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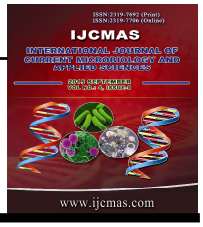

Original Research Article

Identification and characterization of pigment producing strain *Kocuria* KM243757 & JO1 KM216829 from Kharaghoda soil

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Keywords

Halophile, *Kocuria*, Carotenoids, Pigment extraction, 16s rRNA

A B S T R A C T

Orange and yellow pigmented bacteria isolated from the soil samples of Kharaghoda area, Gujarat were used for the present study. These both halophiles, Gram positive bacteria were identified as *Kocuria* spp. by 16s rRNA gene sequencing. These bacteria showed salt tolerance up to 12% NaCl. These gene sequences were submitted to NCBI and they were released by named *Kocuria* KM243757 & JO1 KM216829. The highest extractions of these pigments were yielded in 2:1= 85% Methanol: Acetone mixture among different concentration of different solvents. The Rf value of these extracted pigments were 0.32-0.97 in TLC method. The maximum absorption spectrum was observed at 466 nm by UV-Visible Spectroscopy. Orange/Yellow pigment was characterized as caratenoids which have numerous applications.

Introduction

There is growing interest in microbial pigments due to their natural character and safety to use, medicinal properties, nutrients like vitamins, production being independent of season and geographical conditions, and controllable and predictable yield [1]. Many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, comprise various hazardous effects. To counter the ill effect of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources [2].

The genus *Kocuria* was created from the genus *Micrococcus* on the basis of the phylogenetic and chemotaxonomic dissection of the genus *Micrococcus* [3]. *Kocuria* are coccoid, Gram-positive, non-endospore-forming, aerobic, non-halophilic microorganisms that can be differentiated from other genera in the order Actinomycetales on the basis of their peptidoglycan type (L-Lys–Ala3/4), the presence of galactosamine and glucosamine as their major cell-wall amino sugars, the presence of MK-7(H2) and MK-8(H2) as their major menaquinones, the presence of diphosphatidylglycerol and phosphatidyl-glycerol, the presence of the fatty acid anteiso-C15 : 0 and the G+C content of their DNA, which ranges from 66 to 75 mol%.
Carotenoids are a class of hydrocarbons

(carotenes) and their oxygenated derivatives are known as xanthophylls. Carotenoids are a class of fat soluble pigments responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colours of some birds, insects, fish, and crustaceans.

In plants, algae and photosynthetic bacteria these pigments play a critical role in the photosynthesis process. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may carry out a protective function against damage by light and oxygen.

Although animals appear to be incapable of synthesizing carotenoids, many animals incorporate carotenoids from their diet. Within animals, carotenoids provide bright colouration, serve as antioxidants, and can be a source for vitamin A activity [4].

Some familiar examples of carotenoid colouration are the oranges of carrots and citrus fruits, the reds of peppers and tomatoes, and the pinks of flamingoes and salmon [5]. Some 600 different carotenoids are known to occur naturally [4], and new carotenoids continue to be identified. It is estimated that nature produces about 100 million tons of carotenoids annually (http://www.industrialorganica.com/carotenoids.html).

The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications. Hence, in this study focus was made on pigment producing *Kocuria* spp. for pigment production and its characterization.

Materials and Methods

Selection of the bacterial strain

The Orange pigment producing halophilic isolate (shows up to 12% salt tolerance) and Yellow pigmented isolate were previously isolated from the soil sample of Kharaghodha area, Gujarat and selected for the further characterization study [6].

Identification of the strain

The preliminary identification of the strains was confirmed by Cultural characteristics and Morphological characteristics such as Gram Staining, Cell size, shape, arrangement etc.

Molecular characterization

Further confirmation of the strains was done by molecular characterization (ribo typing) using 16s rRNA gene sequencing. [7]. The sequences were run on NCBI and constructed phylogenetic trees.

