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Screening and Extraction of Microbial Pigment from Organism Isolated from Marine Water

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Abstract: ***Synthetic colors have been widely used in various industries including food, textile, cosmetic and pharmaceuticals. However toxicity problems caused by synthetic pigments have triggered intense research in natural colors and dyes. Among the natural Sources, pigment producing microorganisms hold a promising potential to meet present day challenges. Furthermore natural colors not only improve the marketability of the product but also add extra features like anti- oxidant, anti- cancer properties etc. In this review, we present various sources of microbial pigments and to explore their biological and clinical properties like antimicrobial, antioxidant, anticancer and anti- inflammatory. The study also emphasizes upon key parameters to improve the bioactivity and production of microbial pigments for their commercial use in pharmacological and medical fields. Water samples were collected from different areas under different climatic conditions. A total of five pigmented colonies were isolated that produced intracellular pigments and isolated organism were characterized by the Bergey's Manual of Determinative Bacteriology. The pigments were extracted from the isolates and their antimicrobial and anti-fungal activity was identified. The extracted pigments had inhibitory effect on both Gram positive and Gram negative bacteria as well on fungus. So it was concluded that water has diverse organisms which showed antibacterial and antifungal activity and also some significant anti-oxidant properties.***

Keywords: Pigment, Bio-pigment, Micro-organisms, Antimicrobial

1. Introduction

A pigment is a material that changes the color of reflected or transmitted light as the result of wavelength-selective absorption. This physical process differs from fluorescence, phosphorescence, and other forms of luminescence, in which a material emits light. Pigments are colored, black, white or fluorescent particulate organic or inorganic solids which usually are insoluble in, and essentially physically and chemically unaffected by, the vehicle or substrate in which they are incorporated. They alter appearance by selective absorption and/or by scattering of light. Pigments are used for coloring paint, ink, plastic, fabric, cosmetics, food, and other materials. Most pigments used in manufacturing and the visual arts are dry colorants, usually ground into a fine powder.

The human eye does not see in black and white. Color is one of the first characteristics perceived by the human senses. It is integral to the interface between people and nature. Nature is rich in colors obtained from fruits, vegetables, roots, minerals, plants, microalgae, and so forth, and due to their origin from biological material they are often called "biocolors". Humans have traditionally preferred natural sources to add colors to food, clothing, cosmetics, and medicines. Among the molecules produced by microorganisms are carotenoids, melanins, flavins, phenazines, quinones, and bacteriochlorophylls, and more specifically monascins, violacein, and indigo .Pigments are present in all living matter and provide attractive colors and play basic roles in the development of organisms. Human beings, like most animals, come in contact with their surroundings through color, and things can or cannot be acceptable based on their color characteristics.

Microorganisms are known as a potential source for bio-pigment production due to their advantages over plants in terms of availability; stability; cost efficiency; labor; yield and easy downstream processing. Cultivation of microorganisms can be attained through solid state and submerged fermentation on natural raw material / industrial organic waste. Many of the microbial pigments not only act as coloring agents in various food processing and cosmetics industry but also possess anticancer, antioxidant, anti-inflammatory, anti-microbial activities. Furthermore there is huge demand for coloring agents in industries like textile, plastic, paint, paper and printing. There is an increasing demand for natural colour in the food, pharmaceutical, cosmetics, textile, printing and dye industry. The present review will lead us to explore the potential of microorganisms to produce pigments and further discusses about various strategies for It posses bio-pharmacological activities, Antioxidant, Antimicrobial, Immumo- regulation activities. Applications of these pigment can be widely used in various stream. It can be used as food colourant, as antimicrobial agents, as bio-indicator

Micrococcus luteus is a Gram-positive, to Gram-variable, non-motile, coccus, tetrad-arranging, pigmented, saprotrophic bacterium that belongs to the family Micrococcaceae. It is urease and catalase positive. An obligate aerobe, *M. luteus* is found in soil, dust, water and air, and as part of the normal flora of the mammalian skin. The bacterium also colonizes the human mouth, mucosae, oropharynx and upper respiratory tract. It was discovered by Sir Alexander Fleming before he discovered penicillin in 1928.

