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Isolation and identification of pigment producing bacteria and characterization of extracted pigments

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Abstract
Bacterial pigments have many applications in current day to day life. The pigments produced by chromobacteria can be used for applications in dairy, pharmaceutical, food etc. In the current study, 03 pigmented bacterial isolates obtained from the soil, were used for pigment extraction and study. It was observed that pigment production was increasing as the temperature increased from 10 ºC and maximum production was seen at temperature of 37 °C in 48 hrs. However on transferring the flasks in refrigerator at 4 ºC, the intensity of pigment was increased. The maximum pigmentation was also observed at *pH* of 6 – 7 and at salt concentrations of 4% and 8%. The extracts were prepared using chloroform as solvent and the absorption maximum of each sample was determined using colorimeter. The pigments were further analysed using Paper chromatography. Antibacterial activity of pigments revealed that they had no inhibitory action against *E coli*, *Pseudomonas sps*, *Klebsiella sps*, *Proteus sps*, *Staphylococcus sps.* Germination against *Vigna radiata* seeds (green moong) revealed that these pigments had no specific effects on the seed germination. Thus the current study can be a useful step for large-scale production of pigments, their purification and application in various industries.

Keywords: Bacterial pigments, chloroform extraction, colorimeter, seed germination

1. Introduction
Color is an integral part of both human culture and in human life. Colors have been used to enhance the aesthetic value of everyday human life. It is the most important characteristic of food, clothes in everyday life. Objective of color in food is to make things appealing and recognizable. Biocolor is any dye obtained from any vegetable, animal, mineral or micro-organism, that is capable of coloring food, drugs, cosmetics or any part of human body. These natural colors come from variety of sources such as seeds, fruits and vegetables, leaves, algae & insects. A suitable natural color can be achieved by keeping in mind the factors such as pH, heat, light, storage conditions and interaction with other ingredients of the formula or recipe. The storage conditions for natural colors depend on the particular need of the product. The biocolors have been classified into 3 types:
1. Natural colors: The natural colors used as additives are the chlorophylls which give green color, the carotenoids which give yellow to red color and the flavonoids with subclass of anthocyanins imparts red to blue color to the flowers and fruits respectively. Recent years have seen the much growing interest in carotenoids, especially β-carotene as it is a direct source of vitamin A in the body and has antioxidant properties.
2. Browning colors: These are the colors produced during cooking and processing and thus may not be of any importance in foods directly. For e.g., sugar caramelization, baking etc.
3. Additives: Colors which are added in food are based on anthocyanins which are derived from natural sources like red grapes or beet but the first additive colors were synthetic dyes. They were named as Vitamins of 21st century as they were extensively used as food colorants and also due to their impressive medical and health benefits. (Sharma D, 2014) [14].
The applications of biocolors in human life are diverse and still growing. The importance of biocolors from plants and microorganisms is gaining importance due to its factors of inhibiting mutagenesis, tumor, photo oxidation and its ability to enhance immune system. Microbial pigments are a promising alternative to other color additives which are extracted from vegetables or animals as they are considered natural, give no seasonal production problems and show a higher productivity. These pigments have been used widely in the field

of pharmaceutical industry, dairy industry, fish industry, printing industry and textile industry (Sun *et al*., 2011) [15].

The various types of pigments produced by microorganisms are carotenoids, melanins, flavins, monascins, violacein and indigo (Duffose, 2009) [5]. Among them all carotenoids are the most widely observed and studied pigments. Carotenoids are nothing but lipid soluble classes of molecules associated with the lipid fractions which are sensitive to oxygen, heat, and light (Ciapara *et al*., 2004) [3]. These pigments have an important function to act as protective agents against oxidative damage (Scolnik *et al*., 1995) [13]. Inhibition of various types of cancers and enhancement of the immune response can be done by carotenoids (Krinsky *et al*., 2005) [10]. Due to their antioxidant activity and pro vitamin A function, they protect us from "life style –related" diseases such as cardiovascular disease and age related macular degeneration (Young *et al*., 2001) [17]. These pigments are also used in maintaining the immunity by maintaining membrane receptors (Bendich, 1989) [1]. In the food industry carotenoids are used as colorants in food to pigment salmon, trout and poultry and also in the identification of egg yolk colour (Johnson *et al*., 1995) [8]. They also have been used in beverages such as orange drinks, confectionary, and other prepared foods (Vandamme, 1989) [16]. Recent study in HIV-infected women reported lower serum concentrations of lycopene, α-carotene, and β-carotene, especially in those with low counts of CD-4 helper cells. Thus, acting as a bioindicator of HIV (Coodley *et al*., 1995) [4]. Usage of synthetic dyes at an increasing rate actually gave rise to the problem of environmental pollution. Due to mismanagement of the synthetic dyes they posed a threat to all the biotic and abiotic factors on the earth. The intervention of these pigments has brought down the problems of toxicity and carcinogenicity.

The market of carotenoids is rising from its last estimated sale to be approximately US$500 million. The increasing health concern among consumers is likely to trigger an increase in the demand for carotenoids in the food industry which is expected to lead to rapid sales in the growth of carotenoids (Johnson *et al*., 1995) [8]. The future prospects of carotenoid market is expected to reach a value of $1.2 billion by 2018, which is in turn driven by the never ending demand from end-use applications such as food, animal feed and pharmaceuticals.

