CHAPTER-3
MATERIALS AND METHODS

3.1 Isolation and screening of potential pigment producing actinomycetes

3.1.1 Source of actinomycetes

Actinomycets strain, which have ability of pigment production were isolated from the rhizosphere soil of different regions of Madhya Pradesh.

3.1.2 Collection of soil samples

The soil samples were collected from 8-10 cm depth from surface of soil with help of spatula and transferred to a sterile polythene bag and stored in laboratory. The soil samples were air-dried for 4-5 days at room temperature and crushed in mortar and pestle to make fine particles.

3.1.3 Pretreatment of soil samples

The soil samples were dried at room temperature and finally given pretreatment of heat at 45°C for 24 hrs. Heat treatments of samples were done to select actinomycetes and eliminate contamination presented in the form of lower bacteria, mites fungal spores etc (Williams et al., 1972).

3.1.4 Isolation of pigment producing actinomycetes

One gram of soil sample was 10 fold serially diluted in sterile distilled water and plated on Starch Casein broth (SCA), Starch broth (SA), Yeast extract-malt extract broth (YEMA), Actinomycetes isolation agar (AIA), Inorganic starch salt broth (ISSA), Tyrosine Agar (TA), Oatmeal agar (OA), Glycerol asparagines agar (GAA) media. The inoculated culture plates were incubated for 5-7 days at 30 °C. All the pigment producing actinomycetes that grown on the plates were picked and purified by streak plate method on respective media (Cappuccino & Sherman, 2007).
3.1.5 Composition of growth media for actinomycetes

The following media (Shirling & Gottlieb, 1966) were used for growth studies:

Yeast extract-Yeast extract malt extract agar

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Malt extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>6.2±0.2</td>
</tr>
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</table>

Oatmeal agar (ISP 3)

<table>
<thead>
<tr>
<th>Ingredients</th>
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</thead>
<tbody>
<tr>
<td>Oat Meal</td>
<td>20.0</td>
</tr>
<tr>
<td>Ferric sulphate heptahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Trace salts-</td>
<td></td>
</tr>
<tr>
<td>Manganese chloride tetrahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Zinc sulphate heptahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.3±0.2</td>
</tr>
</tbody>
</table>

Inorganic salts-starch agar (ISP 4)

<table>
<thead>
<tr>
<th>Ingredients</th>
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</thead>
<tbody>
<tr>
<td>Starch (soluble)</td>
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</tr>
<tr>
<td>Dipotassium phosphate</td>
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<tr>
<td>Magnesium sulphate heptahydrate</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>2.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>2.0</td>
</tr>
<tr>
<td>Ferrous sulphate, heptahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Manganous chloride, heptahydrade</td>
<td>0.001</td>
</tr>
<tr>
<td>Zinc sulphate, heptahydrade</td>
<td>0.001</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>Final pH (at 25°C)</td>
<td>7.2±0.2</td>
</tr>
</tbody>
</table>
Glycerol-asparagine agar (ISP 5)

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
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<tbody>
<tr>
<td>L-Asparagine</td>
<td>1.0</td>
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<td>Dipotassium phosphate</td>
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<tr>
<td>Trace salt solution (ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
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</tbody>
</table>

1 ml of Trace salt solution contains –

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/ L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulphate heptahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Manganese chloride tetrahydrate</td>
<td>0.001</td>
</tr>
<tr>
<td>Zinc sulphate heptahydrate</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Final pH (at 25°C) 7.4±0.2

Starch agar

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/ L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat Extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Starch, soluble</td>
<td>2.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Final pH (at 25°C) 7.2±0.1

Peptone Yeast Iron agar (ISP 6)

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
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</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Proteose peptone</td>
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<tr>
<td>Yeast extract</td>
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</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.5</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.08</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Final pH (at 25°C) 6.7±0.2
Starch casein agar