2. Material and Methodology

2.1 Collection of sample

Water sample was collected in clean bottle from different parts of Marve beach & aksa beach malad, and was transported immediately to the laboratory and processed for bacteriological analysis

2.2 Media used

Luria bertani media and salt mannitol agar media was used for screening.

2.3 Isolation of pigmented bacteria

Bacteria present in the water were isolated by serial dilution and spread plated on LB medium and incubated overnight at 37°C.

2.4 Maintenance of culture

Pigmented Bacterial cultures were grown on nutrient agar and maintained at 2-40°C temperature in refrigerator and sub cultured into respective medium.

2.5 Characterization and Identification of isolated pigmented micro-organisms

Colony characterization of pigment producing bacteria from LB plate (24 hour incubation)was done based on its size, shape, color, margin, opacity, consistency, elevation, Gram staining and motility.

2.5.1 Gram staining

A bacterial smear was prepared and heat fixed on a slide, few drops of crystal violet was put on a smear for 1 minute and washed with water. Then fixed the smear with Gram's iodine for 1 minute and washed again with water and decolorized the stain with 95% ethyl alcohol drop wise, washed with water and counter stained with safranine (45 sec) and again washed with water. After drying examined under oil immersion.

2.5.2 Cultural morphology

Colony characteristics was studied by looking at the colony morphology and was represented in tabular form

2.5.3 Biochemical test

1) Indole test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Tryptone water and incubated at 37°C for 24 hrs. 1ml of Kovac's reagent was added along the side of the test tube. The development of a bright red colour ring at the inter-phase of reagent and broth constitutes a positive test.

2) Methyl Red Test

Glucose Phosphate Broth media was prepared and sterilized. The sterile tubes were taken and the broth was poured. Test organisms were inoculated and the tubes were kept into the incubator for 24 hrs at 37oC. After 24 hrs the methyl red indicator was added to the tubes, and the color change is observed.

3) Voges- Proskauer Test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Glucose phosphate broth and incubated for 24 hrs at 37°C. 1ml of Omeara's reagent was added to the tubes. The development of pink colour was taken as positive for the test.

4) Nitrate Reductase Test

The saline suspension of the isolates were inoculated to the suspension tubes containing St. Nitrate broth and incubated for 24 hrs at 37°C. 1ml of α-naphthylamine and 1ml of sulfanilic acid was added. The development of red colour was taken as positive for the test.

5) Sugar Fermentation Test

For this, the medium, 1% Sugar (Glucose/ Sucrose/ Maltose/ Mannitol/ Lactose) in Peptone water base [Appendix-I] with Andrade's indicator and inverted Durham's tube, is inoculated with a loopful of the isolate culture. The fermentation of the media from yellow ochre to pink within 24 hrs at 37oC when incubated shows positive test result. Also the inverted Durham's tube tests for gas production

2.6 Optimization of various parameters for maximum pigment production

The optimization for the production of pigment from the isolates was carried out with nutrient broth. Optimization was carried out to increase the pigment production as well as growth rate of isolates. Temperature and pH are important parameter for growth of pigment

2.6.1Effect of Temperature on pigment production

Equal amount of the bacterial isolate was inoculated in sterile nutrient broth and incubated at different temperature viz 0, 27,37 and 50°C for 48hrs and then assayed for pigment. The optimum pH achieved by this step was fixed for subsequent experiments.

2.6.2 Effect of pH on pigment production

Equal amount of the bacterial isolate was inoculated in sterile nutrient broth with different pH viz, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 and was incubated at 27°C for 48 hrs. And then assayed for pigment production.the optimum pH was achieved by this step was fixed for subsequent experiments

2.7 Screening of pigment producing bacteria

Isolated colonies of identified cultures were suspended in 2% glycerol containing Nutrient broth in a flask and the flasks were incubated on rotary shaker for 48hrs. Extensive growth of pigment producing bacteria was seen in the flasks after 48hrs

2.8 Extraction of yellow pigmented bacterial isolates:

Different solvents like ethyl acetate, methanol, acetone, hexane was used to check for the maximum solubility of pigments. And the solvent was selected by checking the maximum solubility of pigment in it. After incubation the bacterial cells were washed with methanol and it was transferred to centrifuge tube.

2.9 Detection of Lambda Max

Visible absorption spectrum of the separated pigments in nutrient broth was analyzed with UV-visible Spectrophotometer between the wavelengths of 350-750 nm.