Extraction and purification of pigments from the isolates

The different solvents like chloroform, acetone, ethyal acetate and methanol were screened out for extraction of pigments but a modified procedure for the isolation of pigments was carried out according Wei *et al* (2005) [8]; a 24 h old culture broths (1.5 liters), incubated at 25°C and 200 rpm, were mixed with 4 liters of 95 % (v/v) methanol and mixed vigorously via vortex mixing. The mixtures were then centrifuged at 6854 rpm, 0°C for 10 min. The resulting supernatants were collected and filtered through a 0.2 µm Whatman filter paper. The filtrate was concentrated using a rotary evaporator followed by extraction using 5.0 ml chloroform. The chloroform extract was

reconcentrated using rotary evaporator until minimal volume was obtained. This minimal volume of chloroform extract was then transferred into a glass Petri dish prior to drying in a vacuum drying oven. Dried pigments were dissolved in methanol or chloroform for 2-3 times and then drying it again so, pure pigments were obtained which is confirmed by TLC procedure.

Characterization of pigments

UV-Vis. Spectra absorption

Spectral analysis was made on dried pigments extracted by the above method by dissolving in methanol [9]. Spectral analysis was made on a UV- Visible spectrophotometer and the extracts were scanned in the range of 400 to 700 nm to find out the maximum absorption spectra. Methanol was used as a blank.

Pigment characterization by TLC

The purified pigments was analysed by thin-layer chromatography with silica gel G-60 F25~ (Merck, Mumbai, India). The solvent system consists of chloroform: methanol (95:5; v/v). The chromatography chamber with the solvent was kept for 20 min. for the equilibration.

The sample was spotted on the silica gel sheet using a capillary tube and air dried. The TLC sheet was then dipped in the solvent system. After 45 min. the TLC sheet was carefully removed and the Retention factor *(Rf)* value was calculated according to the following equation from the chromatogram.

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Pigmented bacteria as a visual living art

Prior to inoculating the Nutrient Petri dish with spores or 24 hours grown pigmented culture, freshly sterilize the surface of a designated area and the bristles of the Paint brush using rubbing alcohol. Once the paint brush bristles have dried, the tip of brush can be used to gently lift spores from a source (typically another Petri dish with a lawn of well-sporulated bacteria), and applied to a fresh petri dish as ‘‘paint.’’ The transfer of spores will appear as a colorless residue on the media surface. Since nutrient medium in a petri dish is moderately translucent, an image can be placed under the dish as a template for tracing. During the painting process, if a brush stroke is made in error, a paintbrush dipped in rubbing alcohol can be used as an eraser. If multiple strains are used to produce art work composed of multiple colors, a separate paintbrush should be designated for each strain to avoid contamination. The Petri dish is covered with its lid and placed bottom-side up for incubation at 30°C, usually showing pigmentation in 2–3 days. At room temperature, strains will grow more slowly and will likely produce pigment after 7–10 days. When the painting has developed to a desired pigmented state, the Petri dish can be stored in a refrigerator. Though the agar in the petri dish will continue to dry slowly at the cooler temperatures, the drying rate can be reduced by wrapping the edge of the dish with tape [10].

Results and Discussion

Identification of strain

The strains show orange and yellow pigmented growth on Nutrient Agar tube (Fig. 1). These strains are Gram positive, Cocci shaped, in chain forms which is shown in Figure 1. Both strains are

identified as *Kocuria* sp. but, both have different morphology and color so it is possible that both are from *Kocuria* genus but species or subspecies may be different.

Molecular characterization For Orange Pigmented strain

The Reverse (756 bp) and forward (1055 bp) sequence was used for conting sequence which was as below.