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>10.0</td>
</tr>
<tr>
<td>Casein (Vitamin Free)</td>
<td>0.3</td>
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<tr>
<td>Potassium nitrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>2.0</td>
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<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.02</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Actinomycetes isolation agar

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium caseinate</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td>4.0</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.001</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td><strong>Final pH (at 25°C)</strong></td>
<td>7.1±0.2</td>
</tr>
</tbody>
</table>

3.1.6 Screening of pigment producing actinomycetes

The desired pigment producing actinomycete was selected based on diffusible color producing ability. The selection of potential strain was based on production of intense color producing ability in both solid and broth medium. After screening of 85 actinomycetes, the isolate ARITM02 was selected which showed the considerable red pigment in both solid and broth medium (Krishna *et al.*, 2008).

3.1.7 Selection of suitable growth medium

The selection of suitable growth medium is an important aspect for pigment production. Eight media was tested on solid and broth form to select best medium for pigment production and growth of selected isolate. Cultures were inoculated on above
mentioned media plates. Plates were incubated for 6-7 days at 35°C and observed for appearance of growth, sporulation, colour of substrate mycelium, aerial mycelium and production of diffusible pigment (Shirling & Gottlieb, 1966; Williams et al., 1989).

3.1.8 Antimicrobial activity of selected isolate

The selected isolate ARITM02 was tested against microorganisms to check its biological activity by agar overlay method and well agar diffusion method.

3.1.8.1 Agar overlay method

In agar overlay method, the starch casein agar was prepared and poured on petriplates and allowed to solidify medium. The selected isolate was spot inoculated middle of the medium plate and left for incubation for 4 days. After 4 days nutrient agar and potato dextrose agar were prepared for bacteria and fungi respectively and poured on previously incubated petriplates. Now the test bacteria, yeast, molds and fungi are inoculated and spread uniformly on petriplates and incubated. The plates were observed after 24 hours interval (Singh et al., 2006).

3.1.8.2 Well agar diffusion method

For evaluation of antimicrobial activity of broth culture, wells were drilled using a sterile cork borer in fresh test microbial lawn cultures on nutrient agar medium for bacteria and potato dextrose agar medium for fungi. The extracts of the culture broths were then administered to fullness in each well. The plates were put in incubator at 35°C for 24 hours. Bioactivity was determined by measuring the diameter of inhibitory zones (mm) of test microorganisms around the well after incubation. Antibiotic poured in wells served as control. The antibiotic tetracycline was used as a control (7 µg/ml) for bacteria and nystatine (10 µg/ml) used for fungi (Pandey et al., 2011).

3.2 Characterization of pigment producing actinomycete

3.2.1 Colony morphology

Colony morphology of actinomycete was singled out, based on some peculiar features of the colonies: form (round and sometimes slightly irregular), elevation (convex, flat, umbonate), margins (eroded and sometimes whole), texture (powdery, at times opaque), growth intensity, colony color (white, grey, brown, green etc), reverse
color of mycelium, diffusible pigment production, and surface colony appearance were noted. Actinomycetes can be distinguished from other microorganisms by the presence of the substrate and aerial hyphae, the type of colouring of both mycelia and the production of coloured pigments. The trinocular and electron microscopic examination was carried out for morphological identification (Willson and Walker, 2010).

3.2.2 Staining (Gram's staining)

Since Actinomycetes are Gram positive the pure culture was subjected to Gram's staining. The heat fixed smear of the isolates was treated with a series of reagents namely crystal violet (Primary stain), Gram's iodine (Mordant), 95% ethanol (Decolorizing agent) and safranin (Counter stain) followed by observation under oil immersion objective (Aneja, 1996).

3.2.3 Biochemical characteristics

The isolate was subjected to different biochemical tests (Aneja, 1996; Cappuccino and Sherman, 1999).

Citrate utilization test: Simmon’s citrate agar was used.

