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Research Article

Screening and isolation of yellow and orange pigment producing bacteria from different environmental samples

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Abstract

The environment is a treasure for a variety of life forms with characteristics they use for various purposes. One such living form is pigmented bacteria which can produce different types of colourful substances with bioactive properties. The present study aims to screen and isolate such bacteria by exploring different environmental locations. Totally 40 samples were collected and processed for screening of pigmented bacteria. From these samples, 37 yellow and orange pigmented bacteria were collected. Out of 37 bacteria 10 isolates were studied for colonial, morphological characteristics. Among the 2 isolates, one orange (P5) and one yellow (P1) were processed further for various enzymatic tests and molecular identification. The orange was identified as *Rhodococcus corynebacterioides* and yellow was identified as *Micrococcus aloeverae.* Both the isolates were cultivated to produce pigment and checked for different organic solvents to extract pigments. Methanol, acetone and chloroform were tested and methanol together with chloroform was suitable for P5 and only methanol was suited for P1. Thus, it can be concluded that various marine sources can be used for yellow and orange pigmented bacteria and it can be further studied to get better production of pigments by applying various strategies. Moreover, various bioactive properties can also be measured.

Keywords : Bacteria, *Rhodococcus corynebacterioides, Micrococcus aloeverae*, yellow pigment, orange pigment and Solvent extraction.

Introduction

The environment has great diversity which can be explored efficiently with different means to get better life. In case of microorganisms the diversity is maximum and yet to be known by us. There are varieties of bacteria that produce pigments which can be known as chromobacteria (Chandran *et al.,* 2014). The diversity is not limited to bacteria only, it is also seen in pigments produced by them. Different bacteria can produce different pigments such as prodigiosin, carotenes, violacein, phenazine, quinones, tambjamines, melanins and other pigments such as scytonemin and glaukothalin (Nagrajan and Sampath, 2013). These pigments are synthesized as secondary metabolites and not often found in all types of bacteria (Yokoyama and Miki, 1955). Pigments are beneficial to bacteria in providing protection against UV radiation and antibiotics, help in photosynthesis, pathogenesis (George *et al.,* 2005; Abboud *et al.,* 2013; Rashid *et al.,* 2014). Surprisingly, bacterial pigments were proven to have health benefits for which they can be used

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in pharma and food industries. Moreover, they can be also used in textile, cosmetics and paint industries.

Materials and Method

Sample collection

40 soil and water samples were collected from different locations such as fresh water, marine water, garage soil, agricultural field soil, beach sand, rhizosphere soil, etc. 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 a single location. Water samples were collected from the shore and regions away from shore (Rashid *et al.,* 2014). Liquid samples were collected in clean bottles and soil samples were collected in polythene bags. Samples were processed on the day of collection and if required were stored at refrigeration temperature.

Screening and isolation of pigmented bacteria

The samples were subjected to concentration method in which soil sample was mixed in sterile distilled water and centrifuged. The supernatant was collected in another centrifuge tube and centrifuged. Then sediments were used to inoculate agar medium, so that more number of colonies can be obtained. The media of choice for isolation procedure was nutrient agar with 2% glycerol. After inoculation plates were incubated at room temperature for 3 to 5 days. Plates were observed on each day for pigmented bacterial colonies. Plates were screened for yellow and orange colonies.

Purification and Morphological characterization of isolates

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. Pure isolates were transferred on sterile nutrient agar slants with 2% glycerol and stored at refrigeration temperature.

Determination of enzyme profile

The selected bacteria were subjected to testing different enzyme production tests.

Catalase Test

Bacteria were inoculated on a nutrient agar slant containing 2% glycerol and incubated at 28°C for 48 hr. After incubation 3% H_2O_2 was added to the growth of bacteria and checked for effervescence.

Oxidase Test

Bacteria were inoculated on a nutrient agar slant containing 2% glycerol and incubated at 28°C for 48 hr. After incubation, growth was taken and added on to oxidase disc and checked for colour change.

Amylase Test

Bacteria were inoculated on starch agar medium and incubated at 28°C for 48 hr. After incubation plate was flooded with iodine to check the clear zone around the growth of bacteria.

Caseinase Test

Bacteria were inoculated on milk agar medium and incubated at 28°C for 48 hr. After incubation plate was observed in clear zone around the growth of bacteria.

Gelatinase Test

Bacteria were inoculated on gelatine agar medium and incubated at 28°C for 48 hr. After the incubation, plate was flooded with Frazier's reagent to check the clear zone around the growth of bacteria.

Lipase Test

Bacteria were inoculated on tributyrin agar medium and incubated at 28°C for 48 hr. After incubation, the plate was observed to check the zone of hydrolysis around the growth of bacteria.

Urease Test

Bacteria were inoculated into Stuart's urea broth medium and incubated at 28°C for 48 hr. After incubation, the medium was observed for colour change.

