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Fermentative production of melanin pigment from *Streptomyces griseorubens DKR4* from agro waste products

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Abstract
Many pigments are important in food, Pharmaceuticals and cosmetics. Dark diffusible pigment was extracted from agro waste products by using *Streptomyces griseorubens DKR4*. The aim of the present work was to investigate and observed the maximum pigment production in agro waste residues through Solid State Fermentation. A pigment yield was achieved by employing various agro wastes and residues optimized by various parameters such as 50% initial moisture content, and it was incubated at 40 °C. The colour of the pigments was stable at pH 7.5 and it could be a significant point for application purposes. To the best of our knowledge this is the first report on pigment production using various agro wastes and residues in solid-state fermentation (SSF).

Keywords: *Streptomyces,* solid state fermentation, Banana stalk, Rice Flour

1. Introduction

Natural pigments are sourced from plants, animals and many microorganisms. These pigments are used as a food colorants, pharmaceuticals and cosmetics. Many synthetic pigments are hazardous effect to humans, animals and environment. Bacterial pigments have better biodegradability and eco-friendly to the environment. The production of environmental friendly colourings and dye intermediates through fermentation process that can be used as food constituents [1]. Chemical producers have to meet increasingly stringent requirements for the control of heavy metals, pH and colour in process effluents [2]. From the literature, the utilization of a cheaply available substrate through solid-state fermentation (SSF) process, the solid substrates not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells [3]. In recent years, SSF has gained much interest for the production of primary and secondary metabolites. SSF presents a more adequate habitat for fungus, with high pigment productivity in a relatively low-cost process by using agro-industrial residues as substrates [4]. Agro industrial residues and crop such as coconut oil cake, corn cob, rice bran, jack fruit seed, groundnut oil cake, wheat bran were used as substrate this resulting in lower price fermentation and high pigment output [5]. Medium optimization is one of the important processes for getting maximum pigment yield and it involves several factors such as medium components, operating conditions, pH, temperature, aeration and agitation, etc. [6].

Streptomyces are widely distributed in marine and terrestrial habitants. They are responsible for the production of many biologically important secondary metabolites [7-8], antibiotics [9], pigments [10], antitumor agents [11], immunosuppressive agents [12] and enzymes [13]. *Streptomyces* sp. using starch substances, both in solid and submerged fermentation and its give pigments. *Streptomyces* pigment (Melanin) was irregular, dark brown polymers that have the radio protective and antioxidant properties that can effectively protect the living organisms from ultraviolet radiation [14]. Melanins is frequently used in medicine and cosmetics preparations. They showed a broad spectrum of biological roles, including antioxidant [15], antimicrobial activity [16], antitumor [17], antivenin [18], anti-virus [19], liver protective [20] and radio protective [21] etc.

The main objective of this study was to develop a potential fermentation process for the production of pigments employing the solid state fermentation using non-conventional agro-residues and also to study the effect of different parameters in an attempt to maximize pigment production.

2. Materials and methods

(i) Culture collection

A brown pigment producing strain was isolated from Nil Gris soil and it was grown in Tyrosine Starch Casein Agar by standard count method and Tyrosine Starch Casein Broth at room temperature and sub cultured seven days once.

(ii) Inoculum preparation

Seven days agar slope culture was used as a inoculum. The spores were scrapped under aseptic conditions in 10ml of sterile distilled water. ($1.5X10^5$ spores/ml) [22].

(iii) Solid state fermentation

Substrates were purchased from local market at Tiruchirappalli and they were cleaned, sundried for 3 days chopped and used for further studies. Experiments were conducted in 250ml Erlenmeyer flasks containing 5 g substrate (Various agro-industrial residues). The substrate was dispersed with salt solution. After thorough mixing, the wet substrates were autoclaved and cooled to room temperature. It was inoculated with 2ml of spore suspension containing $1.5X10^5$ spores/ml and it was incubated at 37°C for 7 days [23].

(iv) Solubility of the pigment

Solubility of the pigment was tested by using different solvents such as water, methanol, ethanol, Acetone, Ethyl acetate and Chloroform.