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulphate</td>
<td>0.2</td>
</tr>
<tr>
<td>Ammonium dihydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.08</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>6.8±0.2</td>
</tr>
</tbody>
</table>

**Procedure**

The citrate test was performed to differentiate the actinomycete on the basis of their ability to utilize citrate as carbon source. Slants of simmon's citrate agar were prepared and surface of slants were inoculated with appropriate actinomycetes culture and incubated at 27°C for 4 days. Change in colour from green to blue indicated positive reaction. No colour change indicated negative result.
**Hydrogen sulfide production test:**

Sulfur indole motility medium (SIM) medium was used for sulphate reduction and indole production.

### Ingredients (g/L)
- Peptone: 30.0
- Beef extract: 3.0
- Ferrous ammonium sulphate: 0.2
- Sodium thiosulphate: 0.025
- Agar: 3.0
- pH (at 25 °C): 7.3±0.2

**Reagent- Kovac's reagent**

**Procedure**

The nitrogen, carbon, and amino acids sources in SIM medium were provided by enzymatic digest of casein and enzymatic digest of animal tissue. Ferric ammonium citrate and sodium thiosulfate were used to detect hydrogen sulfide production. Hydrogen sulfide (H₂S) gas reacts with ferric ammonium citrate to produce ferrous sulfide, a black ring. Slants of SIM were prepared and isolate was inoculated using a loop and kept for incubation at 35±2°C for 72-96 hours. Then the slants were observed for motility and hydrogen sulfide production.

**Catalase production test:** Nutrient broth was used for this test.

### Nutrient broth

### Ingredients (g/L)
- Peptone: 10.0
- Beef Extract: 10.0
- NaCl: 5.0
- pH (at 25°C): 7.4±0.2

**Procedure**

The breakdown of hydrogen peroxide into oxygen and water was mediated by the enzyme catalase. Tubes containing nutrient broth were kept for incubation of 2-3 days at 27°C after inoculation of isolate and after 3 days tubes were observed for bubble formation on adding 3% H₂O₂ solution.
**Nitrate reduction test:** Nitrate Broth was used.

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Meat extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>1.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.0 ± 0.2</td>
</tr>
</tbody>
</table>

1. **Reagent A** - Sulfanilic acid
2. **Reagent B** - N,N-Dimethyl-1-naphthylamine
3. Zinc

**Procedure**

Nitrites react with an acid solution of sulfanilic acid and alpha-naphthylamine to form a red azo dye. Nitrate broth was taken into test tubes and isolate was inoculated in it. A control tube was also taken in which inoculation was not done. Tubes were incubated at 35°C for 12 to 24 hour. After incubation, tubes were tested for presence of nitrate. One dropper full of sulfanilic acid and one dropper full of N,N-Dimethyl-1-naphthylamine was added to the test tubes along with control and colour change was observed.

**Gelatin hydrolysis test:** Gelatin Agar medium was used.

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
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</tr>
<tr>
<td>Gelatin</td>
<td>120.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.0 ± 0.2</td>
</tr>
</tbody>
</table>

**Procedure**

Gelatin causes liquids to solidify at temperatures below 28°C. At temperatures above 28°C, gelatin is a liquid. Gelatin stab method was done in this method tubes with 12% nutrient gelatin media was inoculated with isolate and control without inoculation was also prepared. Tubes were incubated for 48 hours and then placed in refrigerator for 30 min. The refrigerated isolate inoculated gelatin tubes were examined to see whether the medium is solid or liquid.
**Casein hydrolysis test:** Skim milk agar medium was used.

**Ingredients**

<table>
<thead>
<tr>
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</tr>
</thead>
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<tr>
<td>Skim milk powder</td>
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</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**Procedure**

Casein is the major protein found in milk. It is a macromolecule composed of amino acids linked together by peptide bonds, CO-NH. Casein hydrolysis can be demonstrated by supplementing nutrient agar medium with milk. The medium is opaque due to the casein in colloidal suspension. Skim milk agar media was poured into the plates and after solidifying a single line streak was made with the help of loop across the surface of medium and then plates were incubated for 72-96 hours at 35°C, then the plates were observed for any clear zone around line of growth.