2.10 DPPH (1, 1-Diphenly-2-picryl-hydrazil) free radical scavenging activity

The radical scavenging activity by yellow pigment I.e. its antioxidant property was investigated by using DPPH assay. 5mg/ml of pigment extract was added to 2ml of DPPH solution and absorbance was read photometrically using colorimeter at 500 nm.

2.11 Antimicrobial Activity of Pigment

Antimicrobial activity of the pigments was tested by agar well diffusion method. Five human pathogens (Escherichia coli, Staphylococcus aureus, Candida) were used against extracted pigment to evaluate its antimicrobial activity. Growth was observed after 24hrs, isolated colonies were further characterized for identification.

2.12 Antifungal Test of Pigment

Antifungal test was carried out to check its sensitivity of extracted pigment. It was carried by well diffusion method. Pathogen like Candida and penicillium. Growth was observed after 24hrs and measured activity by zone of inhibition

3. Results

3.1 Isolation of microbial (Micrococcus luteus) pigment

Isolation of micro-organism was carried out using appropriate media. The culturing of organisms was done by using nutrient agar medium and Lura Bertani media. Screening was done for pigmented yellow micro-organisms. Various specific media was used for potential to get yellow colonies of *Micrococcus luteus.*

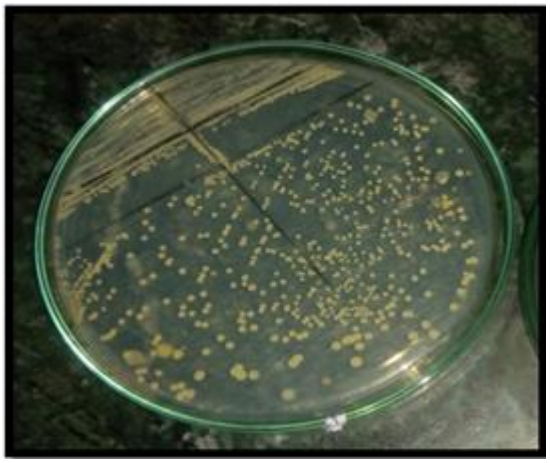


Figure 3.1: Yellow colonies isolated from LB media

3.2 Maintaince of Pure Culture

Pigmented Bacterial cultures were grown on LB agar and slant and it was maintained at 2-40C temperature in refrigerator and sub cultured into respective medium.

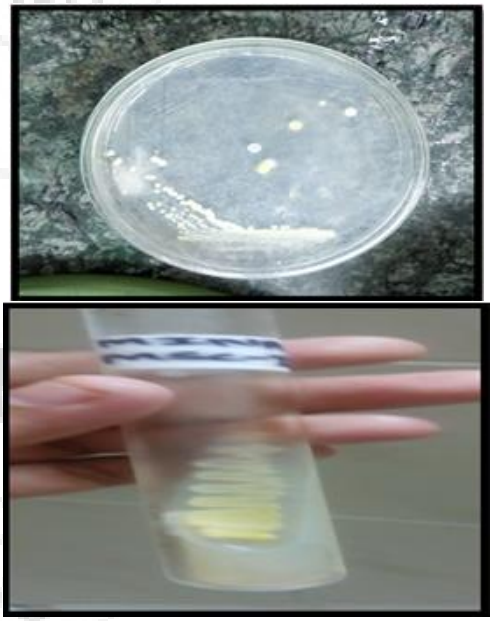


Figure 3.2: Maintenance of pure culture on Luria bertani plate and slant

3.3 Characterization of isolated micro-organisms

Characterization of micro-organism was done by morphologically and various biochemical tests. This test gave us confirmatory results for identification of Micro-organism. Thus given biochemical test followed by referring Bergey's manual gave confirmation of species as *Micrococcus luteus*

Table 1: Colony Characteristics of isolated pigment

Characteristics	Observation
colour	yellow
size	1-2nm
shape	circular
elevation	flat
opacity	opaque
margin	entire
Gram nature	Gram positive rod

Table 2: Biochemical test of isolated pigment

Sr.No	*Tests*	*Results*
1	Methyl red test	-
2	Indole test	-
3	Voges proskaur test	-
4	Catalase test	+
5	Nitrate test	+
6	Oxidase test	+
7	Citrate test	-

