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Optimization of prodigiosin production from a strain of *Serratia marcescens* SR_1 and screening for antifungal activity

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ABSTRACT: Prodigiosin produced by *Serratia marcescens* strain (SR_1) is a promising drug owing to its reported characteristics of having antifungal, immunosuppressive and antiproliferative activity. From a commercial point of view, there is a necessity to select a suitable medium for the enhancement of cell growth as well as pigment production simultaneously, from *Serratia marcescens* (SR_1) was the aim of the work. Compared to the nutrient broth and glycerol-yeast extract media, the casein-enriched medium supplemented with 4% vegetative oil mixture (sunflower, coconut and olive oil) showed a higher yield of prodigiosin (765.1mg l^{-1}) with a cell weight (2.1g l^{-1} dry wt), was observed after the incubation period of 84h at 30 ± 2°C with pH7.0. The antifungal activity of the crude prodigiosin, separated through solvent extraction process and purified by TLC was tested against fungal pathogens following the well–diffusion method, which showed the maximum inhibitory zone against *Helminthosporium sativum, Fusarium oxysporium* and *Rhizoctonia solani* in decreasing order.

KEY WORDS: Antifungal activity, *Fusarium oxysporium*, *Helminthosporium sativum*, Prodigiosin, *Rhizoctonia solani*, *Serratia marcescens*

INTRODUCTION

A number of soil bacteria produce pigments. Among them, *Pseudomonas sp.* (Shellito *et al.*, 1992) and *Streptromyces sp.* (Dastager *et al.*, 2006) are well known. *Serratia marcescens* is a promising organism, producing copious amount of prodigiosin in specialized medium (Furstner, 2003). Prodigiosin is a typical alkaloid produced as secondary metabolite. Chemically, it is (5-[3-methoxy-5-pyrrol-2-xylidene-pyrrole-2-xylidene) methyl]-2-methyl-3-pentyl-1H-pyrrole (Giri *et al.*, 2004). Prodigiosin is valued for medical applications, as it possesses the immunosuppressor, antifungal and antiproliferative properties (Soto–Cerrato *et al.*, 2004). It also induces apoptosis against certain cancer cells (Montaner and Perez–Tomas, 2001). Conventionally liquid media rich with a variety of nutrients is used for the production of prodigiosin (Giri *et al.*, 2004). Vitamin thiamine (Goldschmitt and Willams, 1968) and ferric acid plays major role for the better yield of the same. Bacteria develop prodigiosin on their cell envelope and its yield was enhanced by the addition of silica gel carriers (Giri *et al.*, 2004), surfactant, *i.e.*, sodium dodecyl sulphate (SDS) (Yamashita *et al.*, 2001) and

peanut seed flour (Giri *et al.*, 2004). Luria-Bertani (LB) broth with higher concentration of tryptone and yeast extract along with vegetable oil was shown to be effective medium for growth of *Serratia marcescens* and it was leading to a higher yield of prodigiosin production (Honng *et al.*, 2002 and Wei *et al.*, 2004). The present study was focused on the optimization of the yield of prodigiosin from the strain SR_1 (*Serratia marcescens*) and screening for antimicrobial activity against fungal pathogens.

MATERIALS AND METHODS

Bacterial strain

Strain SR_1 was isolated, purified and identified to be *Serratia marcescens* as per the methodology described by Aneja (2003).

Culture media

1. LB broth consisting of tryptone (10g l^{-1}), yeast extract (5g l^{-1}) and NaCl (10g l^{-1}) at pH7.0 was used for the revival and pre-culture of the strain SR_1 (*Serratia marcescens*) from a frozen stock, maintained at 4°C in a refrigerator. Different media were checked and compared for the better yield of prodigiosin from the strain SR_1 *viz.*, 2. Nutrient Broth (5.0g of peptone, 3.0g of beef extract, 5.0g of NaCl per litter, pH7.2), 3. Glycerol yeast extract agar (5.0ml of glycerol, 2.0g of yeast extract, 1.0g of K_2HPO_4 per liter, pH7.2). 4. Casein enriched medium, (20g of casein, 1.7g of K_2HPO_4, 1.0g of $MgSO_4.7H_2O$ and 1.0g of NaCl per liter, supplemented with the mixture of olive oil, sunflower oil and coconut oil @ the rate of 4%(v/v), pH7.2). All the media were sterilized at 120°C for 20min and the experiments were replicated 3 times.