CAGCTCAGGACGAACGCTGGCGGCG
TGCTTAACACATGCAAGTCGAACGAT
GATCTCCCGCTTGCGGGGGTGATTAG
TGGCGAACGGGTGAGTAATACGTGA
GTAACCTGCCCTTGACTCTGGGATAA
GCCTGGGAAACCGGGTCTAATACTGG
ATACGACCCTCCATCGCATGGTGGGG
GGTGGAAAGGGTTTGACTGGTTTTGG
ATGGGCTCACGGCCTATCAGCTTGTT
GGTGGGGTAATGGCTCACCAAGGCG
ACGACGGGTAGCCGGCCTGAGAGGG
TGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGC
GGAAGCCTGATGCAGCGACGCCGCG
TGAGGGATGACGGCCTTCGGGTTGTA
AACCTCTTTCAGCAGGGAAGAAGCCA
CAAGTGACGGTACCTGCAGAAGAAG
CGCCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGCGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGAGC
TCGTAGGCGGTTTGTCGCGTCTGCTG
TGAAAGCCCGGGGCTCAACCCCGGGT
CTGCAGTGGGTACGGGCAGACTAGA
GTGCAGTAGGGGAGACTGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATAT
CAGGAGGAACACCGATGGCGAAGGC
AGGTCTCTGGGCTGTTACTGACGCTG
AGGAGCGAAAGCATGGGGAGCGAAC
AGGATTAGATACCCTGGTAGTCCATG
CCGTAAACGTTGGGCACTAGGTGTGG
GGGACATTCCACGTTCTCCGCGCCGT
AGCTAACGCATTAAGTGCCCCGCCTG
GGGAGTACGGCCGCAAGGCTAAAAC
TCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGCGGATTAATT
CGATGCAACGCGAAGAACCTTACCA
AGGCTTGACATTCACCGGACCGCCCC
AGAGATGGGGTTTCCCTTCGGGGTCG
GTGGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTCG
TTCTATGTTGCCAGCACGTGATGGTG
GGGACTCATAGGAGACTGCCGGGGTC
AACTCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGTCTTG
GGCTTCACGCATGCTACAATGGCCGG
TACAAAGGGTTGCGATACTGTGAGGT
GGAGCTAATCCCAAAAAGCCGGTCTC
AGTTCGGATTGAGGTCTGCAACTCGA
CCTCATGAAGTCGGAGTCGCTAGTAA
TCGCAGATCAGCAACGCTGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCG
CCCGTCAAGTCACGAAAGTTGGTAAC
ACCCGAAGCCGGTGGCCTAACCCCTT
GTGGGAGGGAGCCGTCGAAGGTGGG
ACTGGCGATTGGGACTAATCT

Phylogenetic tree of orange pigmented strain

The conting sequence was run on NCBI software and prepared phylogenetic tree as shown in figure No.2. From the result of gene sequencing matched with NCBI data, the orange pigmented strain was 99 % matched with *Kocuria sediminis* strain FCS-11, *Kocuria rosea* strain CT22, *Kocuria turfanensis* GJM817, *Kocuria* sp. RM1, *Kocuria turfanensis* HO-9042 which are shown in Fig. 2. This isolated strain having high salt tolerance capacity and it produced carotenoid pigment so, this gene sequence was submitted to NCBI and the accession number for this sequence is JO1 KM216829.

For yellow Pigmented strain: The Reverse (760 bp) and forward (1011 bp) sequence was used for conting sequence which was as below.

GACGAACGCTGGCGGCGTGCTTAACA
CATGCAAGTCGAACGCTGAAGCTCCA
GCTTGCTGGGGTGGATGAGTGGCGAA
CGGGTGAGTAATACGTGAGTAACCTG
CCCTTGACTCTGGGATAAGCCTGGGA
AACCGGGTCTAATACTGGATACGACT
CCTCATCGCATGGTGGGGTGTGGAAA
GGGTTTTACTGGTTTTGGATGGGCTC
ACGGCCTATCAGCTTGTTGGTGGGGT
AATGGCCTACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGTGACCGGC
CACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCT
GATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGAAGAAGCCACAAGTGA
CGGTACCTGCAGAAGAAGCGCCGGC
TAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGGCGCAAGCGTTGTCCGGA
ATTATTGGGCGTAAAGAGCTCGTAGG
CGGTTTGTCGCGTCTGCTGTGAAAGC
CCGGGGCTCAACCCCGGGTCTGCAGT
GGGTACGGGCAGACTAGAGTGCAGT
AGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAG
GAACACCGATGGCGAAGGCAGGTCT
CTGGGCTGTTACTGACGCTGAGGAGC
GAAAGCATGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCATGCCGTAA
ACGTTGGGCACTAGGTGTGGGGGACA
TTCCACGTTCTCCGCGCCGTAGCTAA
CGCATTAAGTGCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGCGGATTAATTCGATGC
AACGCGAAGAACCTTACCAAGGCTTG
ACATCCACCGGACCGCACTGGAGAC
AGTGCTTCCCTTCGGGGCTGGTGGAC
AGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTCGTTCTAT
GTTGCCAGCACGTGATGGTGGGGACT
CATAGGAGACTGCCGGGGTCAACTCG
GAGGAAGGTGGGGATGACGTCAAAT
CATCATGCCCCTTATGTCTTGGGCTTC
ACGCATGCTACAATGGCCGGTACAAA
GGGTTGCGATACTGTGAGGTGGAGCT
AATCCCAAAAAGCCGGTCTCAGTTCG
GATTGAGGTCTGCAACTCGACCTCAT
GAAGTCGGAGTCGCTAGTAATCGCAG
ATCAGCAACGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTC
AAGTCACGAAAGTTGGTAACACCCGA
AGCCGGTGGCCTAACCCCTTGTGGGA
GGGAGCCGTCGAAGGTGGGACCGGC
GATTGGACTATC