3.4 Effect of Temperature on pigment production

It was found that 27°C was optimum temperature for increase growth of pigment production. Therefore Micrococcus luteus can optimally grow 27°C temperature. This study suggest how different temperature can affect its growth and readings of temperature variation for maximum production of pigment was determined by colorimeter technique

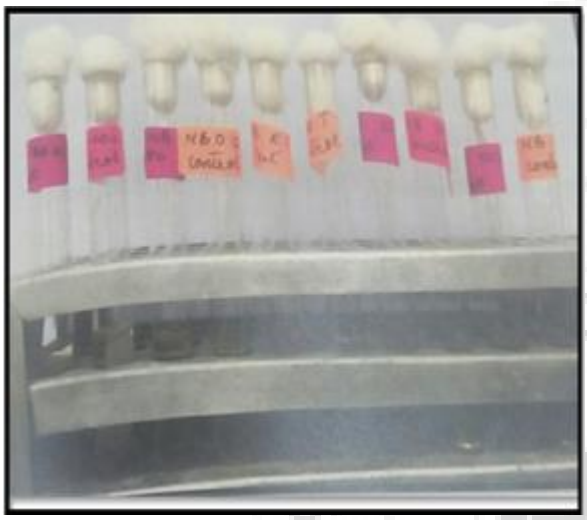
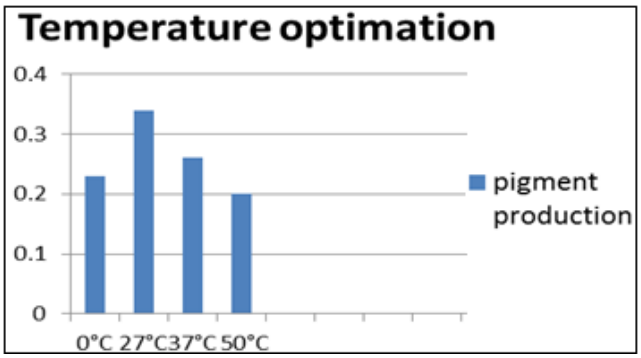


Figure 3.4: Effect ofTemperature on yellowon yellow pigment production of Micrococcus luteus

Table 3: Temperature reading of different Temperature for optimatization

Temperature	O.D
0	0.23
27	0.34
37	0.26
50	0.20



Graph 1: Temperature Optimization

3.5 Effect of pH on pigment production

The effect of pH 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 and incubated at 37 ºC for about 24-48hrs. Investigation on this study, *Micrococcus luteus* show highest production recorded on pH 7.5 .Hence pH 7.5 was maintained optimization studies. This suggests importance of pH in the media since its altered value can either increase or decrease the amount of pigment. Micrococcus was considered to grow optimally at pH 6.4-7.4. Their growth was maximum at neutral pH and pigmentation was observed maximum when kept under refrigeration.

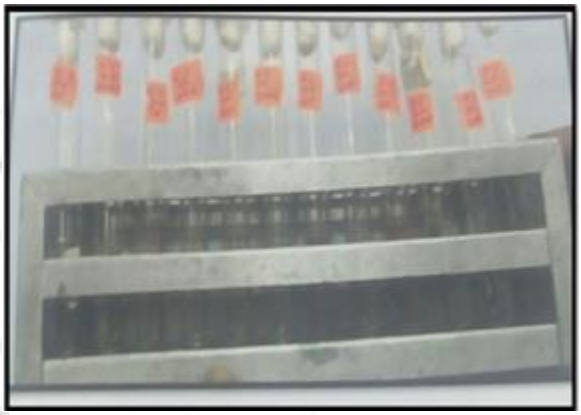
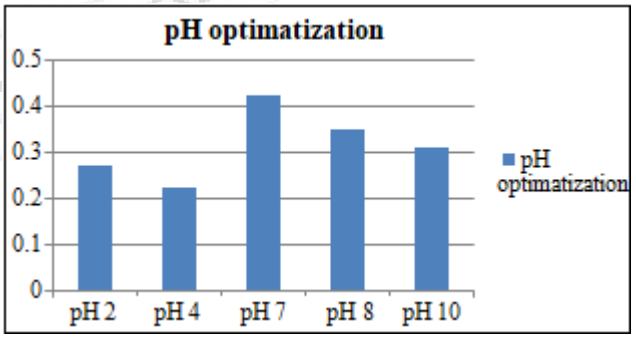