Batch fermentation

An overnight grown culture of strain SR_1 of *S. marcescens* was inoculated into the sterile liquid media (working volume, 100ml) in shake flasks at a 0.5% (v/v) inoculum and incubated at different temperatures (28°C, 32°C and 37°C) with different pH (4.5, 7.0 and 9.0) and were agitated at 200rpm. Samples were taken at designated time intervals to optimize the cell concentration and pigment production. Cell concentration was determined by measuring dry cell weight (DCW). For DCW measurement, the culture broth was filtered through a weighed micro pore filter (0.2mm). The cell loaded filter was then washed with distilled water and dried (105°C) followed by reweigh to determine the cell mass. For pigment production, the culture broth (0.5ml) was mixed with an equal volume of 2% (w/v) alum placed in a vial. Four milliliters of methanol was added to the vial and the mixture was vigorously vortexed. The solution was then centrifuged at 1200xg for 10 min. A fixed amount (0.8ml) of the supernatant was further mixed with 0.2ml of 0.05N HCl / methanol mixture (4:1 v/v). The optical density of the resulting solution was measured at 540nm ($O.D_{540}$). The $O.D_{540}$ was converted to mass concentration via appropriate calibration, using the purified prodigiosin (sigma) product as the standard.

Purification of pigment

Serratia marcescens (SR_1), grown in casein enriched broth, supplemented with vegetative oil mixture was centrifuged at 10000rpm for 15min. using centrifuge (Remi, India) and the supernatant was extracted with ethyl acetate. The pigment from the cell pellet was extracted with acetone. Then the extraction was centrifuged at 10000rpm for 15min, the white pellet was discarded. The pigment extracted acetone fraction was mixed with ethyl acetate and dried with sodium sulphate. The extracts were evaporated using a rotary evaporator under reduced pressure at 70°C. A wavelength scan was done from 200 to 700nm using UV-VIS to confirm the prodigiosin by a spectrophotometer (Systronics). Purification of the crude pigment was done by thin layer chromatography (TLC). A solvent mixture of dichloromethane, chloroform and acetone (2.5: 2.5: 0.5) was used for the effective separation of the impurities extracted along with the pigment by thin layer chromatography.

Antifungal activity of prodigiosin

Antifungal activity of pigment prodigiosin was tested against fungal pathogens by well

diffusion assay method. Diameter of the inhibition zone (mm) showed against the growth of the specific target organisms was taken as the antimicrobial activity *of* strain SR_1. *Fusarium oxysporum, Alternaria alternata, Curvularia lunata , Rhizoctonia solani.* and *Helminthosporium sativum* were used as fungal pathogens

RESULTS AND DISCUSSION

Serratia marcescens strain SR_1 having the characteristics of culture morphology, gram negative, production of cell associated prodigiosin, lipase positive and inability of lactose fermentation on MacConkey plates. The results were identical as observed by Giri *et al.* (2004). The results showed the maximum yield of pigment in casein–enriched media supplemented with 4% of vegetable oil mixture (sunflower oil, coconut oil, olive oil) compared to nutrient broth and glycerol-yeast extract broth, incubated at different time intervals (Table 1). This suggests that the addition of vegetable oil may provide more accessible carbon source for cell growth and pigment production by *S. marcescens*. A previous study (Ang *et al.*, 2001) showed that *S. marcescens* has lipase activity and is thereby capable of hydrolyzing oil substrates to liberate fatty acids as carbon and energy sources. A variety of plant seed oils have also been used as carbon substrates for prodigiosin production and displayed stimulatory effects on the production of prodigiosin by *S. marcescens* (Giri *et al.*, 2004). Wei and Chen (2005) reported that Luria-Bertani (LB) broth, frequently used for prodigiosin biosynthesis with *S. marcescens* was modified by increasing the concentrations of tryptone and yeast extract with addition of vegetable oils (2-6% v/v). Similarly, Song *et al.* (2006) studied the red pigment content in *Serratia sp.* KH-95 was enhanced using casein–enriched medium.

