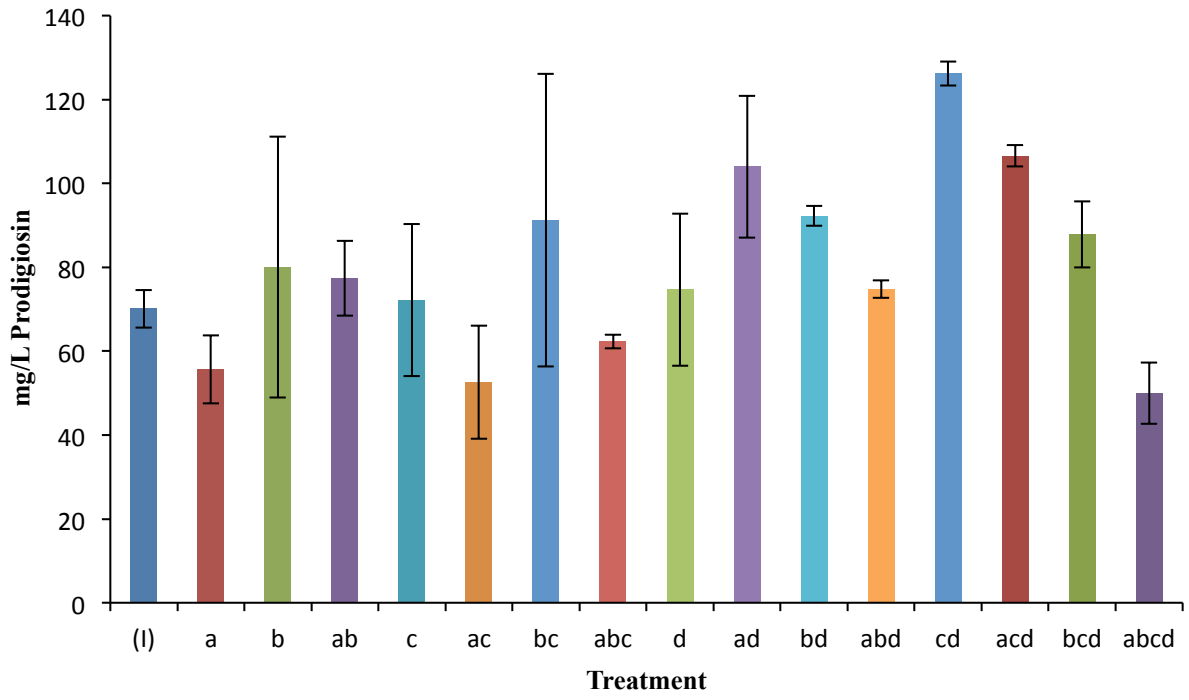

Figure 2. Results for prodigiosin production from the factorial analysis by treatment (Table 1).

