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(RESEARCH ARTICLE)

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Identification, isolation and extraction of pigments producing bacteria from egg and use for dyeing

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Abstract

The present study is aimed at isolating bacteria from eggs white that can produce pigments and are potentially used in textile industries as dyes. Generally, bacteria produce pigments for various reasons, and it plays an important role. Some pigments produced by the bacteria show antimicrobial activity against pathogens. These antimicrobial agents or substances produced by the bacteria are successfully used for preventing and treating microbial diseases. Pigments like carotenoids, melanin, flavin, violacein, prodigiosin showed distinct antimicrobial effect against many pathogenic bacteria. Contaminated eggs may produce bacteria like *Salmonella spp., Proteus spp., Bacillus spp., Pseudomonas spp. and Staphylococcus spp.,* which have flagella that allow them to penetrate through the pores. These bacteria were extracted by using organic solvents and are purified and characterized by thin layer chromatography and optimized to dyeing parameters. The obtained dye serves as alternative source to chemical dyes.

Keywords: Bacteria; Eggs; Organic Solvents; Thin Layer Chromatography; Dyeing

1. Introduction

1.1. Overview

At present, fabrics are dyed by using synthetic pigments. These pigments can cause allergic conditions in people. However natural pigments are also rarely available due to their natural color tones. Natural pigments from various coloured plants were extracted and used in such areas as cosmetics, perfume and fashion industry. Such products are still expensive. To overcome these problems, bacteria that can produce natural pigments are cultivated on a large scale (Shirata et.al., 2000). These bacteria are identified from waste products like contaminated eggs; they readily produce pigments on suitable cultivation process. Natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH (Choubey et.al., 2017). The advantages of pigment production from microorganisms comprise easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes has been mounting in recent years. In the food industry they are used as additives, antioxidants, colour intensifiers, etc. Microbial colorants play a significant role as food coloring agents, because of their production and easy downstream processing.

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1.2. Pigments

Pigments, any group of compounds that are intensely cultured and are used to color other materials. Pigments are synthetic organic or inorganic compounds that are insoluble in water, although some are soluble in organic solvents. The pigment particles are ground down to a fine state of subdivision (0.5–2mm) and stabilized for use by the addition of dispersing agents and stabilizers (Ratnakaran et.al., 2020).

Table 1 General pigments and their colors

Pigments	Examples of typical colors
Flavonoids	Blue, purple and red
Carotenoids	Yellow and orange
Curcuminoids	Yellow
Betalanis	Red violet
Prodigiosin	Red
Violacein	Purple
Melanin	Brown
Pyocyanin	Blue
Chlorophyll	Green

1.2.1. Pigments producing microbes

Production of natural pigments from microbial sources is gaining much importance due to faster recovery, greater intensity of colour, higher yield of pigment, and cheaper production methods. Microorganisms such as bacteria, yeast and molds produce several kinds of natural pigments depending on their sources of origin**.** Pigments such as Quinones, Carotenoids, Violacein, Indigo and Melanin are produced from microbes (Dawoud et.al.,2019) Pigments extracted from microbial sources have a different range of commercial applications, as shown in table 2. Microorganisms produce a wide variety of natural colours such as anthocyanin's, carotenoids, quinines, violacein, monascins, melanin, etc. (Ursan et.al., 2019)

Table 2 List of pigment producing bacteria and their applications

Bacteria	Pigments	Applications
Micromonospora lupine	Anthraquinone	Antitumor agent
Streptomyces sp.	carotenoid	Food-grade pigment
Chromobacterium	Violacein	antifungal agent
Hymenobactersp.and chryoseobacterium sp.	Carotenoid	Photo sensitive in dye sensitized solar cells
Pseudomonas aeruginosa	Pyocyanin	Anti-microbial agent
Pedobacter	carotenoid	Antioxidant
Vogesella indigofera	Blue pigment	Detect heavy metals

1.3. Bacteria

Bacteria are single-celled microorganisms that lack a nuclear membrane, are metabolically active and divide by binary fission. Medically they are a major cause of disease. Superficially, bacteria appear to be relatively simple forms of life; in fact, they are sophisticated and highly adaptable (Poddar et.al.,2021). Bacteria are classified as Gram-positive or Gram-negative based on the characteristics of their cell wall, as seen under a microscope after stains have been administered, a procedure called Gram staining that was developed in 1882 by Hans Christian Gram.