**Starch hydrolysis test (Amylase production Test):** Starch agar medium was used.

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3.0</td>
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<tr>
<td>Soluble starch</td>
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</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**Reagent:** Gram’s iodine solution.

**Procedure**

The ability to degrade starch was used as a criterion for the determination of amylase production by a microbe. Starch agar media was poured into petridishes and allowed to solidify. Then zigzag streak of isolate was made on the plates and one control without streak was also taken. Then plates were kept for incubation for 72 hours at 35°C. After incubation plates were flooded with gram’s iodine solution. Then plates were examined for starch hydrolysis around line of growth by the change of colour.
Carbohydrate fermentation test: Phenol red carbohydrate broth was used.

Ingredients

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>*Carbohydrate</td>
<td>5.0</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.018</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>15.0</td>
</tr>
<tr>
<td>pH (at 25°C)</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*(Carbohydrates were used like glucose, xylose, fructose, arabinose, sucrose, ribose, galactose, maltose, rhaminose and raffinose).

Procedure-

The end-product of carbohydrate fermentation was an acid or acid with gas production. Various end-products of carbohydrate fermentation can be produced. Broth was taken into different test tubes and isolate was inoculated, un-inoculated tube was also taken as control and then Durham’s tubes were inserted in inoculated test tubes. All the tubes were inoculated for 72 hours at 35°C, then tubes were observed for the acid production was given by A and gas production by G and AG for both acid and gas production.

3.2.4 Physiological characterization

3.2.4.1 Growth temperature

Isolate ARITM02 was inoculated on Starch casein agar (SCA) medium and incubated at different temperatures (15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C). Growth was observed at regular intervals upto the 7th day.

3.2.4.2 Growth at different incubation time

Starch casein agar (SCA) medium was prepared with different incubation time (24, 48, 72, 96, 120, 144 and 168 hours). The isolate ARITM02 was inoculated on the plates and growth observed at regular intervals upto the 7th day.

3.2.4.3 pH tolerance

pH of starch casein broth was adjusted with different buffer solutions to give a pH range (2, 4, 6, 7, 7.5, 8 and 10). The petridishes containing the media were
inoculated and incubated at 30 °C. Selected isolate was examined for growth at regular intervals up to the 7th day.

3.2.5 Molecular characterization

In order to identify the isolate ARITM02 at molecular level, 16s rRNA sequencing was carried out at “Ammnion Biotech Pvt. Ltd.” Bangalore.

3.3 Optimization of cultural parameters for maximum growth of actinomycete

3.3.1 Selection of suitable medium

The selected isolate was inoculated in different broth medium to test the maximum growth of selected isolate ARITM02 in broth culture. The media used were Yeast extract-Malt extract broth, Oatmeal broth, Inorganic Starch-Salt broth, Glycerol asparagines broth, Starch casein broth, Starch broth and Peptone yeast extract iron broth, Actinomycetes isolation broth and Tyrosine broth. One loopful culture was inoculated in each medium and allowed for incubation up to seven days. The optical density was measured after regular interval of 24 hours at 480 nm.

3.3.2 Optimization of cultural parameters

The optimization of various parameters for pigment production by actinomycete isolate was carried out in 100 ml Starch casein broth in 250 ml conical flask. Parameters namely temperature, pH, carbon sources, nitrogen sources and incubation time were carried out using the protocol of with minor modifications. All experiments were carried out in triplicate and mean optical density and standard deviation were recorded.