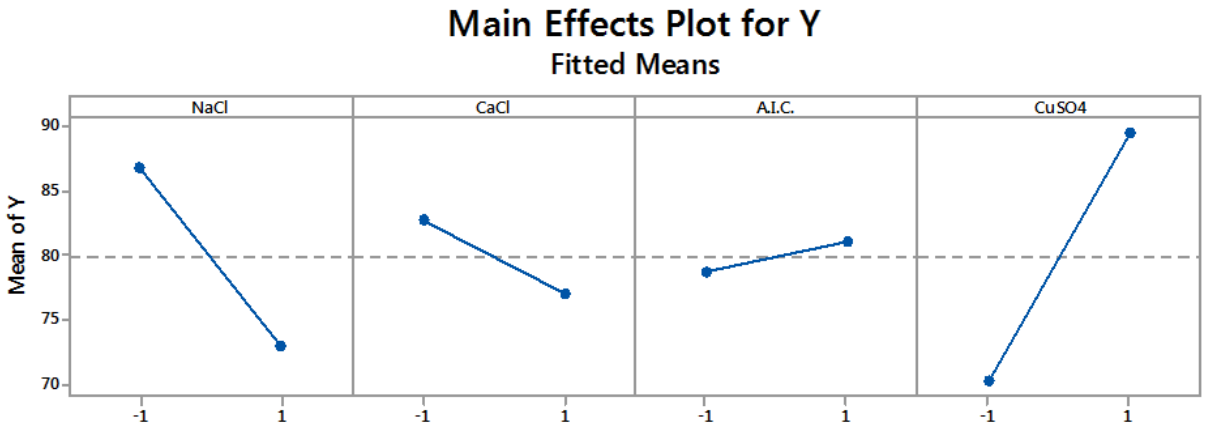

Figure 3. Effect of the four minerals NaCl, CaCl, Ammonium Iron Citrate (A.I.C.) and $CuSO_4$ on prodigiosin production, low level (-1) and high level (1) used in factorial design.

solubility of the compound in organic solvents as indicated by its XLogP of 4.1 (ACD/Labs Software V11). The back-extraction method proposed achieves a reduction of the 90% of the original Triton X- 114 in weight with a prodigiosin recovery around 1 mg/mL, in the concentrated cleaned extract; this represents an estimated recovery of 63% of the prodigiosin from coacervate phase.

The chromatographic method employed showed a single resolved prodigiosin peak (Figure 1) at 31.6 min at 535 nm, distinguished from the surfactant triton X-114 (34.9 min at 223nm). An absorption spectrum of the peak is presented as insert in Figure 1 and identified as purified prodigiosin when compared with the spectrum reported by other authors like Hardjito *et al*. (2002) and with the actual commercial standard.