Recent developments in the Molecular biology for carotenoid biosynthesis from organisms that produce different types of carotenoids have provided a variety of genes (Sandman, 1994) [11] which can be used as tools for a completely new strategy of heterologous expression of genes in different host organisms. Proper engineering of microbial pathway enzyme and also strain improvement can produce high amount of carotenoids in industrial processes.

Bacteria are a good source of pigments, with β carotene as the prominent one. Employment of *Streptomyces Chrestomyceticus* subsp. *Rubescens* for lycopene production and *Flavobacterium* sp. for production of zeaxithin and lutein have been gaining importance. (Joshi *et al*, 2003) [9].

The present study was aimed at isolation and identification of bacterial isolates and characterization of extracted pigments from different soil sources, capable of possible commercial importance. Bacterial isolates were further identified through morphological and biochemical characteristics.

2. Materials and Methods

2.1 Sample Collection: About 20 soil samples from different areas of Hyderabad like Hitech city, Kachiguda, Rajmohalla, Sanath Nagar, Bhoiguda, Banjara Hills, Abids etc were used for the isolation of pigment producing bacteria.

2.2 Media: The media used for enrichment and isolation of pigmented bacteria were Nutrient Agar, Nutrient Broth and Mueller-Hinton Agar which were obtained from Himedia, India.

2.3 Isolation and Identification: From the collected soil samples, soil suspensions were prepared using sterile distilled water. Loopful of soil suspension was streaked on sterile nutrient agar plates and the plates were incubated at 37 °C for 24 hrs. Only the pigmented bacterial colonies were selected and sub-cultured on the nutrient agar plates for further studies. These colonies were observed for Gram's nature and morphological characters such as size, shape, color, texture, opacity, elevation, margin and mobility. They were further identified using biochemical methods as stated in Bergey's manual for characterization which includes Indole, Methyl Red, Voges Prauskaeur, citrate, urease, TSI slants etc. Sugar fermentation test was carried out for glucose, sucrose, mannitol and lactose.

2.4 Effect of *pH* and salt of growth and pigment production: The effect of *pH* and NaCl concentration on the pigment production by every bacterial isolate was determined by inoculating the pure cultures in sterile Nutrient broth. For effect of *pH*, sterile nutrient broth with *pH* 2, 4, 6, 7, 8 and 10 was used whereas for effect of NaCl, sterile nutrient broth with 0.5%, 1%, 2%, 4%, 6% and 8% NaCl concentration was used and incubated at 37 ºC for about 24-48hrs. All sets were performed in triplicates. These cultures were plated on sterile Nutrient Agar plates and incubated at 37 °C for 24-48hrs (Bhat *et al*., 2013) [2].

2.5 Extraction of pigments: The pigments were isolated using liquid-liquid extraction method. The cultures were inoculated in 100ml sterile Nutrient Broth flasks. Two sets were prepared; one flask was kept at static condition in incubator at 37 °C and one in rotary shaker with temperature settings of 37 °C at 90rpm. (Goswami *et al*., 2014) [6]. The color change was observed for about seven days. At the end of 7 days incubation, the pigment was extracted by using cold centrifuge with conditions set as 6,000rpm/ 12 ^{0}C/15minutes. The supernatant was collected and pellet was discarded.

Further the pigmented supernatants were mixed with equal quantity of chloroform and they were separated by using separating funnel. The colored supernatant was separated and filtered through Whatman no.1 filter paper (Sasidharan *et al*, 2013) [12].

2.6 Antibacterial Assay: To check if the extracted pigments have any antibacterial property, agar cup method was performed using 5 lab cultures namely *E.coli, Staphylococcus aureus, Proteus, Pseudomonas and Klebsiella* using Mueller Hinton agar.

2.7 Chromatography Analysis

Paper Chromatography

The analysis was used to characterize the pigments based on partition coefficient principle. The Whattman Filter Paper No.1 was used and the solvent system used was Chloroform: methanol (9:1) and methanol (Sasidharan *et al*., 2013) [12].

2.8 Colorimetric Analysis: The pigments were analysed for the detection of λ_{max} by using colorimeter.

2.9 Germination effects: 10 moong seeds (*Vigna radiata)* were used to test the effect of pigments on germination. The seeds were placed on circular whattmans filter paper in a petri plate and 1ml of pigment was added accordingly for 10 days. Everyday analysis on the root and shoot length was done by measuring them.

3 Results and Discussion

Results

3.1 Isolation and Identification

Identification of pigment producing bacteria was carried out by referring Bergey's Manual. Morphological, cultural and biochemical tests were carried out (Table 1, 2 and 3). All the 3 isolates were found to be Gram negative coccobacilli.