Figure 3.5: Effect of initial pH on yellow

Table 4: Different pH reading for pH optimization Pigmentproduction of *Micrococcus luteus*

pH	O.D
2	0.27
4	0.22
7	0.42
8	0.35
10	0.31



Graph 2: pH optimization

3.6 Extraction of Biopigment

The yellow pigment was obtained in the broth after incubation period of 7 days. The pigments were isolated using liquid-liquid extraction method.

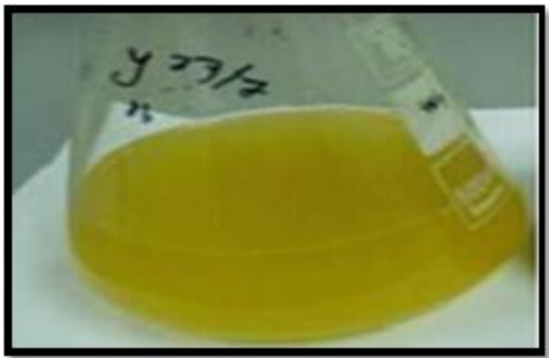


Figure 3.6.1: Culture broth of *Micrococcus luteus*

Figure 3.6.2: Pallet showing yellow pigment

3.7 Antimicrobial and Antifungal Activity

Antibacterial activity against 2 organisms of gram positive and gram negative organisms like Escherichia coli, Staphylococcus and Bacillus sp. All concentrations of samples were resistant to *Staphylococcus sp.* The antibacterial activity of pigment produced from *Micrococcus luteus* showed promising results against *staphylococcus aureus a*nd bacillus species and conclude that the isolated strain *M. luteus* is able to act against both gram positive and gram negative bacteria

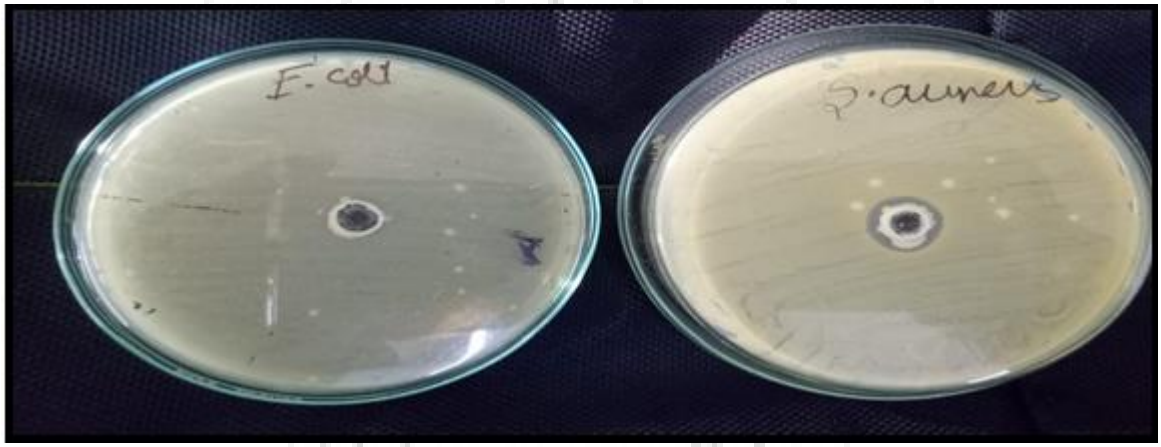


Figure 3.7.1: Antimicrobial activity against gram positive (S.aureus) and gram negative (E.coli)

Figure 3.7.2: Antimicrobial activity against Bacillus

Figure 3.7.3: Antifungal activity against pencillin

This indicates *Micrococcus luteus* is effective against gram positive and gram negative organisms.it also show effectively against fungal like *penicillin spc.* thus it has wide range of application in pharmacy world

3.8 DPPH (1, 1-Diphenly-2-picryl-hydrazil) free radical scavenging activity

Results of reading are as follow:
DPPH – 0.37DPPH with extracted sample- 0.21
DPPH scavenging effect (%) = [(A0-A1) /A0] x 100
= [(0.37-0.27)/0.37] x 100
= 27.027 %

Therefore, it was found that *Micrococcus luteus* showed radical scavenging activity of about 27%.The readings was determined by colorimetrically with the solution of DPPH.