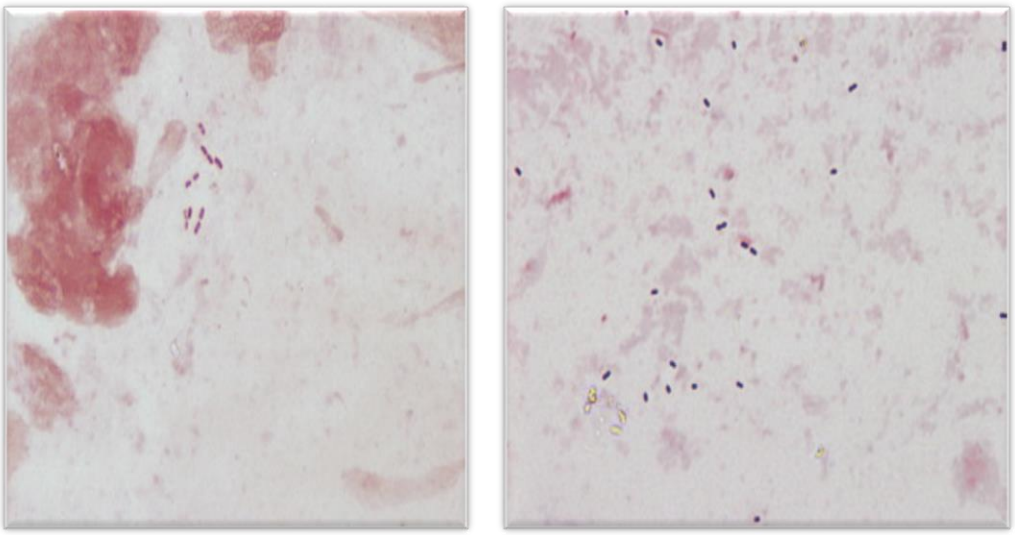

Figure 1 Gram strains of gram-positive bacteria and gram-negative bacteria

1.3.1. Pigment producing bacteria

Bacteria produce pigments for various reasons, and it plays an important role. Some bacteria such as *cyanobacteria* have phycobilin pigments to carry out photosynthesis. Other example for pigment-producing bacterial strains includes *Serratia marcescens* that produces prodigiosin, *Streptomyces coelicolor* (prodigiosin and actinorhodin), *Chromobacterium violaceum* (violacein) and *Thialkalivibrio versutus* (natronochrome and chloronatronochrome). These bacteria can be isolated/ cultured/purified from various environmental sources such as water bodies, soil, on plant, in insects and in man or animal (Ahmad et.al.,2012). Various pigment producing bacteria is already shown in table 2.

1.3.2. Use of pigments of bacterial sources

The pigments produced from bacterial cells are used in many applications like food industry, textile industry and paint manufacturing etc. Various bacteria with their pigments are shown in table 3.

Table 3 Various bacteria with their pigments

Name of the microorganism	Pigment bacteria	Colour
Bacillus subtilis	Riboflavin	Yellow
Bacillus thuringiensisH-14	Melanin	Dark Brown
Brevibacterium	Canthaxanthin	Orange red
Chromobacterium violaceum	Violacein	Violet
Dietziamaris	Canthaxanthin	Red
Janthinobacterium lividum	Violacein	Violet
Pseudomonas aeruginosa	Pyocyanin	Blue green
Serratia sp.	Prodigiosin	Red
Streptomyces sp.	Prodigiosin	Red
Streptomyces virginiae	Melanin	Dark Brown
Streptoverticilliumrubrireticuli	Prodoginine	Red
	MOLD	
Ashbyagossypi	Riboflavin	Yellow
Aspergillus ruber	Physcion	Yellow

Blakslea trispora	Lycopene	Red
Fusarium sp.	Naphthoquinone	Brownish yellow
Monascus sp.	Monascin, Rubropunctatin, Monascorubramine	Yellow, Orange, Red
Penicillium oxalicum	Arpink red	Dark red
Penicillium melinii	Atrovenetin	Yellow
	YEAST	
Cryptococcus sp.	Melanin	Black
Phaffiarhodozyma	Astaxanthin	Orange red

1.4. Dyes

1.4.1. Natural dyes in textile industry

Based on some research conducted by Indonesia government, there are approximately 41 dyeing plants are commonly used in natural textile industries (Poorniammal et.al.,2013) Dyes can be classified based on their chemical structures consists of indigoids (blue), pyridine based (yellow), carotenoids (yellow, orange and red), quinonoid (yellow to red range), flavonoids (pale yellow to dark yellow range, orange and blue). Colouring dyes based on its Source consists of plants (flowers, fruits, seeds, stems, leaves), animals (insecticides that produce red and purple) (Celedón et.al., 2021).Natural dyes act as antimicrobials that can prevent the growth of microbes, reducing to produce fewer odours, loss of colour, reducing skin infections and allergies and various related diseases. Examples of natural antimicrobial dyes are tannis, flavonoids, alkaloids, curcumin. That can also prevent UV light which leads to skin diseases to cancer (Elsahida et.al.,2019)

2. Materials and methods

2.1. Collection of raw materials

Egg samples were collected from local shops. Contaminated egg samples were chosen as already discussed as it contains the pigment producing bacteria. The egg is broken and poured in a beaker. The egg white is separated from yolk and is mixed well. Then this mixture is diluted in the water.