Phylogenetic Tree of yellow pigmented strain

The conting sequence was run on NCBI software and prepared phylogenetic tree as shown in figure No.3. From the result of gene sequencing matched with NCBI data, the yellow pigmented strain was 99 % matched with *Kocuria sp.* CNJ787, *Kocuria* sp. PM0532155, *Kocuria* sp. RM13Y, *Kocuria* sp. G3DM-46, *Kocuria* sp. CNJ770, *Kocuria rocea* CT22 which are shown in Fig. 3. This isolated strain having high salt tolerance capacity and it produced carotenoid pigment so, this gene sequence was submitted to NCBI and the accession number for this sequence is *Kocuria* KM243757.

Extraction of pigments from cultured broth

The pigments were extracted by different solvents with their different concentrations. Acetone, Ethyl acetate, Chloroform and Methanol were used. There was no pigment extraction observed in Chloroform and Ethyl acetate solvent. The Acetone and Methanol are the solvents, which have capacity to extract the pigment from the cell. But the highest extraction of pigment was shown in Methanol. There were different concentrations of Methanol and Acetone used for the extraction which are shown in

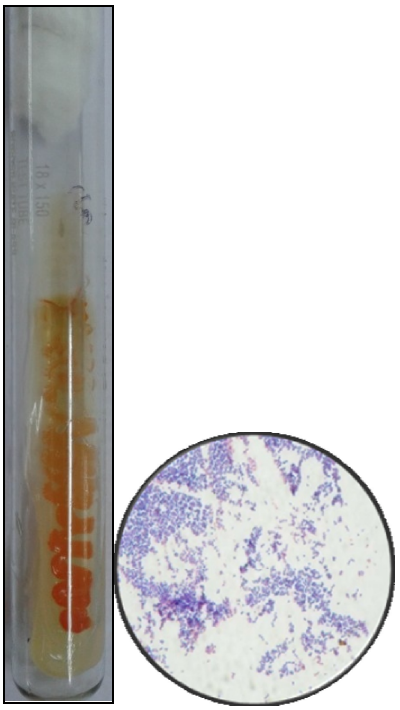
Table 1. The highest extraction of pigment was shown in 2:1 = 85 % Methanol: Acetone.

Orange pigment was extracted from the JO1 KM216829 strain and yellow pigment was extracted from the *Kocuria* KM243757 which is shown in figure No. 4

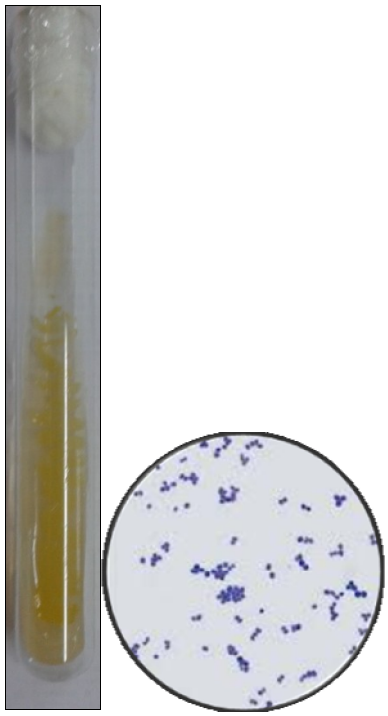
Table.1 Results of extraction with different concentration of solvents

Solvent Concentration		**Absorbance at 466 nm**
(75 % Methanol : Acetone)	1:1	0.640
	2:1	0.596
	3:1	0.601
	5:1	0.805
(85 % Methanol : Acetone)	1:1	0.598
	2:1	**0.989**
	3:1	0.765
	5:1	0.657
(95 % Methanol : Acetone)	1:1	0.876
	2:1	0.734
	3:1	0.802
	5:1	0.899