Table 1: Gram staining, motility and other cultural characteristics

	Size	Shape	Color	Texture	Opacity	Elevation	Margin	Gram Stain	Motility
Pb1	2mm	circular	Bright yellow	Mucoid	Opaque	Convex	Entire	Gram negative coccobacillus	Non motile
Pb2	3mm	circular	Pale orange/creamy	Smooth	Opaque	Convex	Entire	Gram negative coccobacillus	Motile
Pb 3	2mm	circular	Red	Smooth	Opaque	Convex	Entire	Gram negative coccobacillus	Motile

Key: pb 1 = Pigmented bacteria 1, **pb 2** = Pigmented bacteria 2, **pb 3** = Pigmented bacteria 3

Table 2: Biochemical properties

Sr.No	I	MR	VP	Citrate	TSI				Urease	Catalase	Oxidase
					Butt	Slant	H_2S	Gas			
Pb1	+	-	-	+	Yellow	Red	-	-	-	+	+
Pb2	+	+	-	+	Yellow	Yellow	-	-	-	+	+
Pb3	+	-	-	-	Yellow	Red	-	-	-	+	+

Key: "+" = Positive; **"-" =** Negative; **I =** Indole; **MR =** Methyl Red; **VP =** Voges Prauskaeur

Table 3: Results of sugar fermentation test

Sugar Culture	Mannitol	Fructose	Lactose	Sucrose
Pb 1	Non acid Non gas producing	Acid. Non gas producing	Non acid Non gas producing	Acid. Non gas producing
Pb 2	Non acid Non gas producing	Acid. Non gas producing	Non acid Non gas producing	Acid. Non gas producing
Pb 3	Slightly Acid. Non gas producing	Acid. Non gas producing	Non acid Non gas producing	Acid. Non gas producing

3.2 Effect of *pH* and salt on growth and pigment production

3.2.1 Effect of *pH*

Among the 3 isolates the maximum growth was observed by pb 2 at *pH* 10(Figure No. 1). The intensity in pigmentation for all the 3 isolates remained between *pH* 6-7 (Figure No.1, 2, 3, 4 and 5). It was further observed that the intensity of the pigmentation was increased as the duration of time increases under cold conditions at about 4 ºC (Goswami *et al*., 2010) [7]. In Figure Number 2 and 5 there was an increase in pigmentation observed as the colonies were kept below 10ºC after the incubation period suggesting role of lower temperature in pigment production.

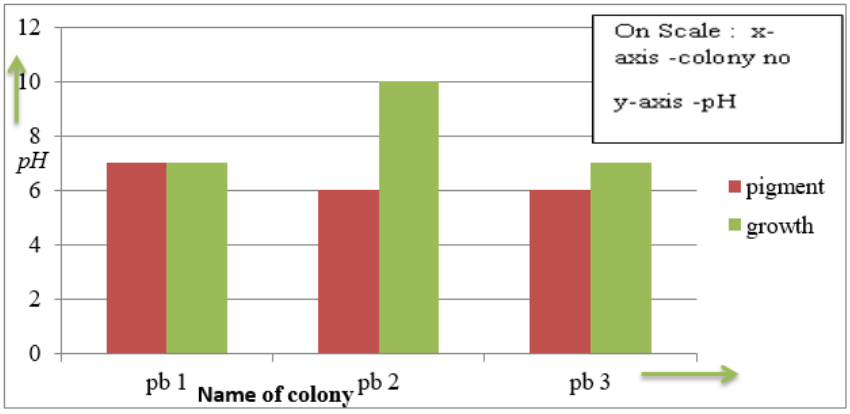

Fig 1: Effect of *pH* on the growth and pigment

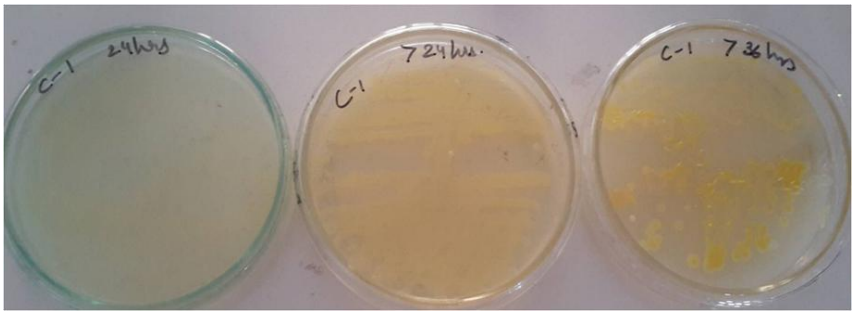

Fig 2: pb 1 (Variation in growth and pigmentation)

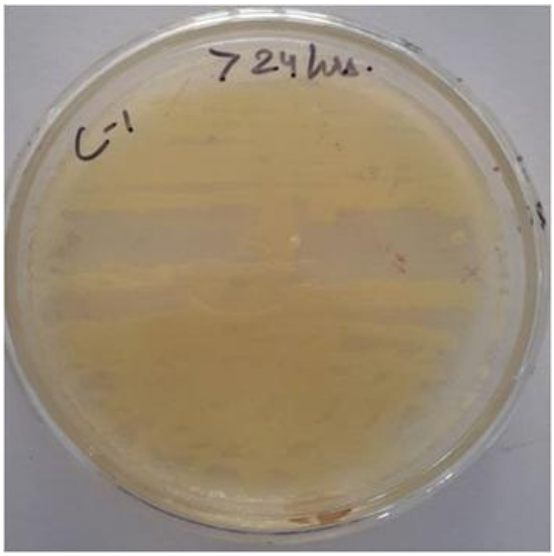

Fig 3: pb 1

Fig 4: pb 3 (Maximum pigmentation)