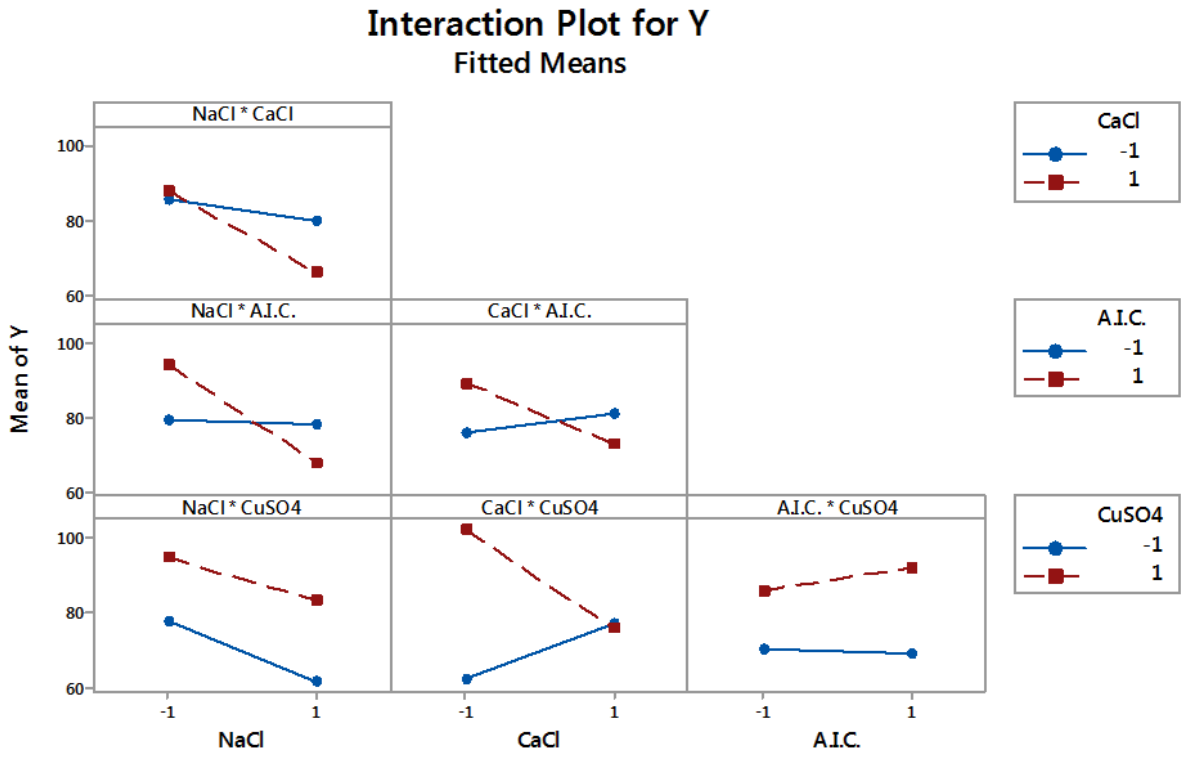

Figure 4. Interaction plot of the four minerals: Ammonium iron citrate (A.I.C.), NaCl, CaCl, $CuSO_4$, used in combination at low level (-1) and high level (1) on factorial design.

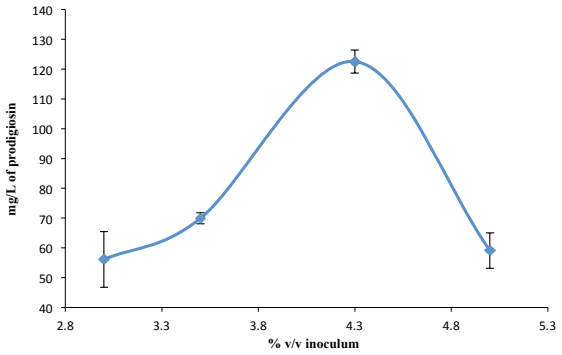

Figure 5. Effect of the % of inoculum on the production of prodigiosin. Media composed of 10 g/L soluble starch, 10.5 g/L casein peptone, 16 ml/L glycerol, 0.125 g/L $FeSO_4 \cdot 7H_2O$, 0.72 g/L $MnSO_4 \cdot H_2O$, 0.62 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CuSO_4$, 0.05 g/L ammonium iron (III) citrate at 28ºC, 250 rpm and pH 7.

The collected peak was further used as a standard with a calculated purity of 99% by equations (2)-(3), the purified prodigiosin was further used to develop the quantification curve.

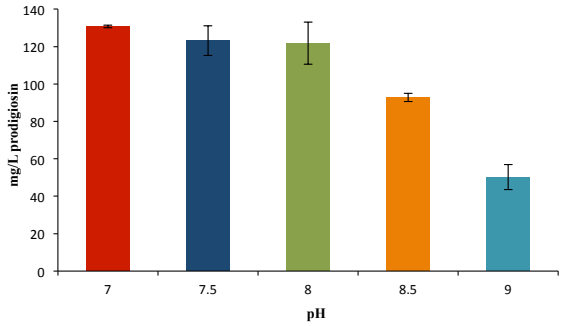

Figure 6. Effect of initial pH on prodigiosin production. All initial pHs show prodigiosin concentration obtained after 18 hrs of incubation (n = 2) using a media composed of 10 g/L soluble starch, 10.5 g/L casein peptone, 16 ml/L glycerol, 0.125 g/L $FeSO_4 \cdot 7H_2O$, 0.72 g/L $MnSO_4 \cdot H_2O$, 0.62 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CuSO_4$, 0.05 g/L ammonium iron (III) citrate.

3.3 Inoculum and pH optimization

A comparative study to elucidate the optimum inoculum size and initial pH was performed. The results, summarized in Figures 5 and 6 respectively,

show that prodigiosin production was higher at an inoculum size of 4.3% v/v in relation with total volume of culture. Taking into account that inoculum was standardized to avoid variability; the bacterial cell weight concentration at the beginning of the culture was calculated to 0.1855 mg/mL as initial dry weight of *S. marcescens*. This resulted in a maximum production of 122 mg/L of prodigiosin.