Table 1. Profiles of (a) cell growth (dry wt g l^{-1}) and (b) prodigiosin production (mg l^{-1}) by *Serratia marcescens* cultivated in nutrient broth, glycerol yeast extract broth and casein enriched broth supplemented with vegetable oils at 30 ± 2°C temperature

Culture Time (h)	Nutrient broth		Glycerol yeast extract broth		Casein enriched medium with vegetable oil	
	Pigment production (mg l^{-1})	Cell dry wt(g l^{-1})	Pigment production (mg l^{-1})	Cell dry wt(g l^{-1})	Pigment production (mg l^{-1})	Cell dry wt(g l^{-1})
0.0	0.0	0.0	0.0	0.0	0.0	0.0
12.0	100.0±8.5	0.2±0.01	120.3±11.2	0.4 ±0.03	143.5±12.2	0.3 ± 0.01
24.0	178.2±14.2	0.85±0.05	190.0±15.2	0.7±0.05	310.3±25.4	0.55 ± 0.03
36.0	350.4±23.3	1.2±0.41	276.0±20.3	1.1±0.03	470.3±34.4	1.5 ± 0.07
48.0	356.2±23.3	1.23±0.41	334.0±30.3	1.4±0.03	596.4±45.2	1.8 ± 0.06
60.0	300.0±21.3	1.1±0.31	350.3±30.3	1.4±0.03	654.3±52.5	2.0 ± 0.08
72.0	230.5±17.2	0.8±0.05	390.4±34.4	1.7±0.2	724.1±38.2	2.1 ± 0.08
84.0	150.2±12.1	0.5±0.03	200.4±17.2	0.68±0.5	765.1±38.2	2.1 ± 0.08
96.0	80.3±7.7	0.13±0.04	135.2±11.1	0.36±0.3	370.8±20.4	1.5 ± 0.07

Table 2. Comparative analysis of prodigiosin production (mg l^{-1}) by *Serratia marcescens* (SR_1) in different media at 28°C, 32°C and 37°C temperatures in 24h old culture

Media Used	28°C (mg l^{-1})	32°C (mg l^{-1})	37°C (mg l^{-1})
Nutrient broth	186.4±12.5	117.6±9.4	86.4±4.6
Glycerol yeast broth	201.4±10.4	223.1±10.4	73.5±4.7
Casein enriched broth with vegetable oils	245.7±12.6	326.4±21.3	61.2±3.5

Table 3. Comparative analysis of prodigiosin production (mg l^{-1}) by *Serratia marcescens* (SR_1) in diffeént media at pH 4.5, 7.0 and 9.0 at 30±2°C temperature in 24h old culture

Media Used	pH		
	4.5	7.0	9.0
Nutrient broth	23.4±2.7	115.1±10.3	55.4±2.3
Glycerol yeast broth	17.3±1.1	227.4±12.5	47.6±2.3
Casein enriched broth with vegetable oils	21.7±1.9	341.3±15.8	102.4±6.4

The highest yield of pigment production was seen in casein-enriched broth, which was ended at the 84th hour. A linear increase in production from the 48th to the 72nd hour was found. In nutrient broth, a maximum production of pigment was seen from 12th to the 48th hour and then started to decline. Previous studies indicated that NaCl in nutrient broth might inhibit the metabolic pathway for the biosynthesis of monopyrrole and bipyrrole, which are the precursors of prodigiosin (Silverman and Munoz 1973; Cang *et al.*, 2000). In glycerol yeast extract broth, the maximum yield of pigment was seen at 72th hour and gradually decreased. As a consequence, glycerol was used as a glucose alternative in a peptone glycerol broth, resulting in a better yield of prodigiosin (Montaner *et al.*, 2000; Bae *et al.*, 2001). The role of temperatures (28°C, 32°C and 37°C) and pH (4.5, 7.0 and 9.0) for the production of prodigiosin pigment by *Serratia marcescens* in different media are shown (Table 2 and 3) and observed that maximum pigment production was seen only at 30°C and at pH7.0 in casein enriched medium as compared to the others. The prodigiosin production was completely blocked at 37°C. Similarly Pryee and Terry (2000) reported that the impact of the physiological role of temperature in blocking prodigiosin production thus seems to vary with medium to different substrate compositions. It was found that glucose may inhibit prodigiosin production due to catabolic repression (Giri *et al.*, 2004) or by lowering the medium pH (Sole *et al.*, 1997).