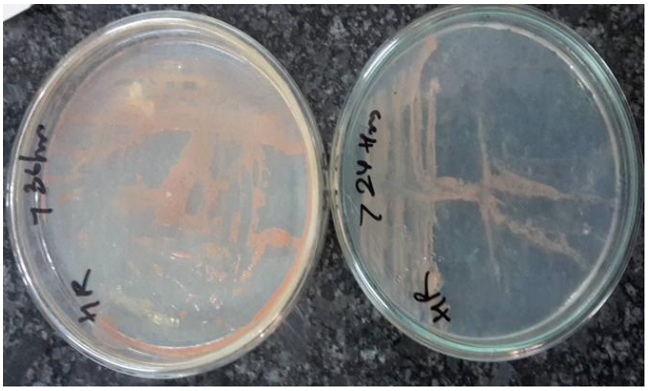

Fig 5: pb 3 (Variation in growth and pigmentation)

3.2.2 Effect of Salt

Among the 3 isolates the maximum pigmentation was observed at 4% for pb 1(Figure No 6) and at 4% and 8% for pb 2(Figure No 7) (Bhat *et al*, 2013) [2]. The growth of pb3 was observed at 0.5% salt concentration.

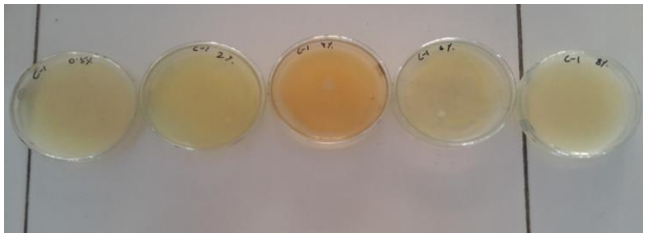

Fig 6: Growth of pb 1 in presence of different salt concentration

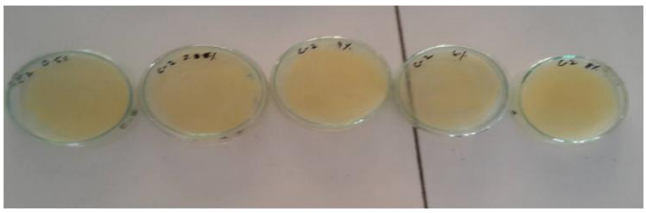

Fig 7: Growth of pb 2 in presence of different salt concentration

3.3 Extraction of pigments

The extraction was carried out by using all the cultures grown at shaker and static (Figure No. 8, 9,10,11,12 and 13). The pigments were extracted by liquid- liquid extraction using chloroform as the solvent. The pb 1 gave a certain bright yellow pigment, pb 2 gave a dull yellow pigment and pb 3 gave a certain red pigment. (Figure No. 14).