(v) Extraction of pigments

After incubation the fermented solid substrates (5mL of different solvent per gram of wet fermented material) were centrifuged at 3,000 rpm for 15 min, equal volume of methanol, chloroform, ethyl acetate and Acetone were added with solid substrates and mixed well. This step was repeated 2 to 3 times. The solvents were evaporated and powdered while the pigment residues were collected. The pigment production was analysed and measured by UV spectrophotometer. The extraction of the pigment in fermented solid substrate is presented in Fig.2.

(vi) Estimation of pigments

Melanin pigment was estimated by taking 2 ml of the culture and 1ml of 0.4% substrate solution (L-Tyrosine (or) L-dopa). The reaction mixture was incubated at 37 ºC for 30 min for L-tyrosine and 5 min for L-Dopa and red coloration resulting from dopachrome formation was observed and read spectrophotometer at 480nm.

(vii) Effect of agro waste on pigment production

To study the effect of various Agro waste and residues such as rice bran, wheat bran, Coconut Husk, Banana stalk, Rice Flour and Dark millet were screened for selecting the best substrate for pigment production. These substrates were taken at a known quantity (5 g, dry wt basis) [23].

(viii) Effect on pigment production by moisture content

Different initial moisture levels (20–50%) were employed in the substrate by adjusting the volume of distilled water to study their effect on pigment production. The pigment production was also monitored in the fermented samples with different initial moisture levels, as per the methods.

(ix) Effect on pigment production by ph & temperature

To study the initial temperature for pigment production and the flasks were incubated at various temperatures (25, 30, 35, 40 and 45 °C). The pigment production was also monitored in the fermented samples with different initial temperature levels, as per the methods. pH of the substrate was changed by adjusting the pH of the salt solution and was optimized so as to get the required pH after autoclaving. The effect of initial pH of the substrate on pigment production was estimated by spectral analysis of the pigment extracts [25].

(X) Antioxidant activity

The purified pigment was analysed by DPPH method and conform the Antioxidant activity. 2ml of purified pigment (100µg/ml concentration), 2.5% linoleic acid prepared in ethanol, 4 ml of 0.05M phosphate buffer (pH 7.0) and it was make up to 10mi with distilled water and mixed well. And this was kept in water bath in the dark at 40°C. Then 100µl of the above mixture was added with 9.7 ml of 75% (v/v) ethanol and 100µl of 30% (w/v) ammonium thiocyanate. After 5 minutes 100µl of 0.02M ferrous chloride in 3.5% (v/v) hydrochloric acid was added to the above mixture and mixed well. The absorbance of mixture was measured at 500nm in every 24 hours interval for one week [26].

3. Result & Discussion

From this study it was concluded that the agro waste and residues are alternative option for pigment production. Fig.1 described the usage of Banana stalk; Coconut Husk, Rice Husk, Dark millet, wheat and Rice flour are the best agro waste for pigment production.

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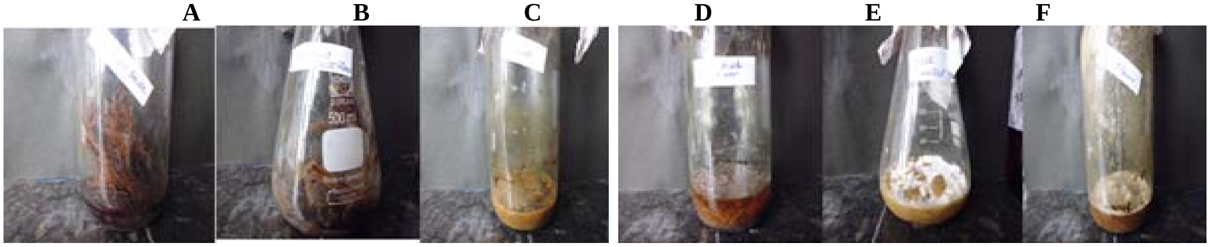

Coconut Husk | Banana stalk | Rice flour | Rice Husk | Wheat | Dark millet

Fig 1: Different substrate for melanin production

3.1 Solubility of the pigment

Fig.2 & 2a showed that the Solubility of pigment was tested using different solvents such as water, methanol and chloroform. From this study, the pigment was completely soluble in Methanol. So, the methanol extract selectable for further studies. Highest pigment production was achieved with Banana stalk (1.893ODUnits), Coconut Husk (1.910ODUnits), Rice Husk (1.741ODUnits) and Rice flour (1.356ODUnits). Wheat (0.774ODUnits) and Darkmillet (0.360ODUnits), showed moderate yields. Hence, Banana stalks; Coconut Husk, Rice Husk and Rice flour were selected and used for subsequent studies.