The effect of the initial pH of the fermentation can be observed from Figure 6. The ANOVA analysis (α=0.05) performed showed that an initial pH between 7.0-7.5 was not affecting significantly the prodigiosin production (p-value=0.255). A slight decrease was observed with the increase in pH to pH 8.0 (p-value= 0.01), but a higher variability was observed and is considered impractical for implementation, these results are coincidental with Hardjito *et al*. (2002) showing pH 7.0 as the optimal to enhance prodigiosin synthesis.

3.4 *Effect of type of starch on cell growth and prodigiosin production*

This comparative study was performed by a 95-hour period to obtain bacterial growth and production profiles for soluble starch as well as two commercial modified starches: hydroxypropylated distarch phosphate Polartex Instant® and a acetylated distarch phosphate Stabitex Instant®, both food-grade modified starches. The profiles presented in Figure 7 show a small biomass peak in the first 10 hours that correspond to a diauxic lag phase due to glycerol used as first carbon source for growth, as similarly reported by Araújo *et al*. (2010). Exponential growth on all the starches was from 10 to 33 hours with a calculated specific growth rate of 0.0.058 h^{-1}, 0.057 h^{-1} and 0.060 h^{-1} for soluble, Stabitex and Polartex respectively. But in comparison with the other starches, Polartex instant maintains a latency phase while the other two starches experiment a third growth phase at 46 hours (Figure 7). This behavior could happen because soluble and stabitex starch suddenly were degraded and assimilated by bacteria, while Polartex instant maintain a stable degradation rate that control bacteria in a latency phase. The maximum prodigiosin production was 570 mg/L at 95 hours with Polartex starch-based medium, this correspond an increase of 8.1 fold in prodigiosin production.

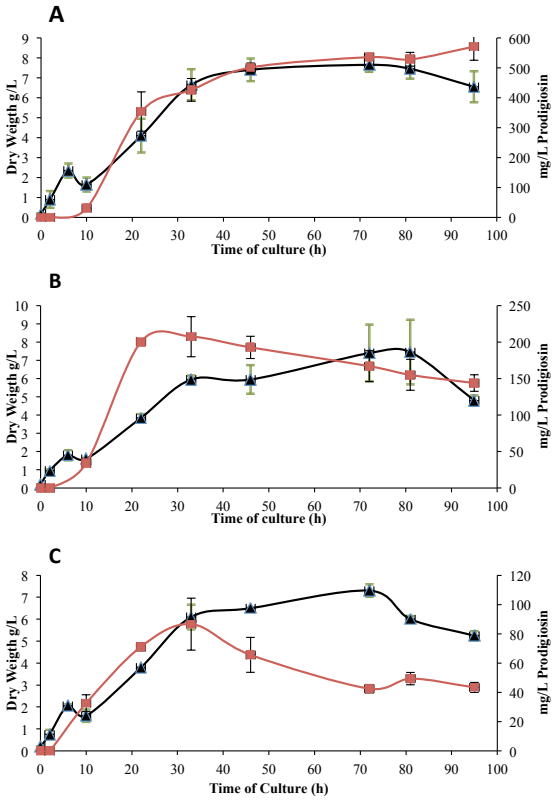

Figure 7. Growth and prodigiosin production kinetics for *S. marcescens* using of three different starches as carbon sources. A. Polartex® instant starch; B. Stabitex® instant starch; C. Soluble starch. (■) Prodigiosin production curve (right axis); (▲) Bacterial growth curve (left axis).

4 Discussion

This study demonstrated that addition of copper sulfate and ammonium iron citrate increase prodigiosin production in our strain *S. marcescens* BS303. Figure 4 shows the effect related with the interactions between mineral salts used, copper sulfate and ammonium iron citrate, displayed a synergistic role between them. When added individually, both minerals did not increased prodigiosin content, in contrast, when both were added, prodigiosin content increased 180% in comparison with the media with no addition.

