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ISOLATION AND IDENTIFICATION OF RED PIGMENTED BACTERIUM FROM SOIL AND STUDY ON ITS PIGMENT PRODUCTION UNDER PHYSICAL AND CHEMICAL STRESS

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ABSTRACT
Soil is a major source of variety of microorganisms which produce variety of products useful to human beings. One such microbial product is Pigment with variety of bioactive properties such as antimicrobial, anticancer, anti-oxidant *etc*. Isolation was carried out from soil samples using concentration method on Nutrient agar medium containing 2% glycerol. Red pigmented bacterial colony was selected and purified. All characteristics such as morphological, colonial and biochemical were determined. For confirmation isolate was subjected to molecular identification.The isolate was found to be *Vibrio rhizosphaerae.* Then cultivation was carried out and pigment was extracted using organic solvents methanol and chloroform. After extraction of pigment absorption maxima (300-800 nm) was measured using UV-Vis spectrophotometer with methanol as blank. The highest absorption was observed to be at 535 nm like Prodigiosin. The degree of pigmentation was measured for the isolate was found to be 1.38. The effect of UV irradiation as well as Ethyl methane sulfonate (EMS) was also determined. In case of UV irradiation, no growth was observed after UV treatment that indicated that bacterium is UV susceptible and in case of EMS treatment bacteria could grow and produce pigment. The degree of pigmentation was again measured for EMS treated cultures and it was found that high EMS (*i.e*. above 1.5 %) concentration can be useful for hyper pigmentation. The newly formulated medium was checked for pigment production and it was found to be more suitable giving 1.41 degree of pigmentation.

KEYWORDS: Vibrio rhizosphaerae, red pigment, UV irradiation, Ethyl methane sulfonate, Degree of pigmentation.

INTRODUCTION

Soil possesses rich microbial diversity. It is an environment where microorganisms interact in both positive as well as negative way. Different microorganisms produce different substances which are helpful to human being one such substance is pigment. Synthetic dyes have detrimental effects if they persist in environment for longer period. Natural pigments are safe hence they are more in demand. Moreover, they can be easily obtained from microorganisms without any difficulties such as seasonal variations (Malik, 2012). Pigments are secondary metabolites and not found in all types of organisms (Yokoyama and Miki, 1955). Microorganisms produce variety of pigments such as carotenoids, flavin, melanin, quinones, prodigiosin, violacein, indigo etc. which possess bioactive properties (Chidambaram *et al*., 2013). Over the past decades various researches have been carried out by scientists to prove bioactive properties of pigments. Advancement has made many ways to change their characteristics by use of mutagenic agents. Sometimes the effect of mutation on bacteria can have positive effect which humans can exploit for betterment of society. Various parameters such as p^H, temperature, aeration rate, moisture content, carbon source, nitrogen source, minerals, *etc*. can be optimized for better growth and pigment production. Moreover, strain improvement methods by physical and chemical mutagenesis and gene manipulation can be applied for high yield of products. For physical mutagenesis gamma-rays, x-rays and ultraviolet irradiation can be used. Ethyl methane sulfonate, nitroso methyl guanidine can be used for chemical mutagenesis. For all organisms, there are not identical mutagens and conditions which give best results, instead they can only be found by trial and error (Sreeju *et al*., 2011). One such approach of pigment production from soil bacterium was carried out by us. The isolated bacterium was red pigment producing and was identified as *V rhizospharae.*

MATERIALS & METHODS

Isolation of red pigmented bacterium

Various soil samples were collected in sterile containers from different locations of Valsad city. Soil samples were collected from surface as well as from the depth. Composite soil samples were prepared by mixing 4 to 5 different samples collected from single location (Rashid *et al*., 2014). Samples were inoculated on nutrient agar plates containing 2% glycerol by spread plate method. Plates were incubated at 28˚C for 24-48 hours.

Screening and purification of isolate

Plates were screened for red pigmented bacterial colony. A single pigmented colony was selected and streaked on another sterile nutrient medium for purification purpose. Once isolate was obtained in pure form its colonial

characteristics were noted down and it was subjected to Gram's staining and motility procedure. And pure isolate was transferred on sterile nutrient agar slants with 2% glycerol and stored at refrigerated temperature.

Biochemical tests

Various biochemical tests were carried out for selected isolate using Biochemical kits (HiMedia). Inoculation and interpretation of tests and test results were done by following instructions given in test kit literature.