3.3.2.1 Incubation Period

To optimize the medium with best nitrogen source, the starch casein broth was prepared and distributed in different seven sets of flasks and various nitrogen sources used in the medium (Peptone, Casein, Beef extract, Yeast extract and Malt extract). The flasks were then properly autoclaved at 15 psi pressure and 121°C for 15 minutes. After sterilization, the flasks were inoculated with 1 loopful culture of ARITM02 isolate and kept at incubator shaker at 150 rpm at 30°C. Growth of the ARITM02 was measured by
UV-Vis spectrophotometer at 480 nm upto seven days at regular interval of 24 hours. Uninoculated medium of starch casein broth (SCB) was used as a control.

3.3.2.2 Temperature
The starch casein broth was prepared and distributed in different sets of flasks. The flasks were then properly autoclaved at 15 psi for 15 minutes. After sterilization, the flasks were inoculated with 1 loopful culture of ARITM02 isolate. The flasks were incubated at different temperature 20°C, 25°C, 30°C, 35°C, 40°C and 45°C and kept into incubator shaking at 150 rpm. Growth of the selected isolate ARITM02 was measured by UV-Vis spectrophotometer at 480 nm upto seven days. Uninoculated medium was used as a control.

3.3.2.3 pH
The starch casein broth was prepared and distributed in different sets of flasks and adjusted to initial pH values ranging from 2, 4, 6, 7, 7.5, 8 and 10. The flasks were then properly autoclaved. After sterilization, the flasks were inoculated with 1 loopful culture of ARITM02 isolate and kept at incubator shaker at 150 rpm at 35°C. Growth of the ARITM02 was measured by UV-Vis spectrophotometer at 480 nm upto seven days at regular interval of 24 hours. Uninoculated medium of neutral pH was used as a control.

3.3.2.4 Carbon Source
To optimize the medium with best carbon source, the starch casein broth (SCB) was prepared and distributed in different seven sets of flasks and various carbon sources used in the medium were glucose, dextrose, sucrose, fructose, starch, lactose and maltose. The flasks were then properly autoclaved. After sterilization, the flasks were inoculated with 1 loopful culture of ARITM02 isolate and kept at incubator shaker at 150 rpm at 35°C. Growth of the ARITM02 was measured by UV-Vis spectrophotometer at 480 nm upto seven days at regular interval of 24 hours. Uninoculated medium of SCB was used as a control.

3.3.2.5 Nitrogen Source
To optimize the medium with best nitrogen source, the starch casein broth was prepared and distributed in different seven sets of flasks and various nitrogen sources used in the medium were peptone, casein, beef extract, yeast extract and malt extract).
The flasks were then properly autoclaved. After sterilization, the flasks were inoculated with 1 loopful culture of ARITM02 isolate and kept at incubator shaker at 150 rpm at 30°C. Growth of the ARITM02 was measured by UV-Vis spectrophotometer at 480 nm up to seven days at regular interval of 24 hours. Uninoculated medium of starch casein broth was used as a control.

3.4 Extraction and purification of pigment

3.4.1 Extraction of pigment from broth medium

The starch casein broth was prepared for extraction of pigment. Actinomycete isolate was inoculated into medium and incubated under standard optimized conditions. As maximum production was observed on the 4th day of incubation, fermentation was terminated after 96 hours and stored in BOD at 2-3°C temperature. The broth was centrifuged at 10,000 rpm for 20 minutes to separate the crude pigment. Pigment was separated by solvent extraction method using different solvents from culture supernatant. The crude pigment was separated, collected and dried in vacuum oven at 40°C overnight. The residue obtained (crude pigment) was subjected for purification (Krishna et al., 2008).