Molecular Identification

Molecular identification was carried out at Saffron Life sciences Laboratory, Bilimora, Gujarat. DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. A Fragment of 16S rDNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. The DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA sequence was used to carry out BLAST with the database of NCBI genebank databases. Based on the maximum identity score, first ten sequences were selected and aligned using multiple alignment software programs.

Production and Extraction of pigment

The P1 culture was inoculated in 50 ml nutrient broth medium in 250 ml flask and incubated on shaker (120 rpm) at 28°C for 3 days. Once the growth was obtained, the cell pellet was separated by centrifugation and washed with distilled water and collected in clean tube. After washing 3 different organic solvents (Methanol, Acetone and Chloroform) were checked for pigment extraction from cells (Ahmed *et al.,* 2012). The same was carried out for culture P5. The excess lipid was removed by chloroform treatment so that it cannot interfere with the drying process of pigment.

Comparative study

The comparative study between yellow and orange isolates was done in context to their sources, Gram reaction and motility. Also, the approximate comparison was made between pigmented and non-pigmented bacteria from each sample.

Result and Discussion

Sample collection

List of samples collected from various environments are given in the Table – 1.

Table - 1. List of environmental samples

S. No.	Source	No. of samples
1	Marine water	8
2	Marine soil	2
3	River	5
4	Pond	2
5	Soil	15
6	Air	4
7	Fish	1
8	Skin	1
9	Plant leaf	2

Screening and Isolation

Each sample was processed and screened for isolation of pigmented bacteria. It was observed that numbers of pigmented bacteria were found less than non-pigmented bacteria. Table 2 shows the number of types of pigmented and non-pigmented bacteria. Different types of yellow and orange coloured bacteria are presented in Figure -1.

Purification and morphological characterization

Totally 37 yellow and orange pigmented bacteria were obtained. Figure 2 represents the percentage of yellow bacteria compared to orange bacteria out of total 37 isolates. Isolates were obtained in pure form by four flame streaking method. All isolates were given code "P" with series numbers. Figure 3 represents some of the plates having isolates in pure form.

Colonial characteristics

All isolates were small to medium in size. Out of total 37 only 5 yellow and 5 orange isolates

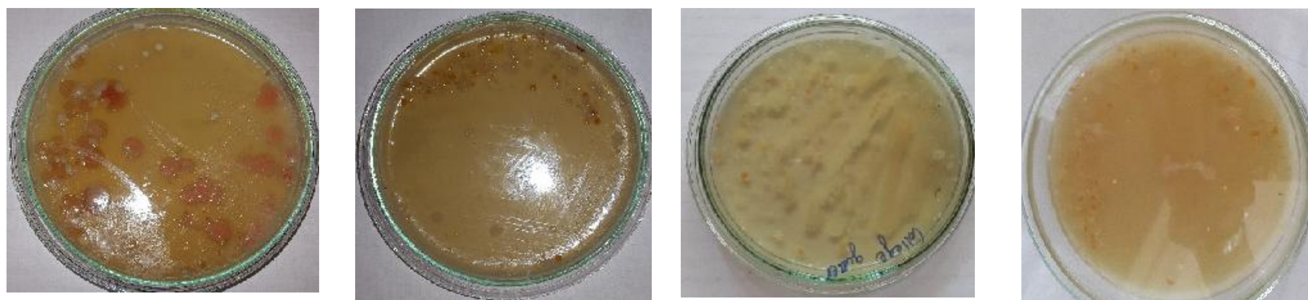

Fig. -1 Nutrient agar plates having different pigmented isolates.

Table - 2. Number of types of pigmented and non-pigmented colonies

Sl. No.	Source	Pigmented colonies		Non-pigmented colonies
		Yellow	Orange	
1	Marine water	5	3	10
2	Marine soil	2	0	6
3	River	3	1	12
4	Pond	2	1	3
5	Soil	9	4	19
6	Air	2	2	5
7	Fish	0	1	1
8	Skin	0	1	3
9	Plant leaf	1	0	5

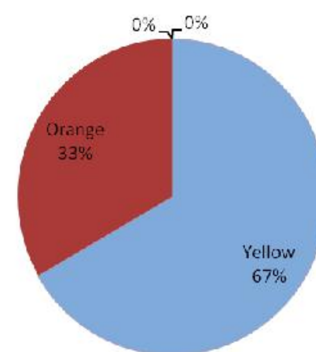

Fig.-2. Percentage of yellow and orange isolates out of total isolates

Table – 3. Colonial characteristics of selected isolates

Sl. No	Code	Source	Size	Shape	Surface	Edge	Elevation	Opacity	Pigmen-tation
1.	P1	Marine Water	Small	Round	Smooth	Entire	Convex	Opaque	Yellow
2.	P3	Marine Water	Small	Round	Smooth	Entire	Slightly raised	Translucent	Yellow
3.	P19	Soil	Small	Flower	Rough	Irregular	Raised	Opaque	Yellow brown diffusible
4.	P21	River	Small	Round	Mucoid	Entire	Slightly raised	Translucent	Yellow
5.	P36	Soil	Small	Round	Smooth	Entire	Flat	Opaque	Yellow
6.	P5	Marine Water	Small	Round	Smooth	Entire	Convex	Opaque	Orange
7.	P7	Marine Water	Small	Round	Mucoid	Entire	Flat	Translucent	Orange
8.	P12	Soil	Medium	Round	Mucoid	Entire	Raised	Opaque	Orange