Molecular Identification of selected dark pigmented isolate:

The cultures were sent to Saffron Life science laboratory Bilimora for molecular identification. DNA was isolated from the culture and its quality was evaluated on 1% Agarose Gel. Fragment of 16s rDNA gene was amplified by PCR. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 8F & 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730x1 Genetic Analyzer. The 16s-rDNA sequence was used to carry out BLAST with the database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Production and extraction of pigment:

The isolate was grown in 250 ml flask containing 100 ml of nutrient broth with 2% glycerol at 28˚C at 120 rpm for 3 days. After cultivation cells were separated by centrifugation at 3000 rpm for 20 minutes. The pellet and supernatant were separated in two different clean tubes. Pellet was washed with sterile distilled water and was centrifuged and collected. Methanol was added to cell pellet and vortexed and centrifuged to get colored supernatant. The step was repeated until the pellet turned colorless. Then the previously separated supernatant was mixed with equal volume of chloroform and centrifuged to get separate layers. The colored layer was collected in clean centrifuge tube and centrifuged. After centrifugation, the colored pellet was washed with distilled water and pigment was extracted using methanol. The methanol extract of the pigment was then filtered and collected in clean glass bottle. The λmax was measured using UV-Vis spectrophotometer of extracted pigment (Modified method Manish *et al*., 2015).

Degree of Pigmentation calculation:

The degree of pigmentation was measured by Ratio of OD of pigment extract to OD of cell growth (Boontosaeng, 2016).

Degree of Pigmentation= $OD_{\lambda max}/OD_{660}$

Effect of UV irradiation:

Cells were grown in 50 ml of standard broth medium (Nutrient broth with 2% glycerol) at 28˚C for 18 hours under shaking condition (120 rpm). Cells were harvested by centrifugation at 3000 rpm for 20 minutes. Then pellet was suspended in sterile saline and vortexed for 20 seconds. Then suspension was transferred into sterile empty petri plate and exposed under prewarmed UV lamp which was placed 15 cm above the surface. 1ml of irradiated solution was withdrawn after 10, 20, 30, up to 120 seconds and serially diluted and plated on standard agar medium (Nutrient agar with 2% glycerol). Non-exposed cells were taken as control. Plates were incubated in dark to avoid photo reactivation for 72 hours and screened for colonies with hyper pigmentation. An isolated colony was selected and inoculated in nutrient broth and incubated at 28˚C for 3 days. After incubation cells were harvested and pigment was extracted by following the procedure described above and absorbance of extracted pigment was measured and compared with non-treated culture (El-Bialy Abou El-Nour, 2014).

Effect of Ethyl Methyl Sulfonate (EMS):

Cells were grown in 50 ml of Nutrient broth medium with 2% glycerol. Cells were collected, washed and resuspended in 50 ml phosphate buffer (pH-7). Then from this 2 ml of solution was transferred to a screw capped tubes containing different concentration of EMS (0, 0.1, 0.2, 0.3 up to 0.5 and 1, 1.5 %). The solution was vortexed and placed on rotating platform and incubated for an hour at 28˚C after an hour the reaction was terminated by adding 200 µl of 5 % sodium thiosulfate (inactivates the mutagenic agent). Then EMS treated cells were harvested and diluted by serial dilution and plated on nutrient agar medium with 2% glycerol. Plates were incubated at 28˚C for 3 days and screened for hyper pigmented colonies. An isolated colony was selected and inoculated in nutrient broth and incubated at 28˚C for 3 days. After incubation cells were harvested and pigment was extracted by following the procedure described above and absorbance of extracted pigment was measured and compared with non-treated culture (El-Bialy Abou El-Nour, 2014).

Production and extraction of pigment in Peptone Yeast Extract Glycerol (PYG) medium:

The isolate was tested for growth and pigment production in another formulated medium. The medium was prepared by substituting beef extract by yeast extract and glucose was added which was absent in medium used originally. Moreover, Salt concentration was changed to 2%. Cultivation conditions were same and extraction procedure was same as stated earlier.

RESULTS & DISCUSSION

Various coloured bacterial colonies were obtained out of which dark red pigmented colony was selected and purified as shown in Fig.1. The dark red pigmented colony was obtained from the soil sample collected from salted soil sample from beach which was covered with Mangroves. The finding of our study matched with the one result that also represented the presence of this type of bacterium from area covered with mangroves (Natrajan R., and Nair S., 2009). Morphological, colonial characteristics are shown in Table 1.