Figure 2 Contaminated egg

2.2. Cultivation of microbes

The egg mixture sample is subjected to serial dilution (up to 10-9) in each test tube and label them. From that odd test tubes were selected, and the spread plate method is performed by using nutrient agar as a medium (Yusof et.al.,2012). After the cultivation of the sample, growth of microorganisms is found by incubating the plates at 37^0 C for 48 hours (about 2 days). The clear colony is isolated among all and sub-cultured using nutrient agar (Berebichez et.al.,2018). A small colony is isolated and cultivated in Eosin Methylene Blue agar. The colonies are isolated and sub-cultured in the same agar. The gram nature of the microbes was studied by Gram Staining method.

Figure 3 Serial dilution

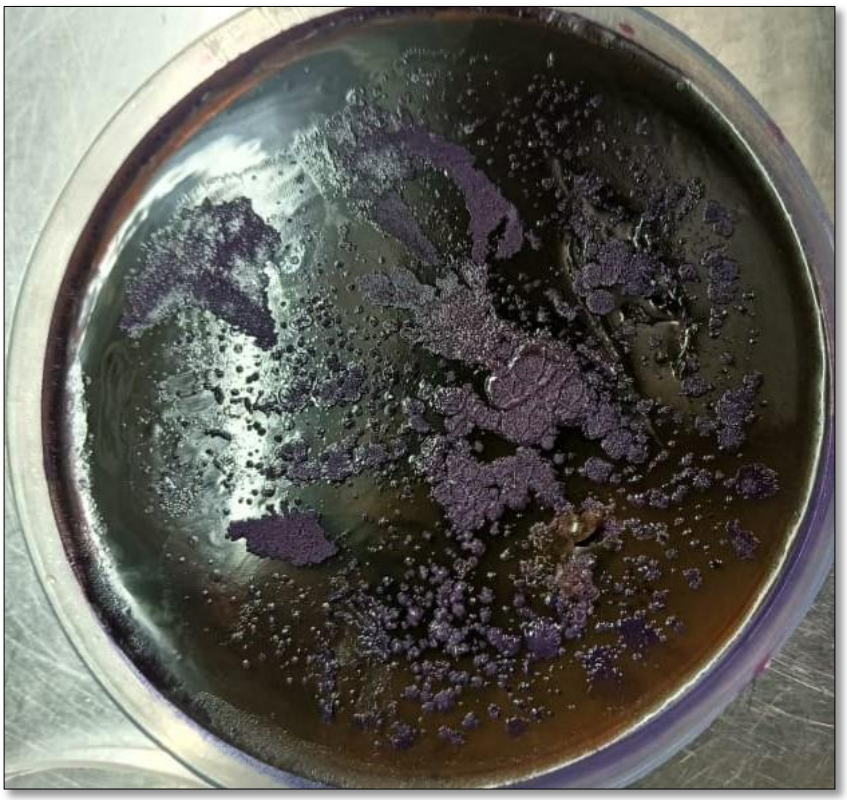

Figure 4 Pigments produced in culture plate

2.3. Extraction of pigments from culture plates

After the desired growth of bacterial cells, harvest the cells by centrifugation. The isolated colonies with bacterial cells are transferred to the centrifuge tube for solvent extraction method. Totally six different organic solvents were used namely acetone, ethanol, methanol, toluene, hexane and dimethyl sulfoxide. Each centrifuge tube is filled with bacterial cells and organic solvents in the ratio of 1:5 respectively. Then tubes are subjected to centrifugation at 10000rpm for 20mins (Garcia-Vaquero et.al.,2020). The cell pellets were deposited in bottom where the pigments are absorbed by supernatant. Transfer the supernatant to the clean container, continue this process until the pellet becomes colourless i.e., complete pigment extraction has been achieved, subsequently collect the supernatant. From six solvents, ethanol and DMSO shows the best results for extracting the purple and pale pink colours respectively, others didn't work well for this