9.	P18	Soil	Medium	Irregular	Rough	Irregular	Raised	Opaque	Orange
10.	P26	Pond	Small	Round	Mucoid	Entire	Slightly-raised	Trans-lucent	Orange

Table – 4. Gram's reaction and motility

Sl. No.	Code	Gram's Reaction	Motility
1.	P1	Gram positive cocci tetrads, cluster or single	Non-motile
2.	P3	Gram negative short rods, single	Motile
3.	P19	Gram positive filamentous	Non-motile
4.	P21	Gram negative short rods, single	Motile
5.	P36	Gram positive short rods, single	Non-motile
6.	P5	Gram positive cocci, single	Non-motile
7.	P7	Gram positive rods	Motile
8.	P12	Gram negative rods, single	Non-motile
9.	P18	Gram positive rods single or in chain	Motile
10.	P26	Gram positive rods, single, pair	Motile

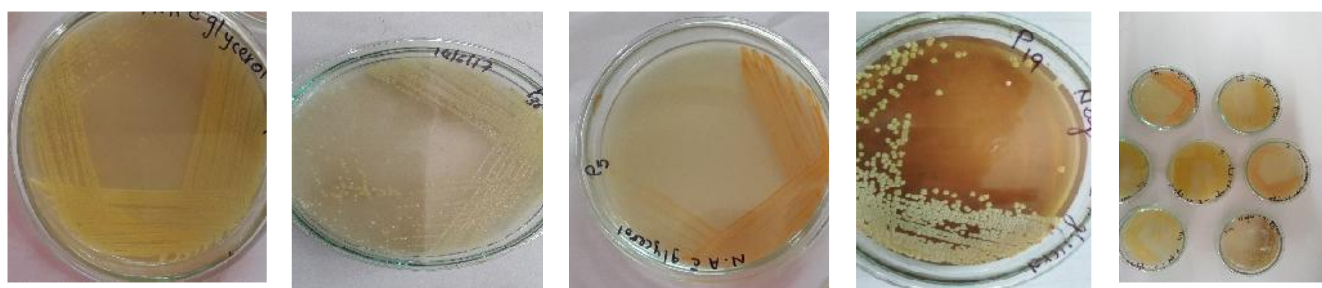

Fig.- 3. Plates having isolates in pure form

Fig.-4. Growth of isolates P5 & P1

Fig. -5. Extracted pigments from P5 & P1 (Methonal extract).

were selected for further studies. Table 3 shows colonial characteristics of selected isolates.

Gram Reaction and Motility

Out of 37, 23 were Gram positive and 14 were Gram negative. Table 4 gives information about Gram's reaction and motility.

Enzymatic profile of isolates

Out of 10 selected isolates, two isolates one yellow and one orange were selected for further studies. Table 5 shows enzymatic studies of 2 isolates and P5.

Table - 5. Results of enzymatic tests

Sl. No.	Enzyme	Isolates	
		P1	P5
1	Catalase	+	+
2	Oxidase	-	-
3	Amylase	-	-
4	Caseinase	+	-
5	Gelatinase	+	-
6	Lipase	-	+
7	Urease	+	-

Molecular identification

Sample labelled as P1 was found to be *Micrococcus aloeverae* based on nucleotide homology and phylogenetic analysis. Sample labelled as P5 was found to *be Rhodococcus corynebacterioides* based on nucleotide homology and phylogenetic analysis.

Production and Extraction of pigment

After incubation the growth can be observed by the change in colour of the broth as seen in Figure -4. After addition of acetone no change in solution was observed. When chloroform was added it turned out to separate two layers one colourless and one coloured which contained cells in case of P5. After separation, methanol was added to coloured part and pigment was extracted. However, it did not work for P1 as it gave a hazy appearance upon addition of chloroform. But upon addition of methanol the supernatant turned coloured and pellet got colourless, which indicated the successful extraction of pigment from bacterial cell pellet. The best solvent for extraction of pigment was determined to be Methanol for both pigments. But, for P5 it was methanol together with chloroform and in case of P1 it was alone methanol. Extracted pigments are shown in Figure 5.

Conclusion

From the present work it can be concluded that marine region can be explored for yellow and orange pigmented bacteria and they can be exploited for the preparation of agents which can be used to apply in high salt conditions. Moreover, further research can also be carried out to know its use as effective bio-control agent and resistant pathogens can be tested against such substances which may be proven to have great effects. Their roles in physiological processes can be determined. Marine environments would be the best choice because of having great diversity but less explored.

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