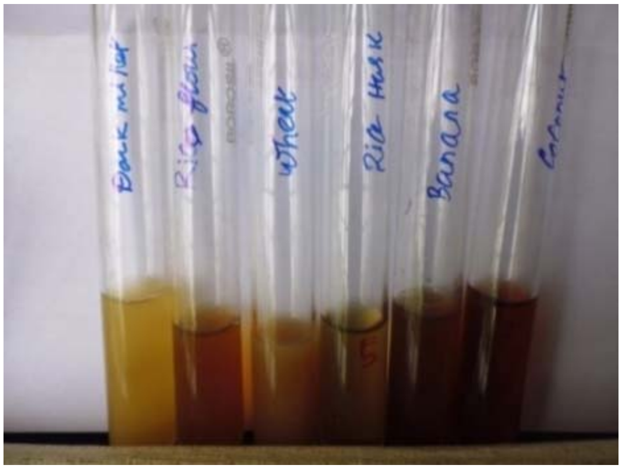

Fig 2: Extracted pigment from agro waste

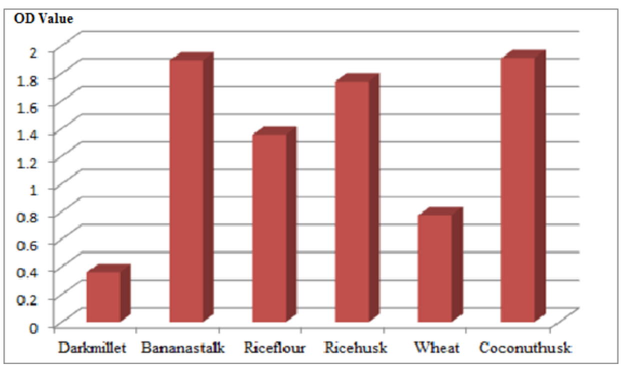

Fig 2a: Effect of solid substrate on pigment production

3.2 Effect of pigment production by moisture content

For solid state fermentation, moisture is a key parameter to control the growth of microorganism and metabolite production. The effect of initial moisture content of the substrate is presented in Fig.3. Maximum pigment production was observed in 50% of the solid state agro waste products like Banana stalk, Coconut husk, Rice husk and Rice flour. The initial substrate moisture content less than 40% gave less pigmentation, but that of 50–56% could give the highest pigmentation (Johns and Stuart, 1991). Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer process and decrease in initial moisture level results in reduced solubility minimizes heat exchange, oxygen transfer and low availability of nutrients to the culture.

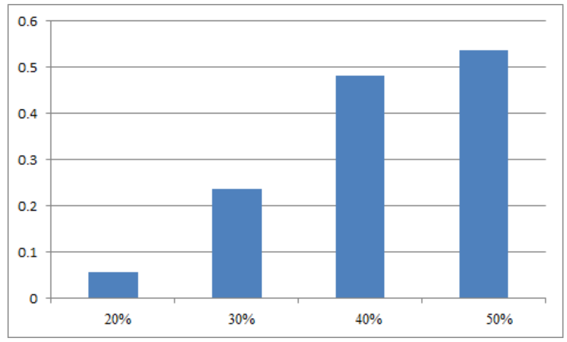

Fig 3: Effect of initial moisture on pigment production

3.3 Effect of pigment production by ph & temperature

In present study, various pH ranges were subjected for pigment production in agro waste and residue. From the spectral analysis (Fig.4.a & b) the maximum absorbance was observed in pH 7.5 & 6.5 at 480nm. For pH 4.5 to 7.5, the agro waste and residue Rice flour, Rice husk, Banana Stalk and Coconut Husk pigment yield was increased. An another observation was made from the Fig.4.c, Effect of various temperatures like 25 °C, 30 °C, 35 °C and 40 °C were studied for the maximum production of antimicrobial compound. The results indicated maximum yield of pigment production is 40 °C the optimum temperature.