3.4.2 Purification of crude pigment

The crude pigment was screened for number of components by Thin-Layer Chromatography (TLC) plates using Methanol: acetone: water: (4:4:2), Chloroform: methanol (9:1), Chloroform: methanol (6:4), Ethanol: water: chloroform (4:4:2) and Ethanol: water: chloroform (4:2:4) solvent system. The chromatographic chamber with solvent was kept for 15 mints for equilibration. The sample was spotted on ready made silica gel sheet (Merk) with the help of capillary tube and air dried. A control sheet without spot was also used as a blank. The TLC sheets now dipped in solvent system and allowed to run. TLC sheet was carefully removed and air dried. The TLC plates were exposed to iodine vapors, sprayed with vanillin and ninhydrin separately. Retention factor \( (R_f) \) value was calculated according to the following equation from the chromatogram (Willson and Walker, 2010).

\[
R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}
\]
Purification of the pigment was carried out by column chromatography using silica gel (60–120 mesh). Fractions were collected at 20 minutes interval. TLC of each fraction was performed. The fractions having same Rf value were mixed together and the solvent was evaporated at 40°C in a vacuum oven. These fractions were tested for their antimicrobial activity by using the well agar diffusion method. The pure compound obtained was stored in an ampoule at 4°C.

3.5 Characterization of purified pigment

3.5.1 UV-Vis spectrophotometer analysis

The UV-Visible absorption spectra of the bioactive component in solvent extracts were determined with a UV-Vis spectrophotometer (PerkinElmer) at 200-600 nm to determine the Lambda maximum of the band.

3.5.2 Fourier Transform Infra Red (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectroscopy, analysis, is a technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies, which are characteristic to that molecule. During FTIR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the infrared rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analyzed and matched with known signatures of identified materials. The parameters used in the FT-IR analysis were: spectral range: 4000-500 cm⁻¹ Resolution: 0.9 cm). The purified pigment sample was subjected to FT-IR spectroscopic analysis (Perkin Elmer Lambda), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulfate) detector.

3.5.3 Mass spectroscopy analysis

Mass spectrometry (MS) is an analytical technique which identifies compounds based on the atomic sample composition of the molecules and their charge state. Therefore, “blind” analysis of unknown samples is possible since MS does not require detailed prior knowledge of the sample composition. Ideally, the chemical identification
by MS is not limited by analyte pre-selection as, for example, in analysis techniques based on fluorescent or radioactive labeling. This bears the advantage that the analysis technique itself does not make any functional changes to the molecules under investigation. The applications of mass spectrometry range from among others environmental analysis, isotope dating and tracking, trace gas analysis, proteomics, lipidomics, metabolomics to clinical applications and forensics. Purified pigment was analyzed at “Sophisticated analytical instrumental facility” (SAIF), Chandigarh (Willson and Walker, 2010).

3.5.4 Nuclear magnetic resonance (NMR) spectroscopy analysis

Nuclear magnetic resonance spectroscopy most commonly known as NMR spectroscopy is the technique which exploits the magnetic properties of certain nuclei. When placed in a magnetic field, NMR active nuclei absorb at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. Since this frequency shift is proportional to the strength of the magnetic field, it is converted into a field-independent dimensionless value known as the chemical shift. By understanding different chemical environments, the chemical shift can be used to obtain some structural information about the molecule in a sample. The conversion of the raw data to this information is called assigning the spectrum (Willson and Walker, 2010). The 'H-NMR spectra of the purified pigment was analyzed at “Sophisticated analytical instrumental facility” (SAIF), Chandigarh.

3.6 Biological activities of purified pigment

3.6.1 Antimicrobial activity

The antimicrobial activity of pigment was done by well agar diffusion method; wells were drilled using a sterile cork borer in fresh test microbial lawn cultures on nutrient agar medium for bacteria. The pigment dissolved in methanol, was administered to fullness in each well. The plates were incubated at 35°C for 24-48 hours. Bioactivity was determined by measuring the diameter of inhibitory zones (mm) of test microorganisms around the well after incubation. The antibiotic tetracycline was used as a control (7µg/ml) for bacteria and nystatine (10 µg/ml) used for fungi (Aneja, 2003).
3.6.2  Toxicity test of pigment  
3.6.2.1 Cell lines and Culture medium  

