



Original Research Article

Effect of Physical and Chemical Parameters on the Production of Red Exopigment from *Penicillium purpurogenum* Isolated from Spoilt Onion and Study of its Antimicrobial Activity

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ABSTRACT

Keywords

Natural colours,
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Exopigment,
Antibacterial

Most of the synthetic dyes that are used for colouring are not environment friendly. There is a good demand for natural colours. The natural pigments that are available are mostly of plant origin and these have many disadvantages. Thus isolation of pigments from microorganisms is an upcoming field. This study was undertaken to screen microbial pigment producers. Out of the several isolates screened. One fungus with bright red exopigment was selected for this study. On performing microscopy and genetic analysis, the isolate was identified as *Penicillium purpurogenum*. Studies on the effect of various parameters showed that it gave maximum pigment production with 2% Xylose, 2% peptone, pH 5.0 and growth at 27 ° C. Characterisation of pigment on visible spectrophotometer showed a prominent peak at 510nm. FTIR analysis showed the presence of amine groups with double and triple bonds. The antimicrobial activity of the pigment showed that it was effective against gram positive and gram negative organisms but did not show antifungal activity. This study will help in finding out alternatives to the synthetic colours which can be used in various applications.

Introduction

The synthetic colours can be obtained from minerals like copper sulphate and lead chromate and various organic compounds like hydrocarbons. These are mostly recalcitrant and xenobiotic compounds and may cause serious health problems. (FDA/IFIC, 1993). Strong consumer demand for natural products has prompted many researchers to look for alternatives to synthetic pigments.

Natural colors are extracted from plants and microorganisms. They have proved to be safe coloring agents. We must however consider the color, stability, yield and price. Many advances in the development of natural colours have been made over the last 25 years, particularly in terms of harmonized legislation. With the advances in processing and formulation technology, there is scope for future development. It is

thought that the natural colour market will grow on a global scale at a greater rate than synthetic colours owing to a continued consumer pressure to 'Go natural' (Latha and Jeevaratnam, 2010). As against widespread use of synthetic dyes not known to be environment friendly, demand for natural pigments for coloring fabrics, foods/feeds, cosmetics and printing inks are increasing. Food colouring now represents a \$1.2 billion global market, with natural colours capturing 31% of the food market, but growing at a rate of 5% (Gupta *et al.*, 2011).

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. It also has immense potential for various industrial applications (Venil.C.K. *et al* review, 2009). The present study has the objective of optimising the growth conditions for maximum pigment production by the fungus isolated from spoiled onion and studying the antimicrobial property of the pigment. This research work would add to the number of potential pigment producers and it is possible applications as a coloring agent.

Materials and Methods

- 1) Screening of microbial strain: Various samples were used for the screening. The fungal strain used in this study was isolated from spoiled onion and it was found to produce a red exopigment. The onion sample was inoculated in Sabourauds broth and further isolated on Sabourauds agar. For further studies the culture was maintained on Sabourauds agar slants and stored at 4°C.
- 2) Identification: Identification of the isolate was done by conventional and genetic analysis methods. The fungal isolate was studied microscopically by the use of the moist chamber technique, an in situ technique to study fungi. The fungus was also studied on Environmental scanning electron microscope (E-SEM) to study the structural details. The sample was teased and mounted on carbon holder and coated with platinum by sputtering. The genetic identification studies were carried out on Panfungal DNA detection kit and 3500DX genetic analyser by PCR sequencing method. The assay targeted the multicopy genes, the ribosomal DNA (rRNA) genes (18S, 28S and 5.8S) and the intervening internal transcribed spacer (ITS) regions (ITS1 and ITS 2). The fungal identification was done by sequencing of the amplified product followed by BLAST analysis with the sequences in Genebank Database.
- 3) Measurement of pigment: The different parameters were studied with respect to the amount of colour produced and the dry weight using visible spectrophotometer. This was used to measure the AU/gm (Absorbance unit per gram) of the pigment. Prior to this the maximum absorbance was found by visible scan of the sample (Gunasekaran and Poorniammal, 2008).
- 4) Inoculum preparation: The culture suspension was made in saline + tween 80 (1% w/v). Spore count of the culture was taken on hemocytometer and was adjusted to 10^6 spores/ml and used for all the experiments. All the inoculations were done in triplicates.
- 5) Effect of chemical and physical parameters on pigment production. The media was inoculated with 1×10^6

spores/ml of the culture. The flasks were incubated at 27°C for 5 days.