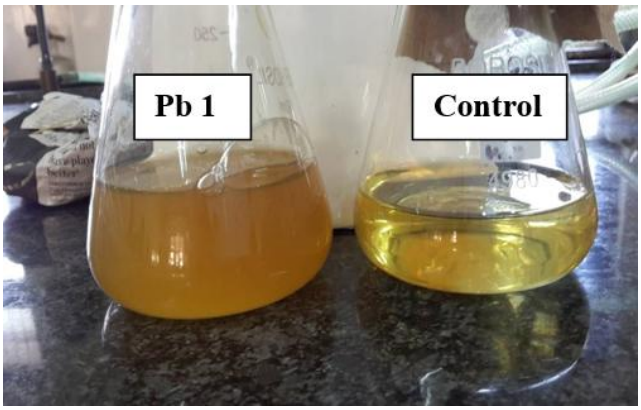

Fig 8: pb 1 Shaker Culture

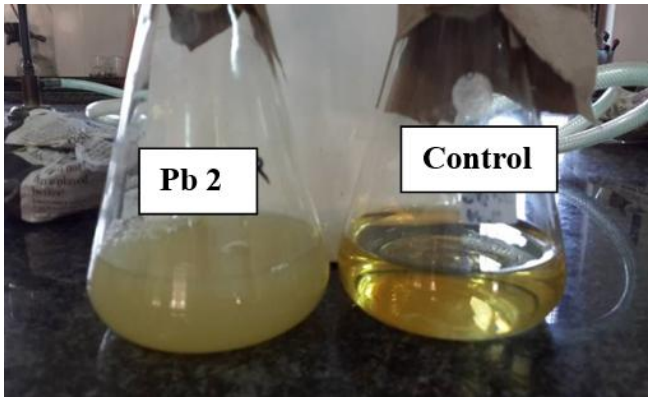

Fig 9: pb 2 Shaker Culture

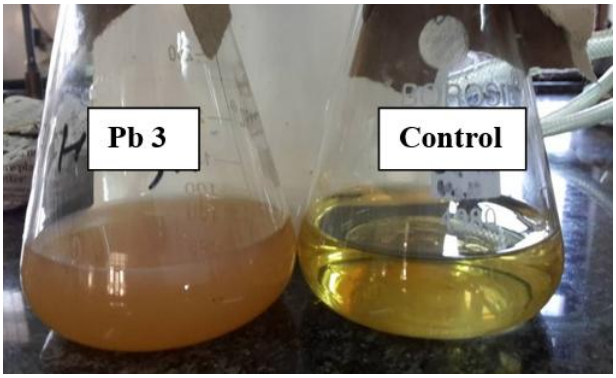

Fig 10: pb 3 Shaker Culture

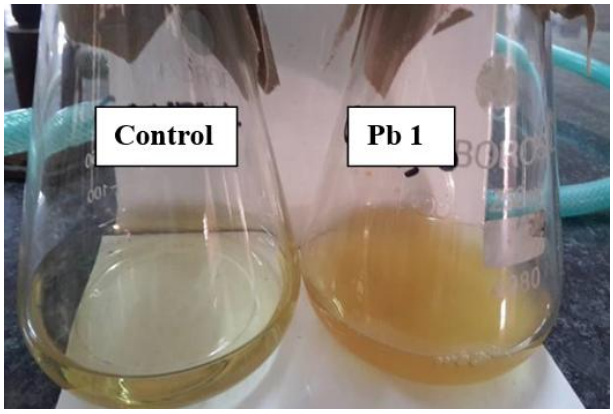

Fig 11: pb 1 Static Culture

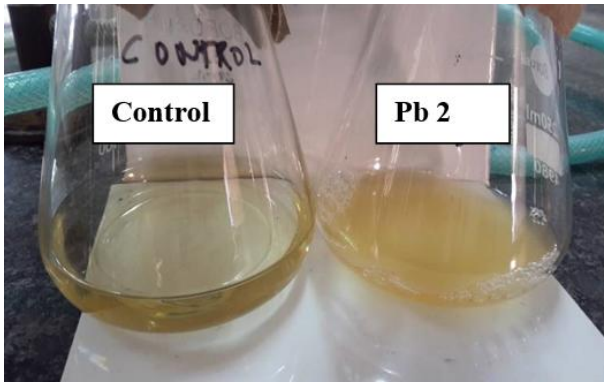

Fig 12: pb 2 Static Culture

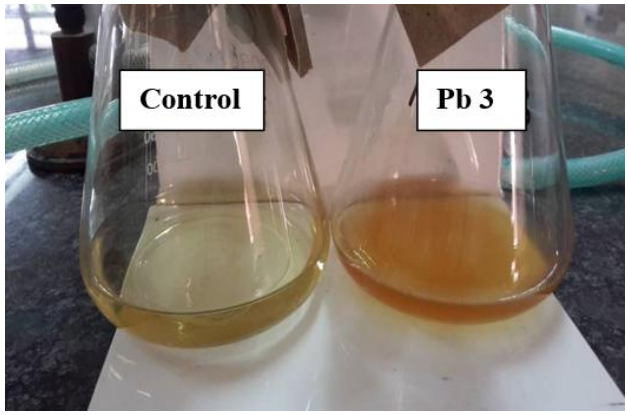

Fig 13: pb 3 Static Culture

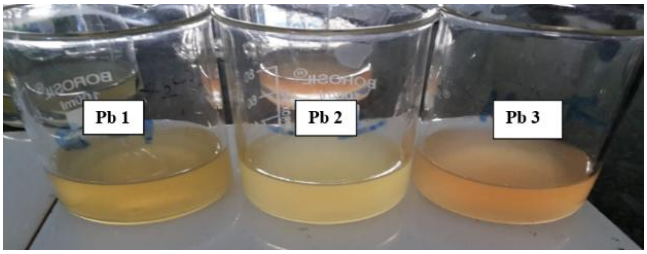

Fig 14: Extracted Pigments

3.4 Antibacterial Assay

The antibacterial assay revealed that there was no zone of inhibition around the wells containing pigments suggesting there was no antibacterial property in the pigments.

3.5 Chromatographic Analysis

Paper Chromatography

The results showed that there was no movement of the pigments suggesting need of other solvent system to be checked

3.6 Colorimetric Analysis

The colorimetric analysis showed that the λ_{max} for each pigment was 440nm (Figure No.15, 16,17,18,19 and 20).