3.9 Applications Using Yellow Pigment

Figure 3.9.1: Paper dying with pigment

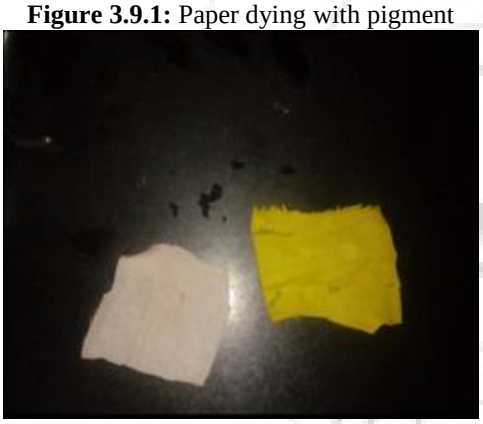


Figure 3.9.2: Fabric Dying with pigment

Images of before and after specimens by using yellow pigment extracted from *Micrococcus luteus* clearly evidence that it can be effectively used as dye for papers and to all kind of fabric materials. Thus it can be used for various arts and crafts use. It have has emerging role fabric industry too.

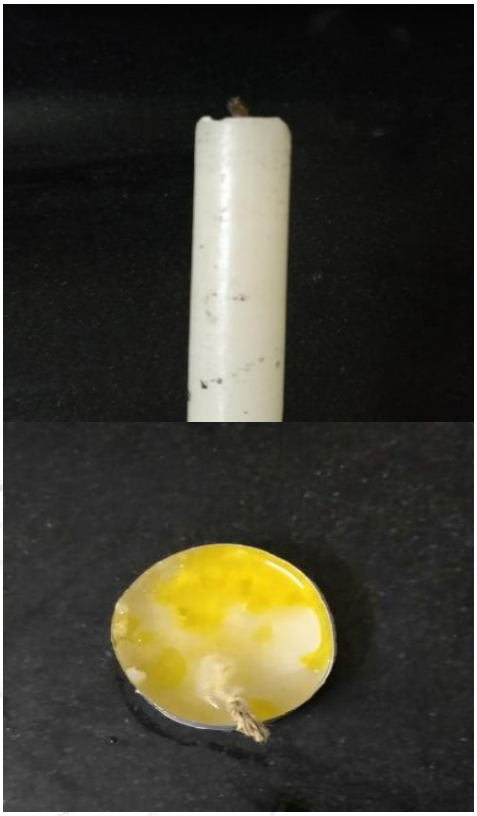


Figure 3.9.3: [A] original candle [B] prepared translucent candle with pigment

Translucent candle was prepared, by figure 3.9.3 it claims that variety of desired type candle can be prepared with the pigment extracted from Micrococcus luteus

