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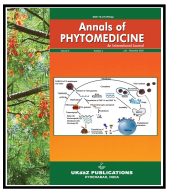

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Production, purification and characterization of prodigiosin by *Serratia nematodiphilia* (NCIM 5606) using solid-state fermentation with various substrate

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Abstract

Agroindustrial wastes such as wheat bran, rice bran, orange peel, sweet lemon peel and pigeon pea peel were examined in order to choose the substrate that support the maximum prodigiosin yield using *Serratia nematodiphilia*. The conversion of agrowaste to a valuable prodigiosin by fermentation process is done to minimize the production cost and environmental risks. Findings revealed that the maximum production yield (1.3075 mg/l) was observed in wheat bran. Sweet lemon and orange peel gave prodigiosin yield of 0.1693 and 0.1495 mg/l, respectively. Pigeon pea and rice bran media gave minimum prodigiosin yield of 0.0082 and 0.0667 mg/l, respectively. Extracted pigment purified by TLC gave Rf value of 0.85. Further, purified pigment was characterized as prodigiosin by absorption sepctra (λmax=535 nm) and FTIR.

1. Introduction

Pigment is the most indispensable trait of any article especially food. Biocolour word comprises of two words bio and color that implies something regular, utilized for pigment reason. Thus, biocolorants can be one of the options in contrast to false quality for expansion into any food material. These are essentially those pigment specialists, which are acquired from the organic sources, for example, plants, animal and microorganisms as well springs of natural color. These natural color are commonly extracted from natural products, vegetables, seeds, roots and microorganisms and are frequently called as 'biocolors' because of their organic source (Sharma, 2014; Rymbai *et al*., 2011). Lately, look for microorganisms delivering non-poisonous metabolites, has been performed by a few analysts (Downham and Collins, 2000). Further, the waste created from food industry can likewise be one of the substrate for development of these biocolors delivering microorganisms.

Natural colors produced as metabolites by the microorganisms serve as potential substitute for harmful synthetic dyes. Unlike, the conventional colors derived from plants and animal skin, microbial pigment are finding tremendous application in food industries, owing to their easier accessibility, simpler extraction and adequacy. *Serratia* spp. are gram-negative microbes grouped in the extensive group of *Enterobactericeae* producing a dark red pigment termed as prodigiosin (Samrot *et al*., 2011).

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The prodigiosin is natural product and belonging to a family of tripyrrole red color that contains a typical 4-methoxy 2, 2 bipyrrole ring framework. The biosynthesis of the color is a two steps in which mono and bipyrrole antecedents are blended independently and after that gathered to shape prodigiosin (Boger and Patel, 1988). The biosynthesis of the color is a bifurcated procedure in which mono and bipyrrole antecedents are blended independently and after that gathered to shape prodigiosin. Prodigiosin is multifaceted secondary metabolites having chemical formula $C_{20}H_{25}N_3O$, possessing an unusual structure with three pyrrole rings and a pyrryldipyrrylmethane group comprising three rings where two rings are directly linked to each other, and the third is linked through a methane bridge.

Prodigiosin is produced by numerous microorganism including *Serratia marcescens, Vibrio psychroerythrus*, *Sreptomycin griseoviridis* and *Hahella chejuensis* with antibacterial, antimycotic, immunomodulating, against tumor and antimalarial properties (Frustner, 2003; Giri *et al*., 2004). Kobayashi and lchikawa (1991) and Matsuyama *et al*. (1986), deduced that prodigiosin is synthesized as extracellular vesicles or present as intracellular granules in *Serratia* sp. Colored compounds such as pigments absorb light of characteristic wavelength, determined spectrophotometrically (Cerdeño *et al*., 2001). Prodigiosin can exist in two different forms depending on the hydrogen ion concentration of the production media. In acidic medium, colored pigment shows characteristic spectra at 535 nm. The pigment production depends on type of fermentation and the cultural conditions. It has been previously reported that SSF shows efficient like high volumetric profitability (Duenas *et al*., 1995). Agrowaste utilization of compelling use of agroindustrial wastage as substrates that even

copy the common living surface of organisms and economy (Murugesan *et al*., 2007) because of its static nature. This reutilization of agrowaste is refreshing with regards to reasonable advancement. In recently SSF has picked up significance in the creation of microbial pigment because of a few monetary favorable circumstances over customary submerged fermentation (Sathya *et al*., 2009).