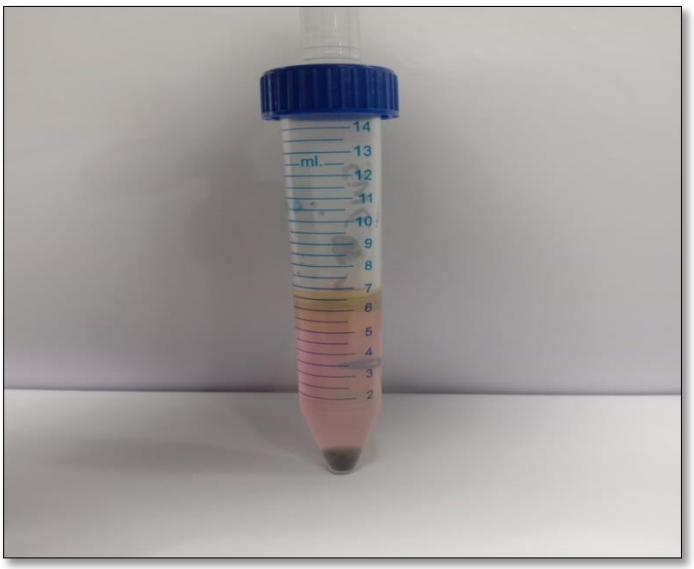

Figure 5 Solvent extraction method-DMSO

Figure 6 Collected Supernatant as DMSO and ethanol as organic solvent

2.4. Formulating the pigments

Generally, the collected supernatant was concentrated by using rotatory evaporator. Here a water bath and hot air oven are used for concentration. It is done by placing the sample solution in water bath and leave for 1hour at 90°c. This makes the organic solvent present in the sample solution evaporate leaving the pigment in the bottom of the plate. Then they are placed in hot air oven to remove any excess moisture. This step leaves behind a residue of purified pigments. Store the pigment in an appropriate temperature to prevent degradation (Joshi et.al.,2019).

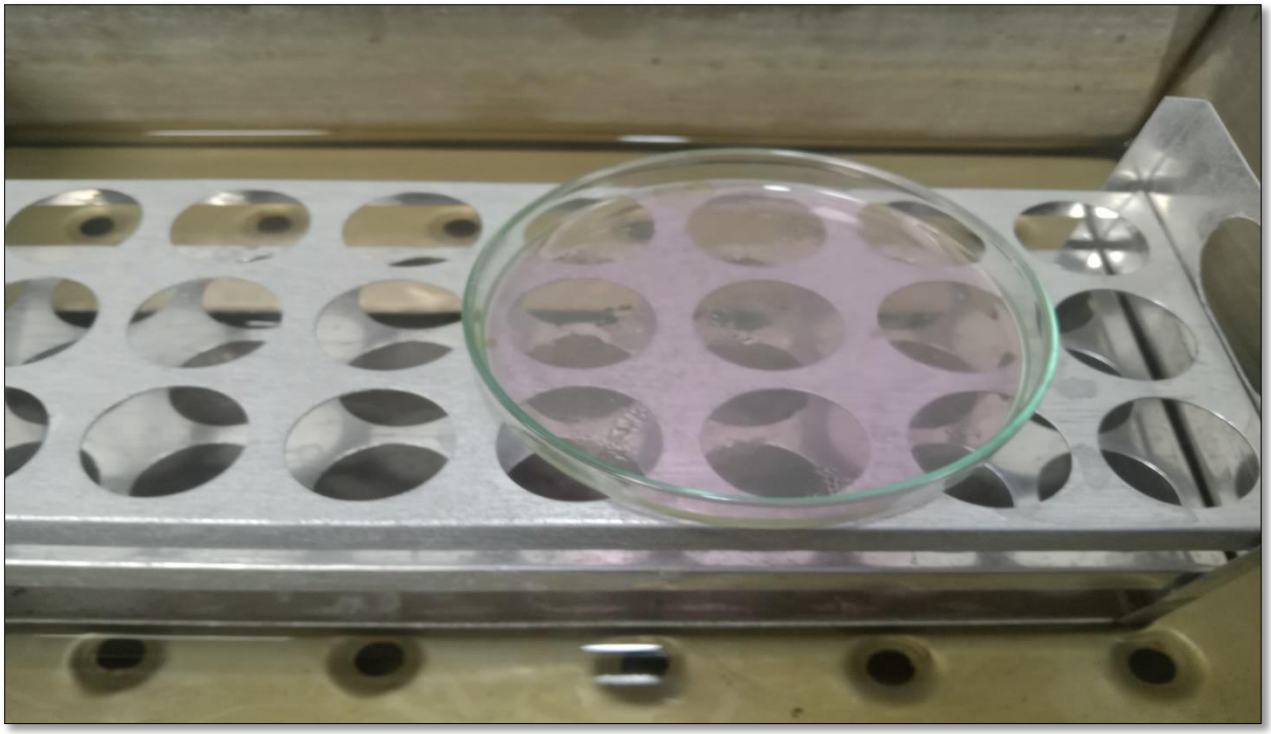

Figure 7 Concentrating the sample by water bath

2.5. Dyeing process

2.5.1. Boiling with the extract solution

The dyeing process was attempted by using organic solvent solution of the pigment. After the extraction of pigment from bacterial cells and air-drying process a fixed amount of dry pigment is mixed with various organic solvents. Desired fabric is immersed in the various solvents and extent of dyeing is compared. **(**Akira et.al.,2000).