Fig.1 Macroscopic as well as Microscopic view of Pigmented strains



JO1 KM216829 growth on N.agar tube and its microscopic view



Kocuria KM243757 growth on N.agar tube and its microscopic view

Fig.2 Phylogenetic tree of orange pigmented strain

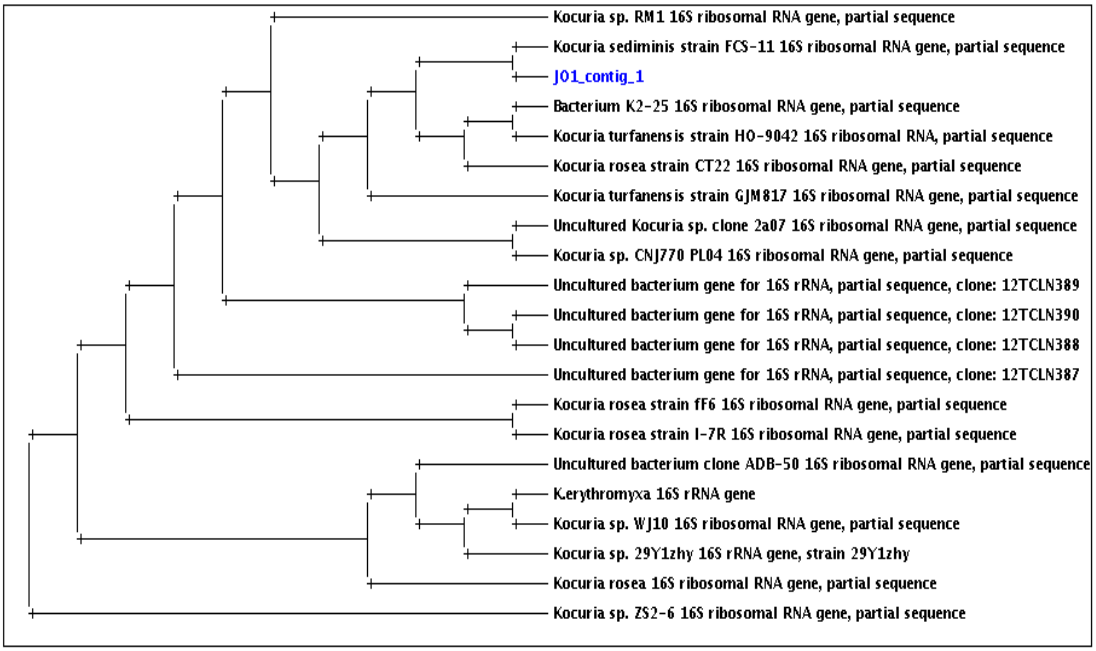


Fig.3 Phylogenetic Tree of yellow pigmented strain

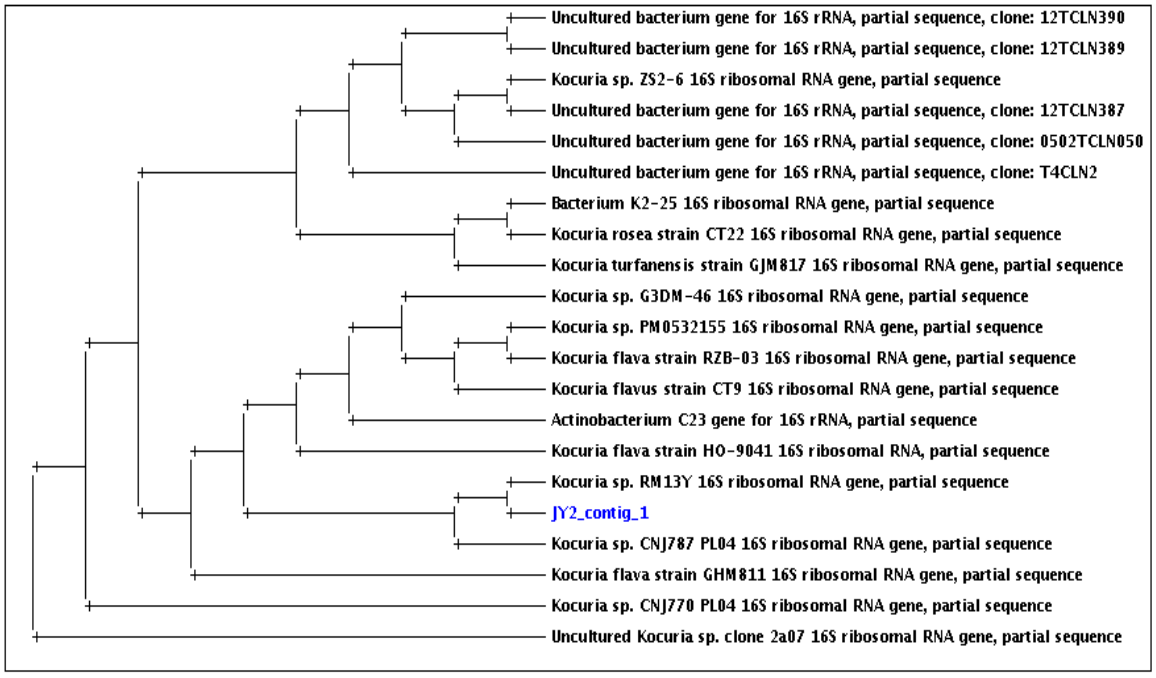


Fig.4 Extraction of Pigment

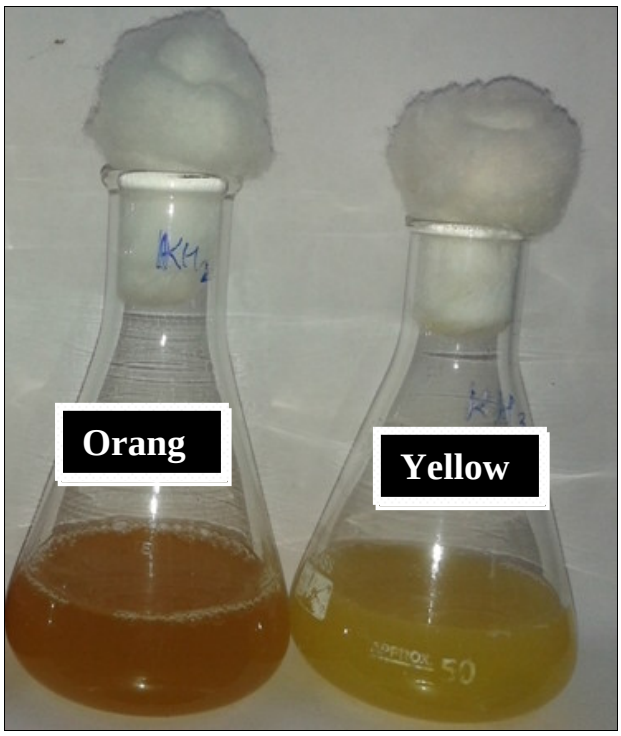


Fig.5 Visual Living Art

Characterization of pigment

In Uv-Vis spectroscopy, maximum absorbance of Yellow and orange pigments was obtained as 466 nm. The maximum absorbance of yellow/orange pigment value was matched with Reddy *et al.* (2003)[11]. So it can be concluded that the value shows orange and yellow pigment belong to carotenoids group. In a study done by Ong and Tee, 1992[4], the carotenoids pigment had 465nm maximum absorbance which

was nearest to this result of orange and yellow pigments.

In TLC profile, there were yellow to green clearly 4 bands with Rf values 0.97, 0.78, 0.62, 0. 32 respectively. In Reddy (2003) study [11], Rf value of carotenoids were in the range of 0.92 to 0.34 which are matching with our results, so Orange-yellow pigment was Carotenoids.

Pigmented bacteria as a Visual Living Art

Below visual living art was made by three isolated colourful bacteria (Fig no. 5). On Nutrient agar plate, this art was made by using different pigmented culture by wire loop. It was very preliminary art but, any type of art can be drawn by using of more colourful bacteria and sterile paint brush.

In conclusion, the selected halophilic strain JO1 KM216829 which produces orange colored pigment and *Kocuria* KM243757 which produces yellow colored pigment which are Carotenoids. This is natural pigments which have numerical applications.

Acknowledgement

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