- i. pH –50 ml of sterile Sabourouds broth was adjusted to pH 3,4,5,6,7,8,9. The pH was adjusted with 0.1 N HCl and 0.1N NaOH.
- ii. Temperature- 50 ml of sterile Sabourouds broth (pH 5) was inoculated and incubated at 4°C, 27°C, 37°C, 55°C for 5 days.
- iii. Agitation - 50 ml of sterile Sabourouds broth (pH 5) was inoculated and incubated at 27°C on rotary shaker for 5 days. Also a control flask at static conditions was maintained to compare the yield.
- iv. Media components.
 - a. **Carbon sources** – 2% glucose, 2% fructose, 2% Xylose, 2% Ribose, 2% arabinose, 2% Maltose, 2% lactose, 2% sucrose, 2% cellulose, 2% starch, 2% Xylan in 50ml sabourouds broth were inoculated and incubated.
 - b. **Nitrogen sources** – Different organic (1% beef extract, 1% yeast extract, 1% meat extract, 1% peptone and 1% peptone + yeast extract) and inorganic nitrogen sources (0.02% sodium nitrate, 0.02% ammonium phosphate, 0.02% ammonium chloride, 0.02% potassium nitrate, 0.02% ammonium sulphate and 0.02% ammonium nitrate) were used in 50ml of sabourouds broth were inoculated and incubated.
 - c. **Trace elements and salts** - 50 ml of sterile Sabourouds broth (pH 5) containing 1% NaCl, 1% KCl, 0.01% Magnesium sulphate, 0.01% Manganous chloride, 0.01% Cobalt chloride, 0.01% Molybdenum chloride, 0.01% Copper sulphate, 0.01% Nickel chloride, 0.01% mercuric chloride, 0.01% Zinc

sulphaite, 0.01% silver nitrate were inoculated and incubated.

- 6) Extraction: Extraction of the pigment with different non- polar solvents like benzene, chloroform, petroleum ether, butyl acetate, ethyl acetate. Before the extraction the samples were acidified with 1% sulphuric acid and pH adjusted to 2 (Cho *et al.*, 2002).
- 7) Pigment characterization: Preliminary identification of the pigment was done based on the Visible and IR spectra of the pigment (Unagul *et al.*, 2005; Palanichamy *et al.*, 2012).
- 8) Antimicrobial activity of pigment extract: Antimicrobial activity of the pigment was done on gram positive (*Staphylococcus aureus*, *Corynebacterium diphtheriae*), gram negative (*Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*) and fungi (*Aspergillus niger* and *Candida albicans*) by agar diffusion method. Butyl acetate controls were maintained.

Results and Discussion

Screening of pigment producers - 25 organisms were isolated based on visual identification. Some of producers produced extracellular pigments and others intracellular (Table 1). The bright red colour of the pigment and its extracellular nature was important in the choice of the culture to be chosen for further experiments.

Identification and characterization of fungus: The results of moist chamber technique and E-SEM images showed that the hyphae and sporangium were typical of *Penicillium* (Fig. 1). Further identification by genetic analysis showed 98 % similarity

to *Penicillium purpurogenum*.CASMB-SEF

7 (Accession number: JQ663996) (Fig. 2).