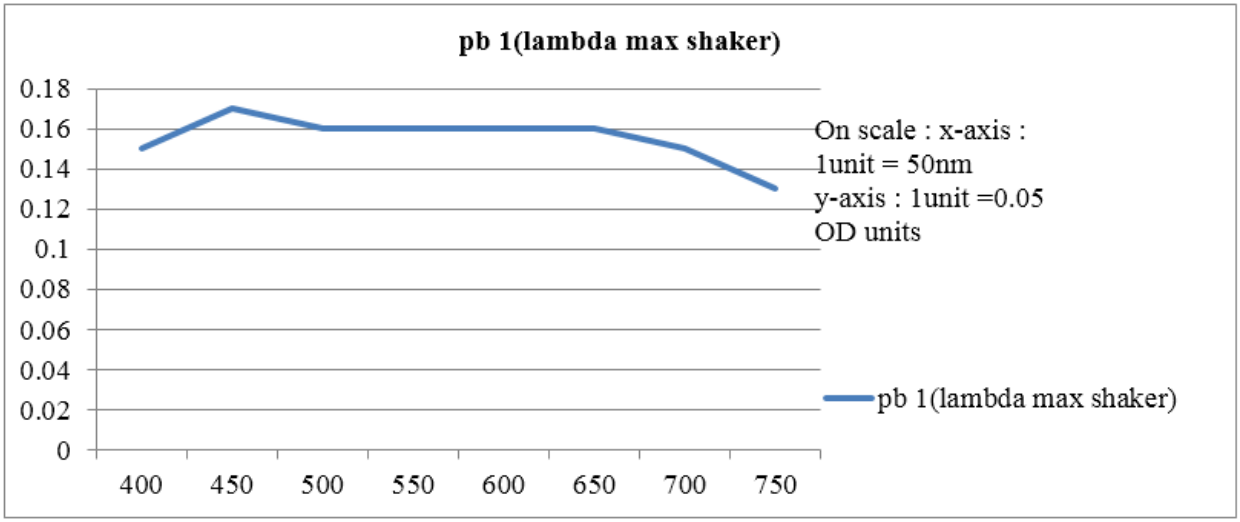

Fig 15: pb 1 λ_{max} shaker

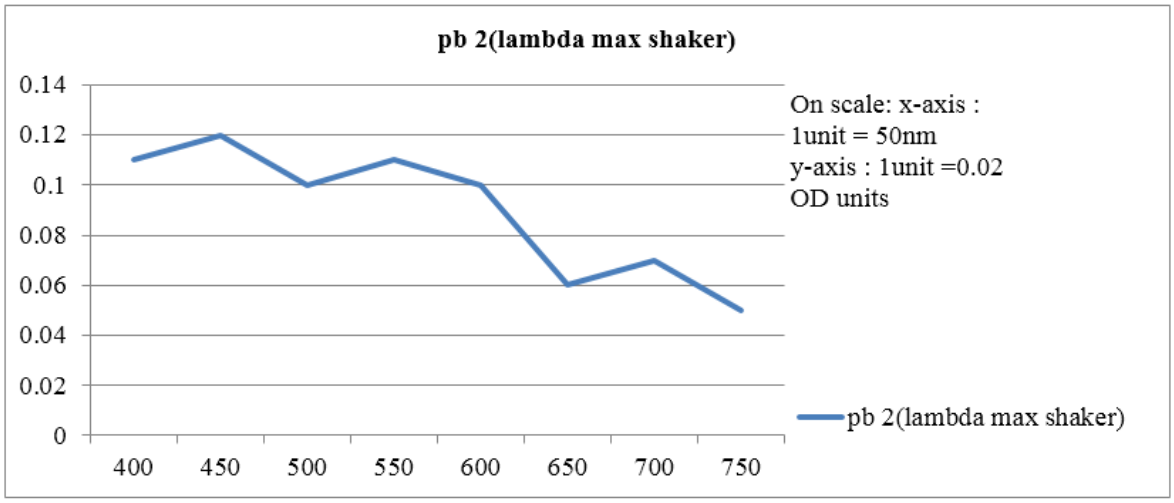

Fig 16: pb 2 λ_{max} shaker

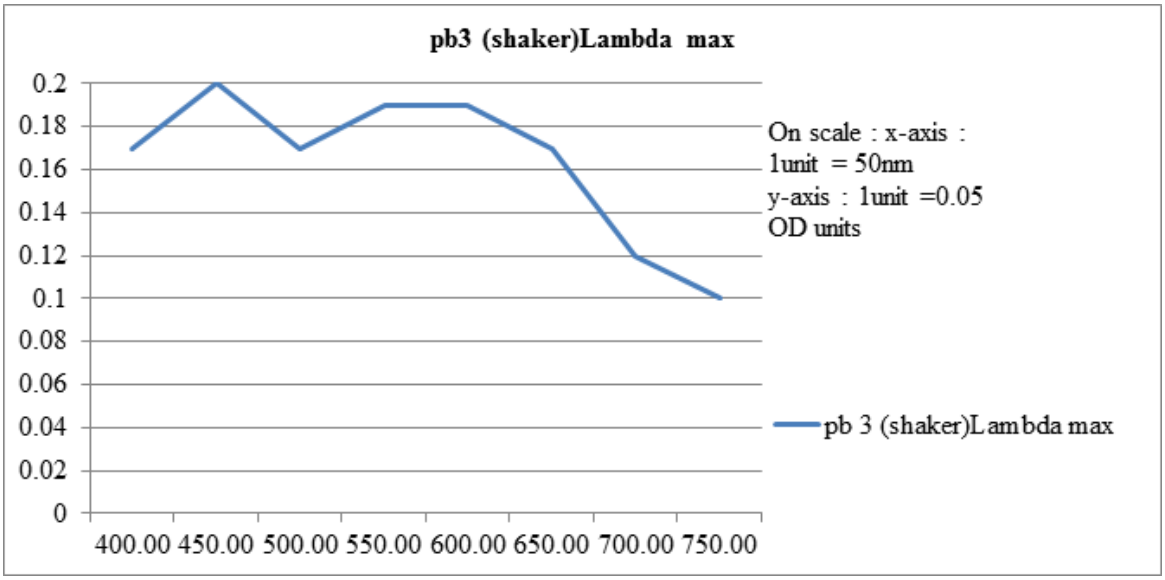

Fig 17: pb 3 λ_{max} shaker

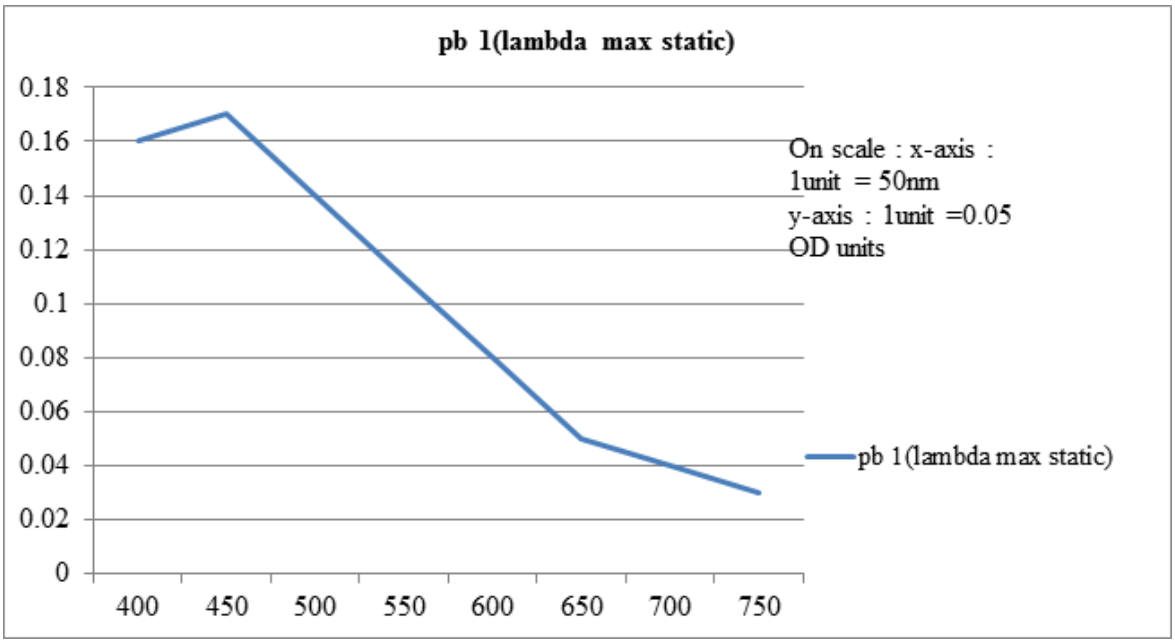

Fig 18: pb 1 λ_{max} static

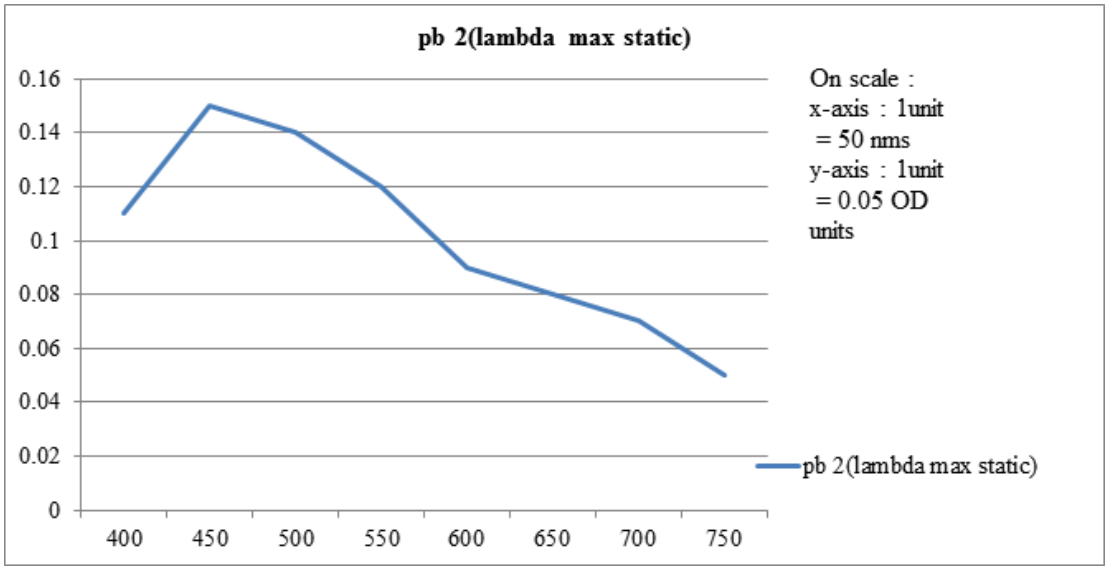

Fig 19: pb 2 λ_{max} static

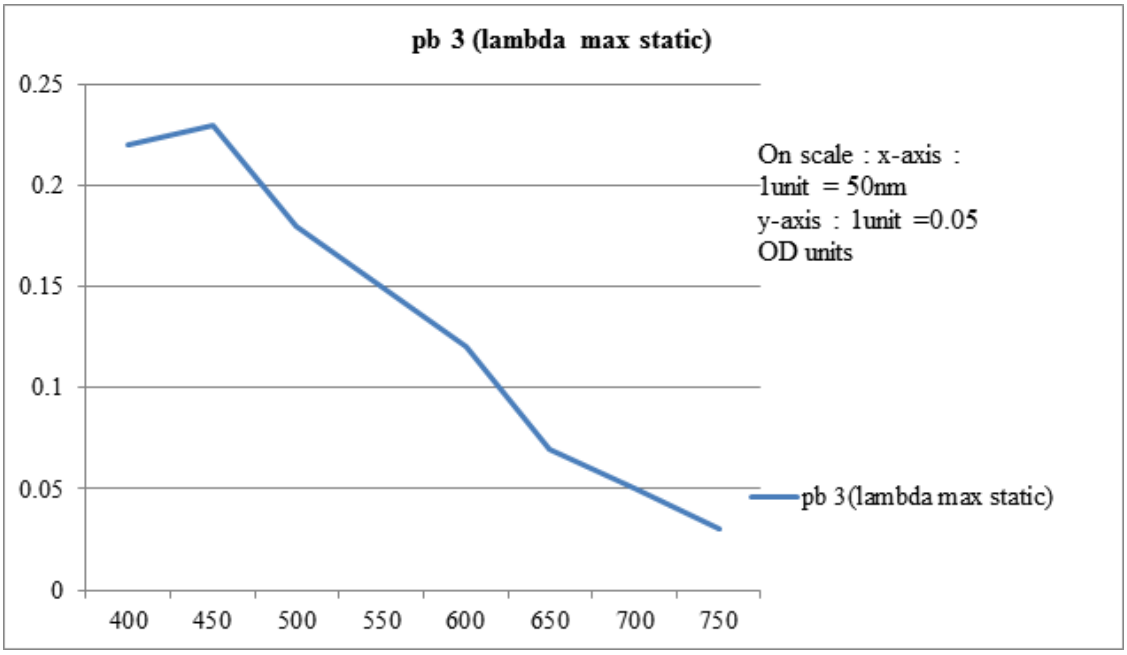

Fig 20: pb 3 λ_{max} static

3.7 Germination effects: The root and shoot length for the *Vigna radiata* seeds were measured. The maximum growth was observed in the pigments kept at shaker conditions. Pb 3 was the only culture which allowed maximum seed germination in both static and shaker conditions. The least was shown by pb 2 isolate in both conditions (Table 4).

Table 4: Germination Effects

Colony	Static /Shaker	Root Length	Average Root length	Shoot length	Average Shoot length
Control	Static	6cm, 10cm, 9cm, 10cm, 11cm, 8cm, 7cm, 9cm, 10cm, 7cm	8.7cm	15cm, 13cm, 9cm, 12cm, 11cm, 11cm, 14cm, 9cm, 10cm, 12cm	11.6cm
Pb 1	Shaker	8cm, 9cm, 10cm, 12cm, 6cm, 5cm, 7cm, 8cm, 9cm,10cm	8.4cm	14cm, 16cm, 9cm, 14cm, 11cm, 13cm, 11cm, 8cm, 10cm, 13cm	11.9cm