2.5.2. Boiling dye with bacterial cells

Here the bacterial cells were transferred to a long vessel along with the medium and boiled after the addition of more than 10 times of water. After the solution was subjected to boiling, the fabric to be dyed is immersed into them and allowed to simmer for about 20mins. Then the fabric was washed with water and dried under shade. The intensity and stability of the dyed fabric is determined (Akira et.al.,2000)

3. Results

Pigment extraction from bacterial cells is influenced by the type of solvent. Generally, both the mixture ethanol and DMSO extracted the pigment very well. The ethanol mixture gives the purple color and DMSO gives the pale pink color. The bacterium called *Bacillus amyloliquefaciens spp plantarum* CICC 20037 CICC is identified as producing the above-mentioned pigment. The resulting pigment dry powder is suspended in their respective organic solvents.

Figure 8 Dried purple pigment

Figure 9 Suspended pale pink pigment

3.1. Characterization

3.1.1. Bacterial identification:

After the serial sub culturing of the sample culture, the single isolated colony is subjected to bacterial identification at microbiological laboratory, RS Puram, Coimbatore, India. The test report says that the culture grown is *Bacillus amyloliquefaciens spp plantarum* CICC 20037 CICC. MALDI-TOF method is used for bacterial identification. The test report also says the score value 1.72 which is probable for genus identification (Zhai et.al., 2016).

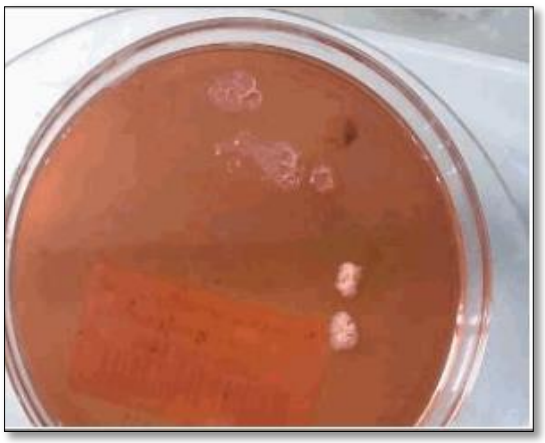

Figure 10 Tested sample

Bacillus amyloliquefaciens ssp plantarum CICC 20037 CICC is a subspecies of *Bacillus amyloliquefaciens* belongs to bacillus (Bacillus) Gram-positive bacterium, be a kind of bacillus mesophilic, aerobic, sporiferous known for its beneficial roles in agriculture, particularly in promoting plant growth and protecting plants from pathogens (Ngalimat et.al.,2021) This bacterium is divided at nature Cloth is extensive, nontoxic to people and animals, free from environmental pollution and fast growth can produce multiple antibacterial substance This subspecies produces indole-3-acetic acid (IAA), which stimulates root growth, and solubilizes phosphate, making nutrients more available to plants. Typically, its colonies on nutrient agar or LB agar are white to off-white, opaque, with rough or irregular edges, and a dry or slightly mucoid texture (Reva et.al.,2004)

3.2. Purification of pigment

3.2.1. Thin layer chromatography

The ethanol and DMSO extracted pigment were eluted using different solvent mixtures on pre-coated thin-layer chromatography (TLC) plates from TLC Silica gel 60 F254, (Merck, India). Different solvent mixtures with varying proportions like methanol: acetone: water (4:4:2); ethanol :water: chloroform (4:2:4) and (4:4:2); hexane: acetone (3:2) and (4:1); petroleum ether: ethyl acetate (9:1) and (1:9);chloroform: ethanol (2:1) and (3:1) were examined as the mobile phase to separate the different compounds of the pigment (Dawoud et.al.,2019). The sample was loaded onto the plate at a height of 1cm (about 0.39 in) from the bottom, and the plate was then placed in the different developing chambers containing the solvent ratios. After the solvent font reached the marked line, the TLC plates were visualized under UV light. The retention factor (Rf) was calculated from the distances traveled by the solvent and the pigment compound following Eq. 1 The elution of the target band was obtained by the best performing ethanol: chloroform (1:3) (Henriques et.al.,2007).TLC was performed for each elutes produced to check the homogeneity and similarity of the compound purified out from the column. The purified compound was then collected in a round bottle and dried in a rotary evaporator to form a powder .A desirable Rf value lies between 0.3 and 0.7, since it is likely that other compounds present in the mixture will be visible on the TLC plate when the Rf is in this range (Melconian et.al.,2014)