Sequence of SR2

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QCCTGCGGAAGGATCATTACTGAGTGCGGACCCCTCGCGGGTCCAACCTCCCACCCG
TGTCTCTTGAATAACCTGTTGCTTTGGCGGGCCACCGGGTGCCTCCCGGTCGCCGGG
GGGCACTGCGCCCCCGGGCCTGCGCCCGCCAGAGCGCTCTGTGAACCCTAATGAAG
ATGGGCTGTCTGAGTGTGATTTTGAATTATCAAACTTTCAACAATGGATCTCTTGGT
TCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCAT
GCCTGTCCGAGCGTCATTTCTGCCCTCAAGCGCGGCTTGTGTGTTGGGTGTGGTCCCC
CCGGTGTGGGGGGACCTGCCCGAAAGGCAGCGGCGACGTCCCGTCTAGGTCCTCG
AGCGTATGGGGCTTTGTACCCGCTCGGGAGGGGCCTGCGGGCGTTGGCCACCCAC
GATAAttttttACCGTTGACCTCGGATCAG
GTAGGAGTTACCCGCTGAACTTAAGCATATCAATAAGCGAAGGAA (585bp)
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Effect of physical and chemical parameters on pigment production

Effect of pH and temperature – The pigment producer was inoculated at various pH and incubated. There is an important role that the pH of the medium plays in pigmentation. From the figure 7, the optimum pH for the biomass and pigment production was found to be 5.0(2.02AU/gm) in Sabourauds broth. The pH of the broth after the incubation and pigment production was found to range from 4.0 to 5.0 showing that the pigment was synthesized under acidic conditions. The organism grew at a lower pH of 4.0 but the pigment production was reduced. Gunashekar et al have reported a pH of 9.0 for another species of *Penicillium*. In a similar study, Cho *et al.* (2002) have reported the pigment production at pH 6.0 for *Paecilomyces sinclairii*. Many fungal pigments are produced under acidic conditions. The temperature effect was studied by incubation at different temperatures. The temperature optimum for pigment and biomass production was 27°C and the pigment produced was 110.04AU/gm (Fig. 3). There was good biomass production at a temperature of 30°C but there was less production of pigment. As the temperature increases the pigment

production ceases. Studies show similar results of temperature for other fungal pigment producers. Since the isolate grew at room temperature, bulk production will not need temperature to be monitored strictly.

Effect of agitation – Agitation allows the oxygen to dissolve in the medium. However for this study, agitation was seen to produce less pigment and biomass as compared to static conditions. The amount of pigment produced was 4.01AU/gm under static conditions. Even if the organism is aerobic, agitation was not preferred for both the biomass and pigment production. When agitated the exopigment either did not get produced or produced in small amounts which may be due damage to the filamentous growth (Fig. 4).

Effect of carbon sources – The effect of different sugars showed that, Xylose was the most utilized (or preferred) monosaccharide for pigment as well as biomass production. From the figure 5 it can be seen that the organism did not give good growth and pigment production in glucose or fructose. Amongst the disaccharides, sucrose and lactose were utilized maximally. Starch and cellulose were not utilised for pigment production. However the organisms could

utilize Xylan with 10.4 AU/gm of the pigment (Fig. 5). The pigment was produced maximum at 2% Xylose concentration (8.41AU/gm). With an increase in the concentration of Xylose, the biomass increased but there was no maximum pigment produced (data not shown).

Effect of organic and inorganic nitrogen sources - Results of maximum pigment production were seen with Sodium nitrate as inorganic source and peptone as organic source at concentration of 2% (Fig. 6). The other sources namely Yeast extract, meat extract, beef extract did not give comparable pigment production. Thus a non synthetic media can be used since peptone may also provide the growth factors for the culture.

Effect of salts and trace metals- From the figure 7, it can be seen that the presence of sodium chloride and Magnesium sulphate enhanced the production of pigment. However potassium chloride did not give maximum pigment.

Extraction of pigments – Esters gave good extraction of the pigment as seen from the results. It was observed that the pigment could be best extracted in n - butyl acetate and ethyl acetate (Table 2) following acidification with 1% sulphuric acid to a pH of 2.0. The amount of pigment has been more in butyl acetate as compared to petroleum ether (Fig. 8). Arai Tepei *et al.* (2013) have used ethyl acetate to extract the pigments from *P. purpurogenum* IAM15392. Cho *et al.* (2002) have shown the extraction of red pigment from *P. sinclarii* with n-butyl acetate in acidified broth.