Earlier studies have demonstrated that copper and ascorbic acid together are toxic to *S. marcescens* due to Cu^{2+} ions reduction to a more toxic Cu^{1+} ion, however, in the same study under aerobic culture, iron ions apparently reduced copper toxicity (Zimmerman, 1966). This interaction between copper and iron

ions can partially explain why the combination of ammonium iron citrate and copper sulfate do not exert a possible toxic effect, but not explain why prodigiosin was enhanced by 1.8 fold. In that case the study performed by Harris *et al*. (2004) demonstrated the relationship between arrangements of certain genes related with prodigiosin biosynthetic enzymes in *S. marcescens*, in particular, the arrangement of the Pig gene cluster. Some strains of *S. marcescens* have a Pig gene cluster flanked by CueR-CopA genes, involving copper ion regulation. This supports the hypothesis of copper ions directly involved on prodigiosin biosynthesis, by boosting transcription of the CueR-CopA genes and co-expression of the Pig gene cluster leading to a higher prodigiosin biosynthesis. The increase in prodigiosin synthesis could be also explained by the presence of certain ions that bind to enzymes as a cofactors, such as Cu, Mn and Fe ions (Chen *et al*., 2013; Harris *et al*., 2004). Mineral supplementation in our case triggered a 1.8-fold increase, this could be explained by a combination of such factors mentioned: transcription induction of the Pig gene cluster and addition of cofactors for enzymes related with prodigiosin biosynthesis. In the other case sodium chloride exert a different response, this mineral supplementation inhibits prodigiosin biosynthesis, in accordance with our results Silverman & Munoz (1973) determined the same inhibitory effect.

To harness the potential of a microorganism like *S. marcescens* is necessary to find the optimal conditions on the medium environment, like inoculum size that is related with cell density. In the case of prodigiosin production Pradeep *et al*. (2013) studied the relation between inoculum size and prodigiosin production and they conclude that a quorum sensing response to cell density was the responsible of an induction of prodigiosin production, and this response could be mediated by elicitor molecules N-acyl homoserine lactones (Fineran *et al*., 2005). Besides the role of inoculum size in prodigiosin production, the role of pH is an important environment factor. Role of pH on prodigiosin synthesis had been corroborated by Solé *et al*. (1994), showing that the synthesis of prodigiosin is limited by pH, due to the amount of available proline, a precursor amino acid integrated to prodigiosin structure during biosynthesis by proline oxidase EC 1.5.5.2. The relation between pH and prodigiosin production could be this proline oxidase enzyme, because the activity of this enzyme changes dramatically with pH, being the optimal around 7.0.

Even if environmental factor like the pH and inoculum size were optimized the product concentration of the medium was limited or scarce, but other techniques were reviewed by authors to overcome product inhibition or enhance metabolite production like the insoluble gels that have been reported as entrapment supports for *S. marcescens* enhancing metabolite production by a cell to cell signaling in an alginate beads entrapped *S. marcescens* (Chen *et al*., 2013). In the case of Bae *et al*. (2001) they reported a modified reactor for the continuous adsorption of prodigiosin in HP-20 resin obtaining 13.1 g/L of prodigiosin and high prodigiosin production was achieved because resin remove the metabolite from the medium and leads to a continuous production of prodigiosin. In our case similar effects could be responsible for the prodigiosin concentration achieved in Polartex starch medium, as a combination of adsorption and a scaffold for bacterial growth. The color of the pellet obtained from MSSGP medium give certain evidence of an entrapment of bacteria in the starch pellet, because the color of the starch in the pellet is of a blood red and the starch hydrolysis performed resulted in a major pigmented pellet of bacterial cells, this could evidence that *S. marcescens* is entrapped inside the structure of the starch, suggesting that besides acting as a carbon source, the slow digestion of the starch structure could also serve as a support for bacterial growth and protection against prodigiosin inhibition (Bae *et al*., 2001). Besides the scaffold property of the starch, its modification could exhort a certain adsorption property because the starch presents a hydroxypropylated functionalization that could act as a resin. Another point to emphasize in our study is the unusual kinetics of prodigiosin production, because the behavior denotes prodigiosin as a primary metabolite, although this is classified as secondary metabolite. Figure 7 shows production related with growth. This point may open the opportunity to implement a primary metabolite-related bioprocess, such as a continuous bioreactor and enhance productivity by this configuration. Process integration represent an attractive opportunity to develop a bioprocess where production and primary recovery can be performed at the same time, with the consequent time and costs savings (Cisneros-Ruiz & Rito-Palomares, 2005). The medium developed have this advantage and the diauxic growth behavior could be the responsible to enforce the bacteria into a growth-related prodigiosin production by a semi-steady state kinetic at 6 to 10 hours of the culture (Figure 7).