In recent years, use of agriculture based product and results acquires significance in bioprocess industries on account of high supplement substance and minimal effort. Changing the waste side-effects to a valuable bioproduct by fermentation diminishes the procedure cost as well as the danger of natural contamination. Customarily, the enhancement is being finished by keeping all parameter consistent and changing different parameters. This does not interaction of one parameter with the other. Our aim is to discover a media that may support the development of the bacteria and in the meantime demonstrate effective to trigger much amounts of color production. In present investigation, we have endeavoured to expand the production of prodigiosin from *Serratia nematodiphilia* (NCIM 5606) by utilizing wheat bran as well as purified prodigiosin is tested for various activities.

2. Materials and Methods

2.1 Microorganism and screening of substrates for prodigiosin production

Bacterial strain *Serratia nematodiphilia* (NCIM 5606) was procured from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The stock culture was maintained at 28°C on nutrient agar slants. Inoculum was prepared by adding a loopful of bacterial colonies in 250 ml conical flasks containing 100 ml of nutrient broth and incubated at 28°C for 72 h.

2.2 Selection of substrate

Substrates of wheat bran, rice bran, orange peel, sweet lemon peel and pigeon pea peel were examined for their suitability for growth of *Serratia nematodiphilia* and prodigiosin production.

2.3 Cultivation conditions for prodigiosin production in solid-state fermentation (SSF)

The substrate, wheat bran, rice bran and pigeon pea were obtained from local market mill located in Varanasi and orange peel and sweet lemon peel collected from BHU fruit shop. All substrate were dried at 50°C, until a constant weight was obtained and then grind with food grinder (Philips) and sieved into particle size of 25-90 µm. Then, 5 g of the dried substrate was then placed in a 250-ml of each conical flask and 10 ml of trace metal solution pH 7 added and autoclaved at 121°C for 15 min and cooled to room temperature. Trace metal solution K_2HPO_4 (2 g/l) and trace ion solution (0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l NaCl, 0.5 g/l $MnSO_4$, 3.4 mg/l, $ZnSO_4 \cdot 4\,H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$ and 2 mg/L $CoCl_2 \cdot 6H_2O$ were prepared. The flask was inoculated with 1 ml *Serratia nematodiphilia* (NCIM 5606) and incubated at 25°C for 6 days Figures 1a and b.

Figure 1a: Before fermentation.

Figure 1b: After fermentation (SSF of different substrate).

2.4 Extraction and determination of prodigiosin

First, we add fermented sample with phosphate buffer at pH 7. Then mixed in shaker at 100 rpm for 24 h at 25°C. 15 ml fermented sample was centrifuged at 10000 rpm for 10 min at 4°C. Pellet was collected. Two phases separation method was used in prodigiosin extraction. Chloroform and sodium hypochlorite was used in 1:1 and extracted pigment was collected in chloroform. Optical density of the resulting solution was determined at 535 nm (OD 535 nm). The total prodigiosin (mg/l) was calculated according to the following formula (Williams and Gott, 1961; Chen *et al*., 2006).

$$\text{TP mg/l} = \frac{\text{ADV1}}{7.07\times10^4\text{V2}}$$

where TP denotes the total pigment yield (mg/l), A the absorbance of chloroform extract at 535 nm, D the dilution ratio, V_1 the volume of methanol added, 7.07 x 10^4 is extinction coefficient of prodigiosin and V_2 is the volume of fermentative liquid.

3. Purification of prodigiosin using TLC

The crude pigment extracted from solid-state fermentation broth after 96 h of cultivation. After extraction, pigment was further purified using preparative thin layer chromatography (TLC) (2.5×30 cm; Kieselgel 60; Merck, Darmstadt, Germany) mesh size: 60-80. Thin layer chromatography was used for separation of the non-coloured impurity (Montaner and Pérez, 2002). The solvent system used for the TLC was chloroform: methanol (5:5) (Figure 2) Spots of pigment refined by TLC were scratched and

separated in chloroform. The extract was transferred into neat and clean vials and then centrifuge in 12000 rpm for 15 min to separate silica and pure red pigment. After separation, supernatant was collected which contained purified pigment. Purified supernatant transfer in porcelain dissipating dishes and evaporated at room temperature till a powdered pigment was gotten.