HDF (Human dermal fibroblast) and U-87 (Human Glial cell) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. HDF and U-87 stock cells were cultured in Ham’s F12 medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India). The cytotoxicity test was carried out at “Radiant Research Pvt. Ltd.”, Bangalore, India. For cytotoxicity studies, weighed test pigment was dissolved in distilled DMSO and volume was made up with Ham’s F12 supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

3.6.2.2 Procedure  

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using Ham’s F12 medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 μl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 hours interval. After 72 hours, the drug solutions in the wells were discarded and 50 μl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hours at 37 °C in 5% CO₂ atmosphere. The supernatant was removed and 100 μl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.
% Growth Inhibition = 100 – \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right)

### 3.6.3 Allergy test

The neutral red uptake (NRU) assay was an *in-vitro* method to determine the skin allergic property of pigment. It is an alternative method of *in vivo* allergy test (Yang *et al.*, 2015). The test was carried out at “Radiant Research Pvt. Ltd.”, Bangalore, India.

#### 3.6.3.1 Cell line and Culture medium

3T3 cell line was cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

#### 3.6.3.2 Preparation of Test Solutions

For Cytotoxicity studies, weighed test drug was separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

#### 3.6.3.3 Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 hours.
interval. After 72 hours, the drug solutions in the wells were discarded, and the plate was rinsed 3 times with PBS, 200μl each well, and then washed. The medium containing neutral red (50μg/mL) was added, 200μl each well. After 3 hours of culture, the medium was removed and the plate was rinsed 2 times with PBS, 200μl each well. The desorbing solution, 100μl each well, containing 1% glacier acetic acid, 50% ethanol and 49% H2O, was added and shaken for 15 minutes with a micromixer in a dark place. The absorbance of colored solution was measured at 540nm with a microplate reader. The concentration producing 50% inhibition for neutral red uptake (NRU 50μg/mL) was calculated.

The concentrations of ≥ 1250, 200 ≤ NRU50 < 1250 and < 200 represented non-/mildly irritant, moderately irritant and strongly irritant (ingredients) respectively,

\[
\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100
\]

3.6.4 Antioxidant test

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm.

Reagents

1. 2,2-Diphenly 1-picryl hydrazyl solution (DPPH, 100 μM):

DPPH was accurately weighed 22 mg and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 μM DPPH solution.

2. Preparation of test solutions:

21 mg each of the extracts was dissolved in distilled DMSO separately to obtain solutions of 21 mg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.
3. Preparation of standard solutions:

10 mg each of ascorbic acid and rutin were weighed separately and dissolved in 0.95 ml of dimethyl sulfoxide (DMSO) to get 10.5 mg/ml concentrations. These solutions were serially diluted with DMSO to get lower concentrations.

Procedure:

The assay was carried out in a 96 well microtitre plate. To 200 μl of DPPH solution, 10 μl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 2000, 1000, 500, 250, 125, 62.5, 31.25 μg/ml. The plates were incubated at 37°C for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader.

3.6.5 Enzymatic and non enzymatic activity of selected isolate
3.6.5.1 Screening for cellulose producing actinomycetes

The selected isolate was cultured on cellulose agar. The composition of cellulose agar (g/L): yeast extract, 1; carboxy methyl cellulose (CMC), 10; KH₂PO₄, 4; NaCl, 2; MgSO₄·7H₂O, 1; MnSO₄, 0.05; FeSO₄·7H₂O, 0.05; CaCl₂·2H₂O, 2; NH₄Cl, 2 and agar, 20; pH 7.3, and then incubated at 28°C for 5 days. The actinomycete plate was flooded with 0.1% (w/v) solution of congo red and left for 30 minutes, then washed with 1 mL NaCl (1 M) and left for 15 minutes. Cellulase producing colony showed clear zones against red colour of non-hydrolyzed medium. Positive isolate was tested again for confirmation.