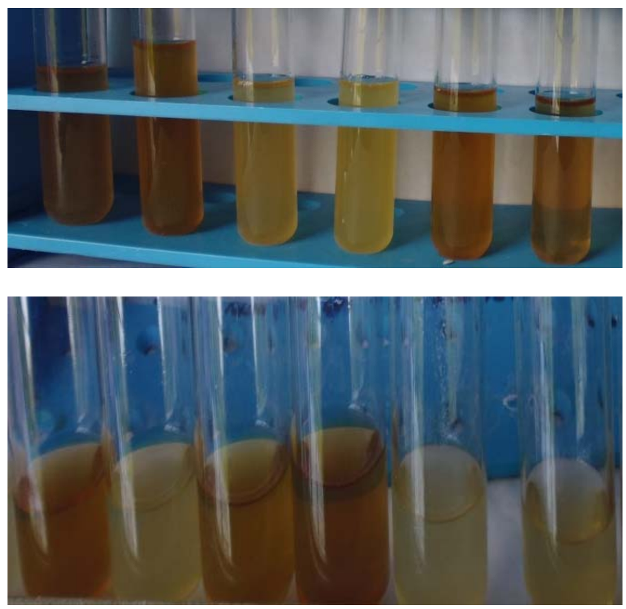

Fig 4a: Effect of pH- 7.5 & 6.5 on pigment production

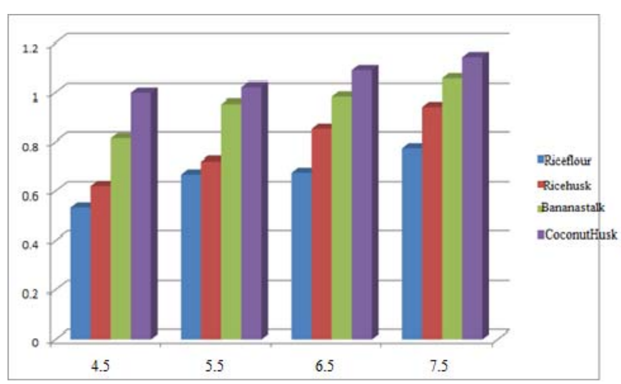

Fig 4b: Effect of pH on pigment production

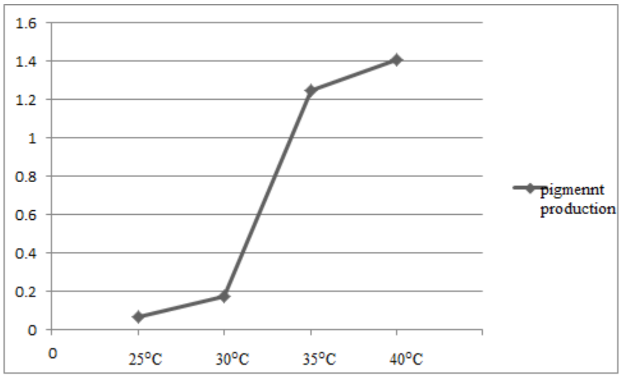

Fig 4c: Effect of temperature on pigment production

3.4 Antioxidant activity of the pigment

The main property of the pigment is ability to antioxidant activity and to trap the free free radical. The standard antioxidant produces peroxide within 24hrs, when the melanin pigment produces peroxide formation for 7 days (Figure 5). This result indicates the antioxidant property of the brown pigment produced strain *DKR4*.

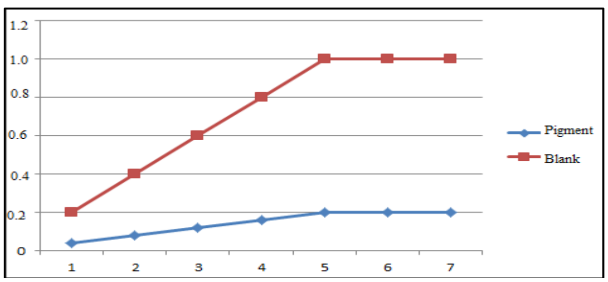

Fig 5: Antioxidant activity of the pigment

4. Conclusion

From this study it was concluded that agro waste and residue could be an alternative option for *Streptomyces griseorubens DKR4* pigment production. The use of Banana stalks; Coconut Husk, Rice Husk and Rice flour substrate was low cost effective and environmental friendly. Maximum production was obtained in solid-state fermentation having initial Moisture content is 50%, pH is 7.5 & temperature 40°C.

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