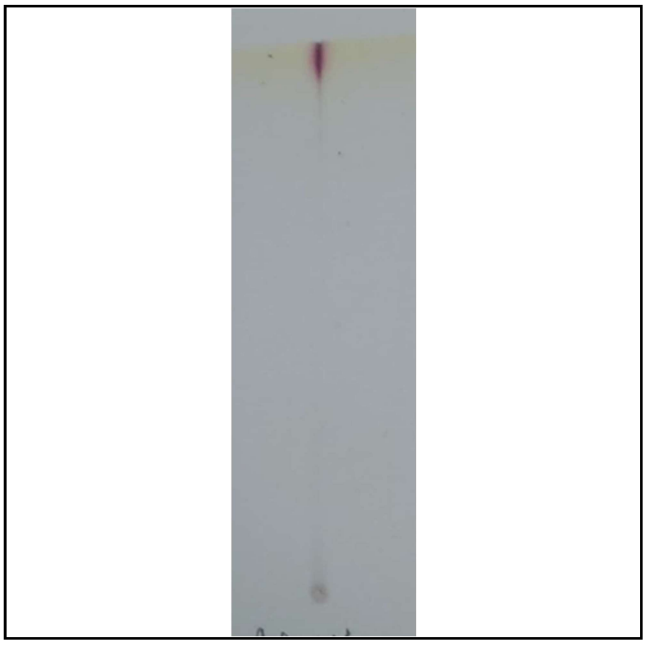

Figure 2: Thin layer chromatography of extracted pigment.

4. Results

4.1 Screening of suitable substrate for enhanced prodigiosin yield

The pigment yield is significantly affected by type of substrate and its amount, fermentation, chosen strain, inoculum size and moisture percentage. In the present study, we investigated the effect of agroindustrial waste on pigment yield under SSF. *Serratia nematodiphilia* (NCIM 5606) was grown in different media (wheat bran, sweet lemon orange peel, pigeon pea and rice bran). Wheat bran showed maximum prodigiosin yield of 1.3075 mg/l after 96 h of incubation (Table 1). Similarly, *Serratia nematodiphilia* grown on sweet lemon and orange peel media gave prodigiosin yield of 0.1693 and 0.1495 mg/l, respectively. Minimum prodigiosin yield was observed in SSF comprising pigeon pea and rice grain as substrate (Table 1).

Table 1: Production of prodigiosin in different substrate in solid state fermentation

Sr. No.	Substrate	Yield mg/l
1	Wheat bran	1.3075
2	Sweet lemon	0.1693
3	Orange peel	0.1495
4	Pigeon pea	0.0082
5	Rice bran	0.0667

4.2 Purification of prodigiosin

The extracted purified red pigment showed Rf value of 0.85 which was comparable to standard prodigiosin (Figure 2). This was in correlation with previous findings of Pathak and Dharmadhikari (2016) who reported Rf values of 0.9 for prodigiosin. Similarly, Raj *et al*. (2009), reported Rf value of 0.9 for the prodigiosin extracted from *Serratia Marcescens* MTCC 97 under similar condition.

4.3 Presumption test for extracted pigment

Presumption test was performed for the confirmation of prodigiosin. Pellet was tested against acidic and alkaline conditions. In acidic condition, red or pink color was obtained and alkaline condition yellow or tan color obtained, that was confirmed a positive presumptive test for prodigiosin.

4.4 Characterization of red pigment

4.4.1 UV-vis spectral analysis

The absorbance spectrum of sample was measured with a Double Beam UV-Visible Spectrophotometer (Electronics India model No-3375) in the range of 350 to 700 nm. Absorption spectra of the red pigment in chloroform showed a maximum absorbance at 535 nm evidenced by the presence of a peak at this wave length (Figure 3). A single peak absorbance at 535 nm depicted the extracted pigment as prodigiosin which was in conformity with previous findings (Giri *et al*., 2004; Montaner *et al*., 2000).

4.5 Characterization of prodigiosin

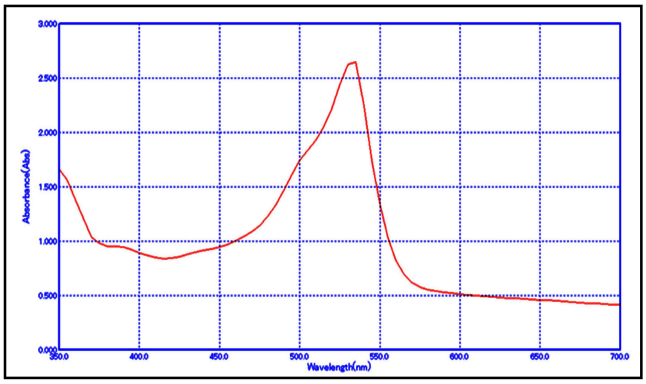

Figure 3: Absorbance spectra of Prodigiosin (535 nm).