The use of cloud point extraction methods is not common, due to the need of a back extraction

procedure to recover the product. However, the high hydrophobicity and possibility of *in situ* adaptation into the bioreactor makes this an attractive option, considering also the product inhibition effect of prodigiosin on *S. marcescens*. Previous reports using Triton X-100 extraction yielded more than 85% of *Monascus sp*. hydrophobic red pigments using a Winsor micro-emulsion back-extraction (Shen *et al*., 2014). The method proposed by the reverse micelle back-extraction could represent a new approach for the continuous recovery of pigments and hydrophobic compounds from surfactant-rich phase at large scales.

Although several authors have reported higher prodigiosin production using complex waste materials as carbon sources, our report is based on a medium employing minimal components for prodigiosin biosynthesis, and using commercially available starch as a cheap carbon source. Waste or natural sources include peanut powder (Giri *et al*., 2004) or cassava waste (Araújo *et al*., 2010) to enrich medium for prodigiosin production. The dependency on the medium composition is clearly evident, given the different nutrient sources and the product yield reported to up to 137 mg prodigiosin/g biomass using a peanut powder-enriched medium and a mutant strain of *S. marcescens* (El-Bialy & Abou El-Nour, 2015). In our experience, the wild type bacteria used was able to maintain a continuous yield of 77 mg prodigiosin/g biomass, this evidence demonstrate the great potential of the simple production medium developed. These optional substrates reported by other authors pose an additional factor given the complexity of the carbon source, which may have a detrimental effect on the primary recovery and further purification strategy that has to be implemented in order to make the bioprocess commercially attractive for this secondary metabolite.

Conclusion

The nutritional requirements of *S. marcescens* for the production of the red pigment prodigiosin were studied through the use of a factorial design, considering the main nutritional requirements. The statistical analysis of the factors studied demonstrates that medium composition affects significantly the production yield for this metabolite and the significant nutrients for prodigiosin production were assessed. The suitable conditions that allowed the production of 570 mg/L of prodigiosin were 10 g/L soluble starch, 10.5 g/L casein peptone, 16 ml/L glycerol, 0.125 g/L $FeSO_4 \cdot 7H_2O$, 0.72 g/L $MnSO_4 \cdot H_2O$, 0.62 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CuSO_4$, 0.05 g/L ammonium iron (III) citrate, pH 7.0 and 4.3% v/v as initial inoculum. This prodigiosin production yield corresponds to 8.1 fold higher than the non-optimized medium and conditions.

Additionally, a modified starch (hydroxypropylated distarch phosphate) was found to be effective as a carbon source to extend the stationary phase of *S. marcescens* for up 50 hours and enhance the prodigiosin production. A further optimization of the concentration of the significant factors may increase the total productivity for this strain using this simple production medium that may permit the establishment of a bioprocess economically attractive with a standardized medium.

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Nomenclature

X	biomass measured as dry weight at time t, g/L
X_0	biomass measured as dry weight at time t_0, g/L
T	time of cultivation at late exponential phase, h
T_0	time of cultivation at early exponential phase, h
Y_0	maximum signal of peak, mAu*s
t_o	elution time of the maximum signal, h
t	elution time at the lowest peak signal, h
Greek symbols	
σ	standard deviation of the peak, h
μ	specific growth rate of bacteria, h^{-1}

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