Rf value of the spot was calculated by using formula:

Retention factor (Rf) = Distance travelled by compound / Distance travelled by the solvent- Eq.1

Rf value for purple colour pigment = 7.6cm/14cm = 0.54

Rf value for pale pink colour pigment = 3cm/14cm = 0.2

Result Analysis

Here the Rf value of purple color pigment (A) is 0.54, which indicates that the compound is moderately polar. It has intermediate interactions with the polar stationary phase, allowing it to travel a moderate distance up the TLC plate.

The Rf value of pale pink color pigment (B) is 0.2 indicates that the compound is highly polar as it interacts highly with polar stationary phase and travels a shorter distance compared to less polar compounds.

Rf value of A is 0.54, therefore, it is less strongly adsorbed as compared to compound B which has Rf value of 0.2. Therefore, A will be eluted first.

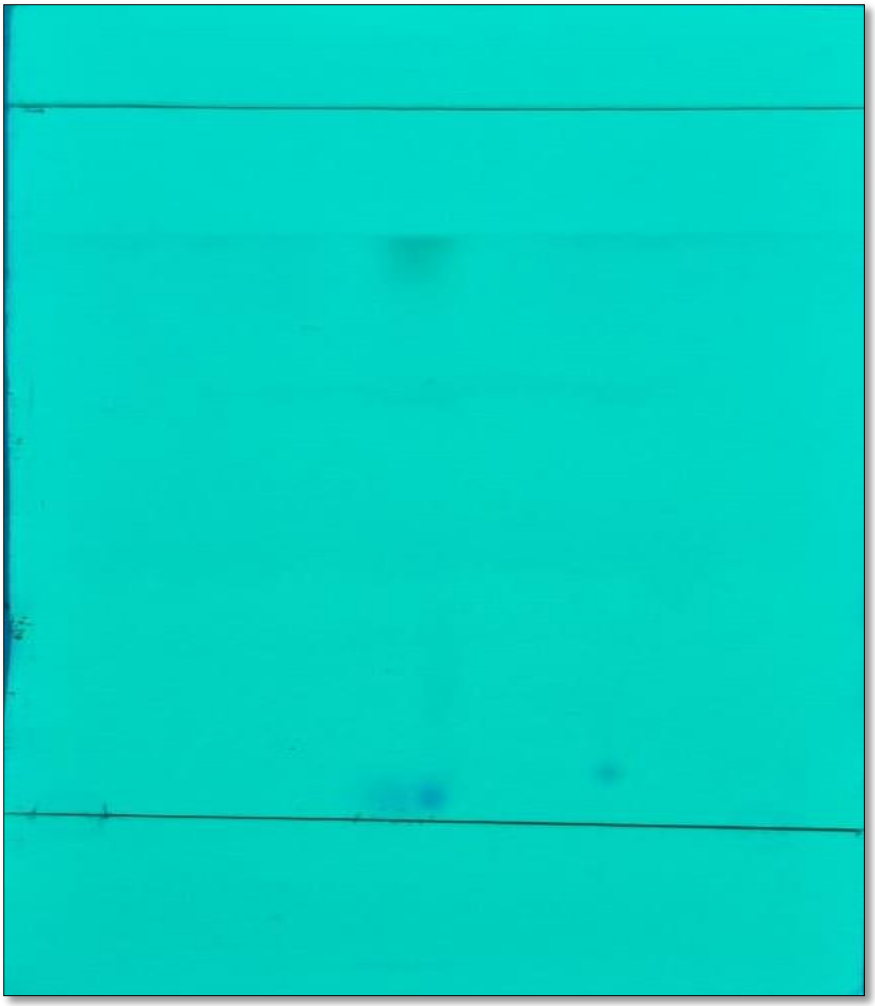

Figure 11 Bands Formed in TLC under UV Light

3.2.2. UV spectroscopy

Spectrometric analysis Absorption spectra of ethanol and DMSO extracts were measured with a UV-1603 spectrophotometer (Shimadzu, Japan). Pigments absorption spectra were measured in the wavelength range of 200 to 410 nm (Mickael Ruivo et.al.,2014). UV/vis analysis (200–500 nm scan; UV 1601PC; Shimadzu) of the purple pigment showed maximum absorption peaks at 278.67 nm and for pale pink pigment showed maximum absorption peaks at 288.56 nm (Ahmad et.al.,2012).