4.5.1 Fourier transform infrared spectrum of pigmented metabolite

Purified pigment was characterized by FTIR as prodigiosin. FTIR spectra showed peaks at wavenumber; 2960.17, 1718.97, 1444.96, 1381.26, 1282.34, 1214.97, 1122.85, 1073.58, 939.68, 749.59 and 666.95 cm^{-1}. The peaks at 2960.17 cm^{-1} are due to asymmetrical and symmetrical stretching of methylene groups. The peaks at 1718.97 cm^{-1} and 1445 cm^{-1} are due to the presence of –NH and methyl groups. The visible peak at 1381.26 cm^{-1} is due to the presence of C-O group in prodigiosin. The fingerprint region for the red pigment was characterized by medium intensity bands at 1073.58, (C-O and C-N) and 939.68 cm^{-1}. The peaks around 1282.34 cm^{-1} and 749.59 cm^{-1} are attributed to carbon-carbon double bond. The characteristic peaks observed at specific wavenumbers deduced

the extracted pigment as prodigiosin (Figure 4). Similar findings were reported by Faraag *et al*. (2017), in FTIR study for characterization of prodigiosin produced by *Serratia marcescens* strain isolated from irrigation water in Egypt.

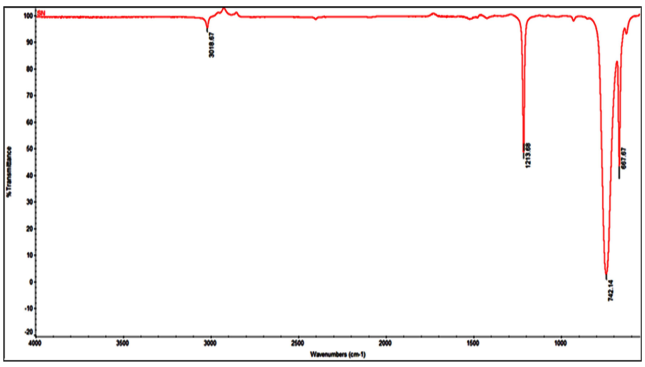

Figure 4: Fourier transform infra-red spectrum of purified prodigiosin.

5. Discussion

The present study provides an evidence for the successfully utilization of agroindustrial waste to enhance prodigiosin production under solid-state fermentation. *Serratia nematodiphilia* (NCIM 5606) was grown in different media (wheat bran, sweet lemon orange peel, pigeon pea and rice bran). But, wheat bran was best substrate for production of prodigiosin in all of them. Results revealed that maximum production of prodigiosin was obtained in wheat bran medium (1.3075 mg/l). Prodigiosin production is totally dependent on nitrogenous substance. Many substrate contain different type of amino acid, but they could not help the production of prodigiosin because specific amino acid utilize in production of prodigiosin. Wheat bran contains 5 types of specific amino acids (alanine, proline, leucine, glycine, cysteine), which was necessary for enhance production, they have showed increase in the production of prodigiosin when using the proline resulted in yield (17.3 mg/l) (Mathlom *et al*., 2018).Wheat bran contains elevated level of these amino acids and gives better prodigiosin yield (Balakrishnan, 1996). Maximum prodigiosin yield of 270 mg/l was observed in MO-1 using ram horn peptone as sole nitrogen source under SMF (Kurbanoglu *et al.,* 2015).

Extracted purified pigment show Rf value which was similar to previous findings by Pathak and Dharmadhikari. (2016). So, this was prove that pigment was prodigiosin.

Presumptive test was performed for confirmation of prodigiosin. In acidic condition, prodigiosin was red in color and alkaline condition pigment show yellow or tan color. This presumptive test proved that pigment was prodigiosin.

The absorbance spectrum of sample show the presence of specific chemical in solution. In sample maximum absorbance obtained 535 nm. Maximum absorbance indicate the presence of prodigiosin in extracted sample. A single peak absorbance at 535 nm depicted the extracted pigment as prodigiosin which was in conformity with previous findings (Giri *et al*., 2004; Montaner *et al*., 2000).

FTIR analysis of crude pigment shows the presence of prodigiosin as active compound. The characteristic peaks observed at specific wavenumbers deduced the extracted pigment as prodigiosin. Figure 4 depicts the different FTIR band and similar findings were reported by Faraag *et al*. (2017), in FTIR study for characterization of prodigiosin produced by *Serratia marcescens* strain isolated from irrigation water in Egypt. So, all discussion proved that extracted pigment was prodigiosin.

6. Conclusion

Serratia nematodiphilia gave a maximum pigment yield of in solid-state fermentation using wheat bran as a substrate. The pigment yield observed in present investigation under solid-state fermentation showed 5 fold better yield in comparison to control. Further, characterisation of extracted pigment by absorption spectra and FTIR confirmed the presence of prodigiosin as a bioactive metabolite. The present investigation deduced that wheat bran can be effectively used as primary substrate in prodigiosin production at commercial level. The usage of agroindustrial waste will minimize the upstream processing cost of novel pigment and can be effectively used in food processing industries as food color. Further, the process may be scale up for its commercial application at industrial level.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. Both the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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