	Static	4cm, 5cm, 6cm, 8cm, 5cm, 6cm, 6cm, 7cm, 8cm, 9cm	6.4cm	11cm, 5cm, 14cm, 5cm, 13cm, 8cm, 9cm, 11cm, 13cm, 10cm	9.9cm
Pb 2	Shaker	5cm, 6cm, 8cm, 9cm, 6cm, 7cm, 8cm, 10cm, 9cm, 6cm	7.4cm	15cm, 18cm, 9cm, 16cm, 11cm, 16cm, 14cm, 9cm, 10cm, 17cm	13.5cm
	Static	2cm, 1cm, 3cm, 1cm, 2cm, 4cm, 4cm, 3cm, 2cm, 1cm	2.3cm	4cm, 5cm, 6cm, 4cm, 7cm, 6cm, 8cm, 5cm, 3cm, 2cm	5.0cm
Pb 3	Shaker	6cm, 11cm, 15cm, 16cm, 12cm, 9cm, 8cm, 10cm, 18cm, 16cm	12.1cm	26cm, 22cm, 20cm, 19cm, 18cm, 19cm, 25cm, 24cm, 19cm, 22cm	21.4cm
	Static	4cm, 5cm, 7cm, 3cm, 6cm, 8cm, 5cm, 10cm, 8cm, 9cm	6.5cm	11cm, 10cm, 9cm, 10cm, 12cm, 10cm, 13cm, 8cm, 9cm, 10cm	10.2cm

4. Discussion

From the results obtained it was observed that the 3 bacterial isolates have a tendency to show maximum pigmentation below 10 ºC. During the antibacterial assay there was no inhibition of growth by the pigments which may be due to absence of antibacterial agents or also due to less concentration of the pigments in the solvent. No separate band formation in paper chromatography suggests need of trying more solvent system combinations in different concentration. Germination results revealed that the growth was observed maximum in the case of pigments extracted from isolates kept at shaker. The seeds in presence of pb 2 pigment did not show much growth that may be due to the presence of some anti-growth factors in the pigments.

5. Conclusion

The study performed on bacterial isolates obtained from soil samples showed that they were Gram negative coccobacilli in nature. These isolates showed sensitivity towards *pH* and salt concentrations. Their growth was maximum at neutral *pH* and pigmentation was observed maximum when kept under refrigeration.

The negative results in antibacterial activity showed that they may be fit for human consumption as they do not interfere with the human biota and can have a potential application as food color additive.

The studies carried out on germination properties showed that these pigments are not toxic to plants but rather influence their growth in increasing concentrations. The isolated pigments were known to be of carotenoids as they showed λ_{max} absorbance at 440nm which is the indication for carotenoids. Thus the current study deals with an approach of developing new sources of biocolors from easily cultivated bacterial species that can be further exploited at larger scale.

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