FIGURE 1: Red pigmented isolate

TABLE 1. Characteristics of red pigmented isolate

Isolates	Characteristics		
	Colony	Gram reaction	Motility
Red Pigmented	Small, round, smooth, entire, slightly raised, opaque, red	Gram negative rods, occurring singly or in chain	motile

Biochemical Tests Results:
Biochemical test results are shown in Table 2.

TABLE 2. Results of Biochemical tests

Sr.No.	Tests	Result of isolate	Result from Bergey's Manual of Systematic Bacteriology	Result from Reference paper
1	ONPG	-	+/-	NS
2	Lysine utilization	+	+/-	+
3	Ornithine utilization	+	+/-	+
4	Urease	-	-	NS
5	Phenylalanine deamination	-	-	NS
6	Nitrate reduction	-	+/-	-
7	Hydrogen sulphide production	-	-	NS
8	Citrate utilization	-	+/-	NS
9	V-P	-	+/-	+
10	MR	-	+/-	NS
11	Indole	-	+/-	-
12	Malonate utilization	-	-	NS
13	Esculin hydrolysis	-	-	NS
14	Arabinose	-	+/-	NS
15	Xylose	-	+/-	+
16	Adonitol	-	-	*
17	Rhamnose	-	+/-	NS
18	Cellobiose	+	+/-	+
19	Melibiose	+	-	+
20	Saccharose	-	+/-	NS
21	Raffinose	-	+/-	NS
22	Trehalose	-	+/-	*
23	Glucose	+	+	+
24	Lactose	-	+/-	+
25	Oxidase	-	+/-	-
26	Maltose	+	+/-	NS
27	Fructose	-	+/-	NS
28	Dextrose	+	+/-	NS
29	Galactose	+	+/-	+
30	Sucrose	-	+/-	+
31	L-Arabinose	-	+/-	NS
32	Mannose	+	+	+
33	Inulin	+	+/-	NS
34	Sodium gluconate	-	+/-	NS
35	Glycerol	-	+/-	NS
36	Salicin	+	+/-	NS

37	Dulcitol	-	-	*
38	Inositol	-	+/-	*
39	Sorbitol	-	+/-	*
40	Mannitol	+	+	+
41	Arabitol	-	+/-	NS
42	Erythritol	-	-	NS
43	Alpha -Methyl- D- glucoside	-	-	NS
44	Melezitose	-	+/-	NS
45	Alpha- Methyl-D-mannoside	-	+/-	NS
46	Xylitol	-	+/-	NS
47	D-Arabinose	-	+/-	NS
48	Sorbose	-	+/-	NS
49	Catalase	+	+	+
50	Amylase	+	+	+
51	Gelatinase	+	+	+
52	Lipase	+	+	+
53	Caseinase	+	+	+

* indicates positive result only in specific conditions. NS-not specified

The biochemical test results were compared with description of *V rhizosphaerae* given in research paper and Bergey's Manual of Systematic Bacteriology. Almost all characteristics were matched (Natrajan R. and Nair S., 2007).

Cultivation of isolate:
Colour change of the broth medium can be observed in Fig. 2 which indicates growth and pigment production.

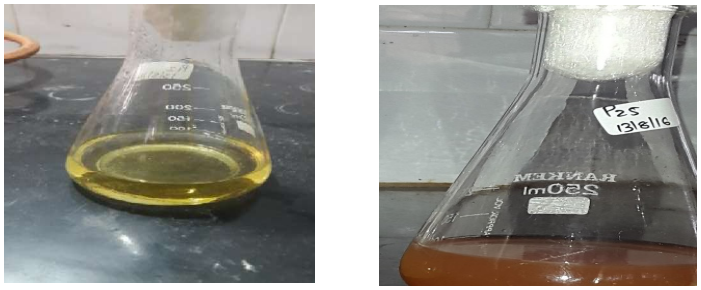

FIGURE 2: Cultivation and Pigment Production

Extraction, Absorption spectrum and Degree of Pigmentation:
The suitable solvent for pigment extraction was methanol. The extracted pigment is shown in **Fig.3.**

FIGURE 3: Extracted pigment

The λ_{max} was found to be 535 nm like Prodigiosin (Ahmed e*t al*., 2012). The degree of pigmentation was measured to be 1.38.
Degree of Pigmentation= $OD_{\lambda max}/OD_{660}$
Degree of Pigmentation= 1.11/0.80 =1.38

Effect of UV irradiation
Growth was inhibited after UV irradiation. Not a single colony was observed on any plate. The experiment was duplicated and similar results were obtained in each experiment. This shows that UV rays inhibit pigment production as well as growth of *V rhizosphaerae*. With this reference method, El-Bialy Abou El-Nour, 2014 used *Serratia marcescens* obtained positive results with hyper pigmented mutants which are contradictory with this presented work. It might be due to different bacterium that is susceptible to UV irradiation which causes lethal and unrepairable mutations.