Table 4. Separation of compounds in pigment prodigiosin using TLC–silica gel coated plates

Separation of compounds	R_f Value
Faint yellow	18.1
Light yellow	58.1
Pinkish	72.7
Dark yellow	90.9

Purification of prodigiosin using TLC Plates

As depicted in Table 4, preparative TLC on silica gel plates separated the prodigiosin complex into 4 fractions. The fractions were detected by observation of the color of the spots or bands, which were observed at different R_f values ranging from faint yellow to dark yellow with intermediate pinkish to red color and subsequently by bioautography against *F. oxysporum*. Similarly, Wagman *et al.* (1976) worked on *Micromonospora sp.*, produced a group of antibiotics, which was tested against *Staphylococcus aureus* ATCC6358P by bioautography.

Pigment identification

Prodigiosin, as reported from *S. marcescens* (Castro *et al.*, 1959) had a maximum absorption spectrum at 537-538nm in 95% ethanol. In this study, the maximum absorption spectrum of prodigiosin purified from SR_1 showed at 540nm in acetone (Fig. 1). Similar results were by Giri *et al.* (2004) showed that the pure pigment gave an absorbance peak at 535nm.

Prodigiosin showed the maximum inhibition zone (diameter) against *H. sativum* (42.0mm) and minimal inhibition zone against *R. solani* (11.0mm) (Table 5 and Plate 1). The result shows that the strain prodigiosin produced by SR_1 is a promising drug owing to its reported characteristics of having antifungal and antiproliferative activity. Nobutaka *et al.* (2001) reported a synergistic inhibitory activity of prodigiosin and chitinolytic enzymes was observed against spore germination of *Botrytis cinerea*.

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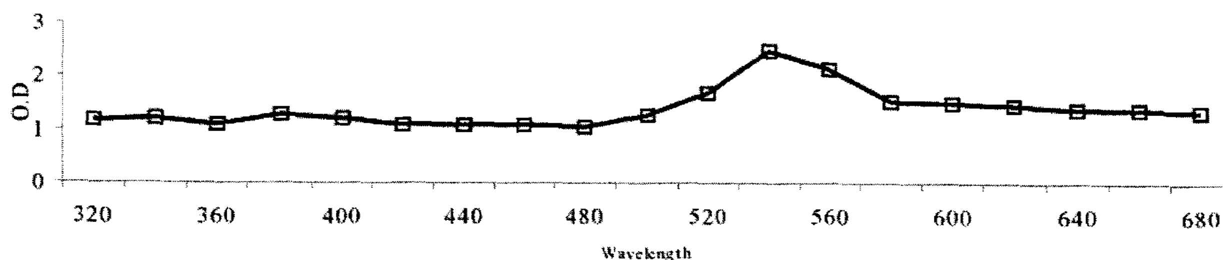

Fig. 1. UV-Vis absorption spectra of red pigment in acetone from strain *Serratia marcescens* (SRI)

Table 5. Inhibition zone of fungal pathogens by the pigment prodigiosin of *Serratia marcescens* on PDA

Target Organisms	Diameter of inhibition zone (mm)
Helminthosporium sativum	42.0 ± 2.3
Curvularia lunata	40.0 ± 2.4
Alternaria alternate	40.0 ± 2.0
Fusarium oxysporum	30.0 ± 1.5
Cercospora apii	24.0 ± 1.5
Rhizoctonia solani	11.0 ± 1.0

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