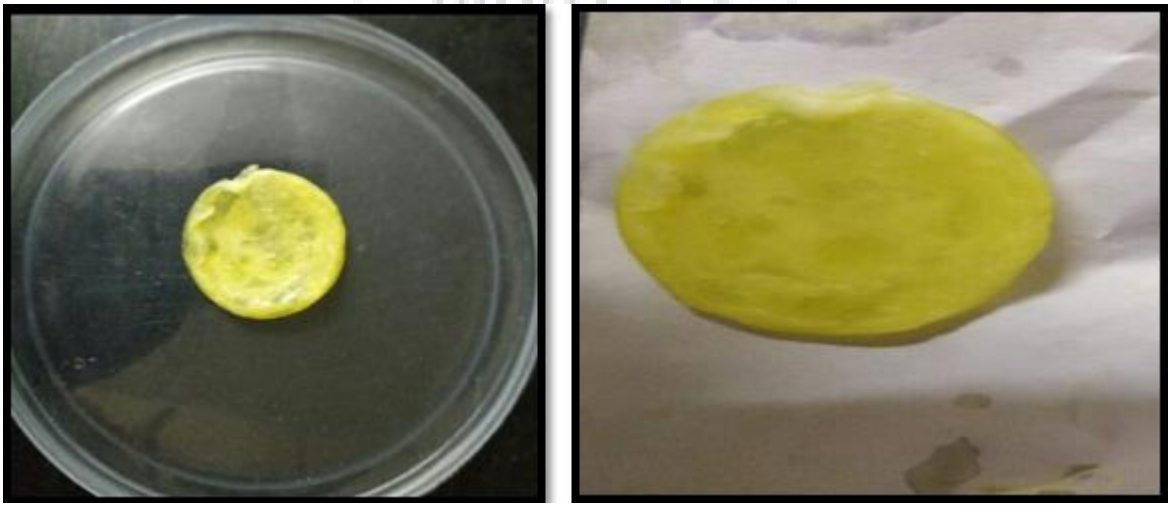


Figure 3.9.4: Soap Preparation with Yellow Pigment Extracted from *Micrococcus luteus*

Prepared soap is an perfect set of example using yellow pigment in effective way. This soap shows its colorful characteristics of soap. Considering its nature, its consisting properties of Antibacterial and Antifungal and addition of glycerin is perfect desired soap for commercial use.

4. Conclusion

The results of this study confirmed that extracted pigment can be used for various purpose and in various fields. As this study showed its anti-bacterial and anti-fungal properties. It also possess anti-oxidant properties. All theses properties are advantageous if subjected for production. While comparing with synthetic colour they are toxic and harmful.

Yellow pigment has given significant zone of inhibition against S. aureus. Since the yellow pigment has shown the antimicrobial activity so it can be used as a potential source for pharmaceutical and other cosmetic industries. Hence it was concluded that soil and water has diverse organisms which showed antibacterial and antifungal activity. More rigorous efforts are required to have a cheap organic substrate for the growth of color producing microorganisms. Also one need to look into the influence of various process parameters on the rate of production of microbial pigments Further, optimization of various parameters needs to be carried out for the maximal production of the pigment so that the above pigment could be exploited in future for various applications like pharmaceuticals and cosmetics. Further the antimicrobial ability of the pigment can be looked for so that it could of great use to the mankind.

Microbial pigments being an important source for natural colors possess wide range of medicinal properties..The above all applications sets best example of using pigment from Micrococcus luteus, as variety of products can be achieved with this. Also it have great influence on market acquiring pigment as naturally. Thus yellow pigment from microbial have many advantages too.

5. Discussion

Soil and water microbial communities are among the most complex, diverse and important assemblages of organisms in the biosphere and they are an important source for the search of novel antimicrobial agents and molecules with biotechnological importance such as microbial pigments that can be used as natural colorants as well as antimicrobial agents in place of antibiotic.

Nowadays there is a great interest of the market for the natural pigments; especially microbial pigments because of widely used synthetic pigments have harmful issues associated with the workers of industry as well as consumer. Microbial pigments have numerous beneficial properties like anticancer, anti-prolifrative, immune-suppressive, antibiotic, biodedabilty etc .Many microorganisms, including bacteria, fungi, yeast and mould etc. are employed for the industrial production of various pigments by using fermentation technology.

Colorants are used in vast majority industries from clothing and textiles. Most of the synthetic colorants are harmful to the environment and are difficult to biodegrade. There is an increasing interest involving microorganisms as an alternate source of synthetic colorants. In this consideration the present study was carried out to reduce the effects of non-bio-degradable pollutants. In the above study soil was used for isolating microorganisms.

Apart from food and textile coloring they have been used in clinical therapy to lower the blood cholesterol concentration, Anti-Diabetic Activity, Anti-Inflammation etc. A few years ago, some expressed doubts about the successful commercialization of fermentation-derived food grade pigments because of the high capital investment requirements for fermentation facilities and the extensive and lengthy toxicity studies required by regulatory agencies. Public perception of biotechnology-derived products should also be taken into account. Nowadays some fermentative food grade pigments are in the market and also the algae-derived or vegetable extracted pigments are successful marketed.

Studies should be more directed towards delineating the mechanism of action behind pharmacological activity of microbial pigments which would be very helpful in designing a novel strategy for the management of dreadful diseases like cancer. Future investigations need to be more focused on the chemical structure of microbial pigments and their structure-function relationship

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