3.6.5.2 Screening for pectinase producing actinomycetes

Pure isolate of actinomycete ARITM02 was cultured on pectin agar. The composition of medium in (g/L): yeast extract, 1; pectin, 5; KH₂PO₄, 4; NaCl, 2; MgSO₄·7H₂O, 1; MnSO₄, 0.05; FeSO₄·7H₂O, 0.05; CaCl₂·2H₂O, 2; NH₄Cl, 2 and agar, 20; pH 7.3, and then incubated at 28°C for 5 days. Plate was then flooded with 1% (w/v) solution of polysaccharide precipitant (cetyl trimethyl ammonium bromide), dissolved in 15% alcoholic solution and then used to detect pectinase production. After 1 h of exposure, colony producing pectinase showed clear zones against an opaque colour of the non-hydrolyzed medium.
3.6.5.3 Screening for xylanase-producing actinomycetes:

The actinomycete ARITM02 was cultured on xylan agar. The composition of xylan agar is in (g/L): yeast extract, 1; xylan, 10; KH₂PO₄, 4; NaCl, 2; MgSO₄.7H₂O, 1; MnSO₄, 0.05; FeSO₄.7H₂O, 0.05; CaCl₂.2H₂O, 2; NH₄Cl, 2; and agar, 20; pH 7.3, and then incubated at 28°C for 5 days. The plate was then flooded with absolute ethanol (99% v/v) and left for 1 hour at room temperature. A clear zone against an opaque colour of non hydrolyzed medium showed if xylanase produced by actinomycete.

3.6.6 Antimicrobial activity of pigment

For evaluation of antimicrobial activity of pigment, wells were drilled using a sterile cork borer in fresh test microbial lawn cultures on nutrient agar medium for bacteria and potato dextrose agar medium for fungi. The pigment was then administered to fullness in each well. The plates were put in incubator at 35°C for 24 hours. Bioactivity was determined by measuring the diameter of inhibitory zones (mm) of test microorganisms around the well after incubation. Antibiotic poured in wells served as control. The antibiotic tetracycline was used as a control (7 µg/ml) for bacteria and nystatin (10 µg/ml) used for fungi (Pandey et al., 2011)

3.6.7 Application studies

In the present study, scope for probable application of the bacterial pigment was evaluated for textile, rubber, paper and plastic industries. The experiments are carried out according to Shirata et al., (2000) with some modifications.

3.6.7.1 Textile Materials

A textile material (cotton) which was commercially available selected for the experiment. Material was cut into equal size of 2 cm². Pigment in methanol was used as the stock solution. From this stock solution 2 ml solution was applied to the cloth material in a warm surface. The cloth material was allowed to dry at room temperature for about 1 hour. A white cloth material was taken as a control.

3.6.7.2 Wash performance

The textile material dyed by pigment was tested for wash performance at room temperature. The dyed textile material was washed with soap solution for 30 mints. at room temperature. The textile material was washed with running tap water and allowed
to dry. The result was observed physically with other dyed but unwashed textile material.

3.6.7.2 Rubber Products

Rubber sheet was purchased and melted at 50°C and pigment dissolved in methanol was applied at 40°C. A control was taken untreated with pigment.

3.6.7.3 Paper Products

A piece of ordinary “bond paper”, commercially available in the market was selected for the study. The paper material was cut into equal size of 2 cm². Pigment in methanol (5 mg/L) was used as the stock solution. From this stock solution 200 µl was applied to the paper material on a warm surface and allowed to dry at room temperature for 15 minutes. Paper material without dye was kept as control.

3.6.7.4 Plastic products

Pigment in methanol (5 mg/L) was used as the stock solution. 10% solution of polymethyl methacrylate (PMMA) was prepared in methanol. 250 µl/ml of pigment was added to PMMA (Poly methyl methacrylate) solution from the stock solution separately and mixed well. Poured into a watch glass and kept for 3 hours at room temperature (28 ± 2°C). Adequate care was taken to cover the watch glass well in order to prevent air contact.