Procedure

Take the pigments that are suspended in the organic solvents (pale Pink in DMSO and purple in Ethanol) set up the UV spectrophotometer. Clean the cuvette with distilled water. Fill the cuvette with the pigment sample. Methanol is used as blank. Place the cuvette into the spectrophotometer. Click the MENU and then select SPECTRUM and set the wavelength range of 260nm-410nm and click START. The concentration of pigments is shown in a graph. The absorbance of purple and pale pink pigment is 1.83 and 2.00 respectively (Ratnakaran et.al.,2020)

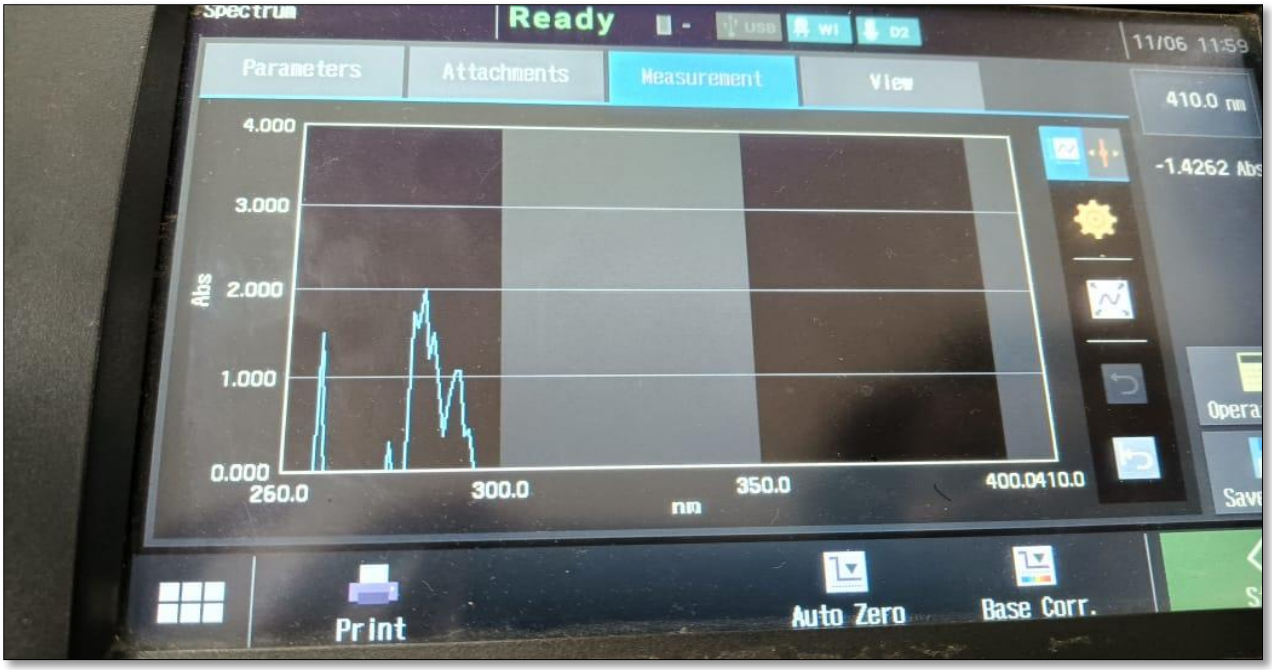

Figure 12 Spectrum DMSO sample

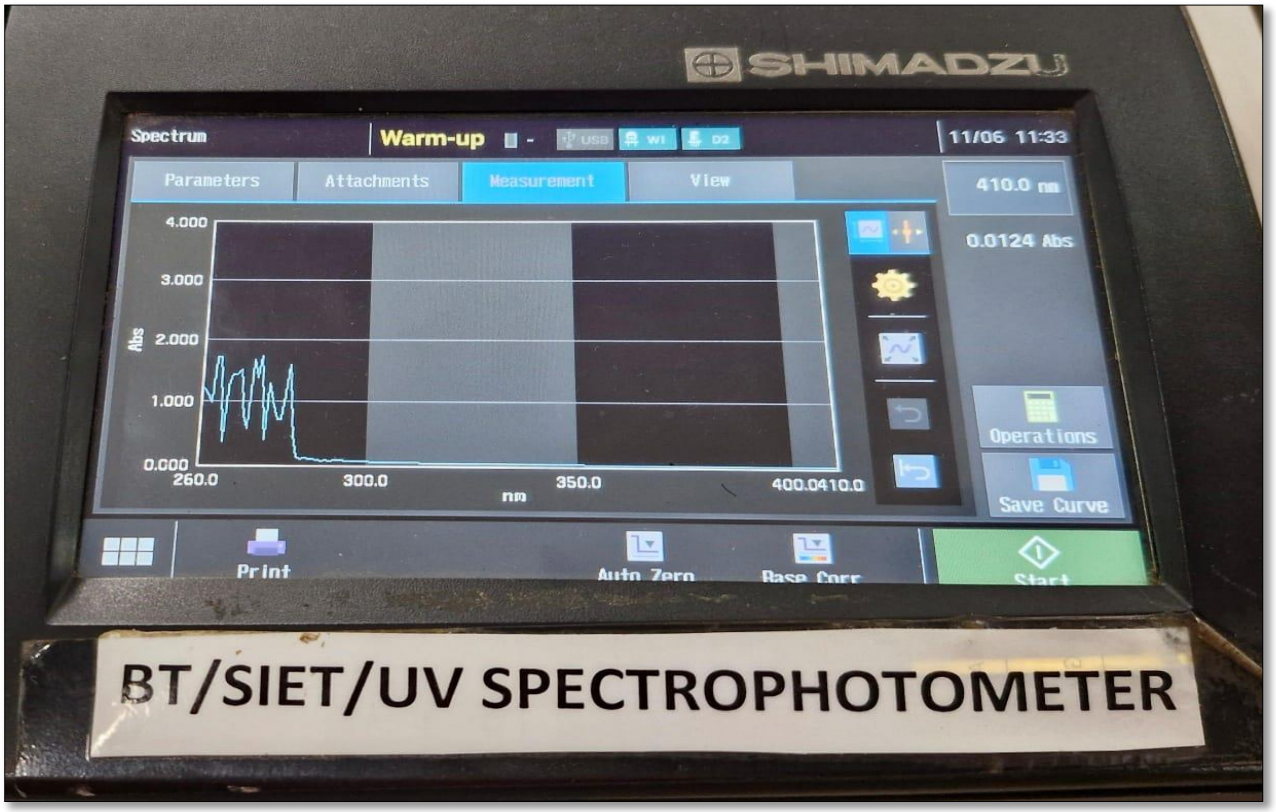

Figure 13 Spectrum ethanol sample

3.3. pH analysis

The purple pigment was tested for stability at various pH ranging from extremely acidic (0.54) to highly basic (13). At extremely low pH values, the purple pigment appeared greenish-blue, bright to dark blue (3.0–9.0) while in highly basic

condition, the pigment was almost decolorized. The decrease in colour intensity at high pH values can be attributed to the deprotonation of nitrogen by NaOH from the three conjugated rings at the pigment structure (**Ahmad et.al.,2012**).

Figure 14 pH analysis of sample

3.4. Morphological and Biochemical characteristics of isolate

After growth of isolate on Nutrient agar the morphological characters were noted. The biochemical characters were performed **(Joshi et.al.,2019)** The result of morphological and biochemical method is mentioned in table 4.

Table 4 Morphological Characters and Biochemical Test for Isolate

Characters	**Observations**	**Biochemical test**	**Observations**
Size	0.1mm	Oxidase test	**+**
Shape	Rod	Indole test	**-**