Effect of EMS
The growth was observed after EMS treatment and absorbance of pigment and cell mass are shown in figures 4 and 5**.**

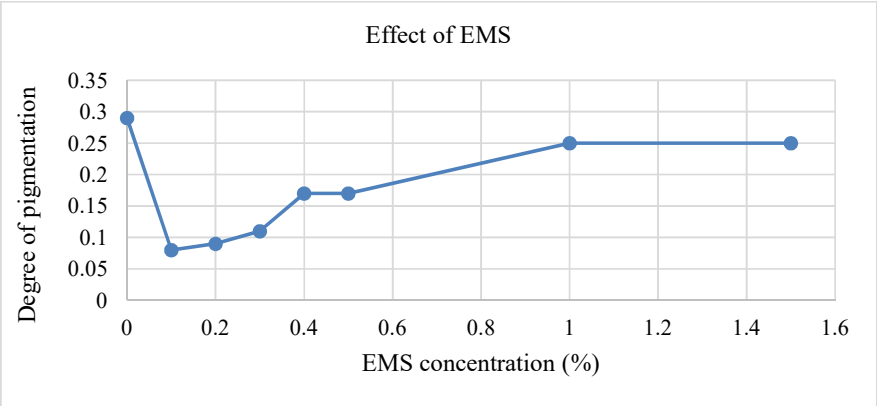

FIGURE 4: Graph of degree of pigmentation against EMS concentration

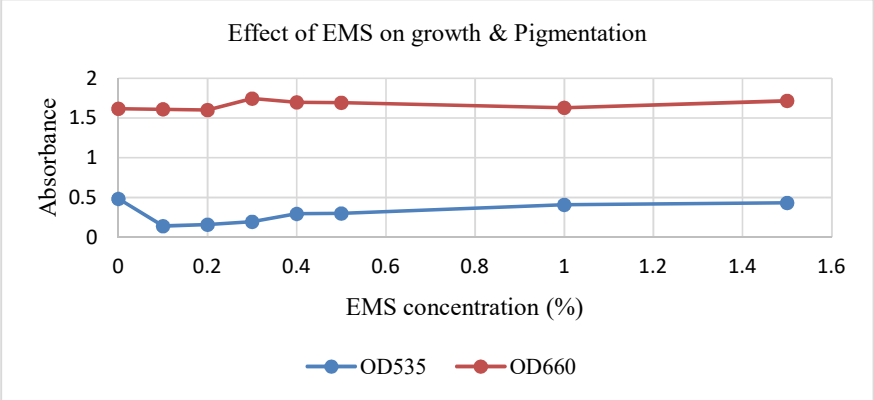

FIGURE 5: Graph of absorbance of growth and pigment of isolate against EMS concentration.

As from the graph EMS concentration increases growth as well as pigment production also increases. So, by raising the EMS concentration beyond 1.5 % might give useful results. In case of chemical mutagenesis also El-Bialy Abou El-Nour, 2014 obtained positive results with hyper pigmented mutants which can be seen in this study that even with *V rhizosphaerae* high EMS concentration treatment can yield hyper pigmented mutants.

Production and extraction of pigment in Peptone Yeast Extract Glycerol (PYG) medium*:*

The increased growth and pigment was produced using this newly formulated medium. The growth can be observed in Fig 6. Moreover, the time for growth and pigment production was minimised using this new medium. It took only 24 hrs. for growth and pigment production while in nutrient broth with 2% glycerol it took 48 to 72 hrs. The degree of pigmentation was measured to be 1.41.

FIGURE 6: Growth in PYG medium

Degree of Pigmentation= ODλmax/OD660
Degree of Pigmentation= 2.594/1.830 =1.41.

CONCLUSION

From the study, it can be concluded that *V rhizosphaerae* can be used for pigment production. It is UV susceptible and can tolerate EMS high concentration so that by manipulation it can be used for hyper pigment production. Moreover, it can be tested for various other applications.

It can be assumed that glucose and yeast extract have positive influence on both growth and pigment production of isolate. Different amino acid composition may influence the growth and pigment production of an isolate as might be assumed from the better result with yeast extract rather than beef extract.

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