Colour	Purple, Pale pink	Catalase test	**+**
Consistency	Dry	Methyl red test	**-**
Gram staining	positive	Voges-Proskauer test	**+**
Elevation	Convex	Urease	**-**

Abbreviations

- spp-Species
- Ph-Power of Hydrogen
- UV-Ultraviolet
- TLC- Thin Layer Chromatography
- DMSO-Dimethyl Sulfoxide
- MALDI TOF-Matrix Assisted Laser Desorption Ionization-Time of Flight
- LB agar- Luria-Bertani
- Rf- Retention factor

SI UNITS

- mm – Micrometre
- Rpm - Revolutions per minute
- Nm – Nanometre
- cm- Centimetre
- ^{0}C- Celsius

4. Conclusion

This current study demonstrates the use of contaminated egg white as a substrate to cultivate pigment producing bacteria which is a cheap and easily available kitchen waste. We were able to isolate a bacterium which was identified as *Bacillus amyloliquefaciens ssp plantarum* CICC 20037 CICC. The bacterial cells were extracted with dimethyl sulfoxide and ethanol for one day and the extract was filtered. Then, after concentrating the extract by subjecting to water bath and hot air oven, even lyophilisation method is applied, it was fractionated by silica gel chromatography (ethanol: chloroform) and pale pink and purple coloured pigment was separated. These bacteria are non-pathogenic. The pigments from these bacteria can be used to dye natural fibres, providing good colour and stability. Mass-culture of the bacteria is possible, the pigment and dye can be produced cheaply. These pigments are subjected to dyeing parameters and the resulted dye serves as the alternative source to chemical dyes.

Compliance with ethical standards

Disclosure of conflict of interest

No conflicts of interest to be disclsoed.

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