Characterisation of pigment: Visible scan of the crude pigment at different wavelengths showed that the pigment absorbed maximum at 510 nm. There was a prominent peak

observed at 510 nm and a small peak at 450 nm (Fig. 9). Unagul *et al.* (2005) have reported 500nm maximum absorbance for red pigment isolated from insect pathogenic fungus. FTIR results showed that there was presence of primary and secondary alcoholic groups and double and triple bonded amine groups in the pigment (Fig. 10). Jagannadham *et al.* (1991) have studied the UV visible absorption spectra of carotenoid pigments from Psychrotrophic bacteria *Micrococcus roseus*.

Antimicrobial activity of pigment extract: Agar diffusion of the butylacetate extracts showed antimicrobial activity against both gram positive and gram negative organisms. However no anti fungal activity was observed (Fig. 11). The butyl acetate control did not show any activity showing that the solvent did not affect growth. Venil and Lakshmanaperumalsamy (2009) studied significant antimicrobial activity with prodigiosin and violacein pigments from bacteria. Gulani *et al.* (2012) has also studied the production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potential. Visalakchi and Muthumary (2009) have studied the antimicrobial activity of endophytic fungus *Monodictys castaneae* SVJM139 pigment on different bacterial strains and the pigment showed inhibition towards all the strains.

The pigments available belong to synthetic chemicals which have a large number of disadvantages. With the understanding of the disadvantages of these synthetic colours there is a constant need to search for biological pigment producers. Hence the use of pigments from natural origin is explored. This study is the preliminary step to identify extracellular pigments from fungi. Fungal pigments have proven to give good results in various applications like food, textile and

pharmaceutical applications. The present study can be extended to finding out the

chemical structure of the pigments and its use in the making of antimicrobial products.

Table.1 Screening of pigmented organisms from various niche

S.no	Organism	Site	Colour	Gram character
1.	SO1	Air flora	Orange	Gram positive cocci
2.	SO2	Air flora	Pale Orange	Gram positive cocci in tetrads
3.	SO3	Air flora	Dark orange	Gram positive cocci in clusters
4.	SY6	Air flora	Yellow	Gram positive diplococci
5.	SO5	Air flora	Orange	Gram positive cocci
6.	SO6	Air flora	Orange	Gram positive cocci
7.	SO7	River water	Orange	Gram positive cocci
8.	SO9	River water	Pale orange	Gram positive cocci
9.	SY5	Air flora	Yellow	Gram positive cocci
10.	SO12	Salt pans	Dark orange	Gram positive cocci
11.	SO13	Salt pans	Orange	Gram positive cocci
12.	SO14	Salt pans	Orange	Gram positive cocci
13.	SY1	Air flora	Yellow	Gram positive cocci in tetrads
14.	SY2	Mangrove water	Turmeric yellow	Gram positive cocci
15.	SG4	Sea water	Yellow green (exopigment)	Gram positive large cocci
16.	SR1	Garden soil	Red	Gram negative short rods
17.	SR2	Rotten onion	Red(exopigment)	Fungi(<i>Penicillium purpurogenum</i>)
18.	SW1	Air flora	White	Gram positive cocci
19.	SW2	Air flora	White	Gram positive rods
20.	SB1	Garden soil	Brown(exopigment)	Actinomycete (<i>Streptomyces hygrosopicus</i> subsp. <i>jinggangensis</i> TL01)
21.	SR3	Mangrove water	Red (exopigment)	Fungi (<i>Lasiodiplodia theobramae</i>)
22.	SY4	Air flora	Yellow	Gram positive cocci
23.	SV1	Petroleum soil	Violet	Gram negative short rods
24.	SB2	Sub zero temperature soil	Brown (exopigment)	Actionomycetes
25.	SB3	Garden soil	Brown (exopigment)	Fungi

Table.2 Absorbance of pigment using different solvents for extraction

Solvent used	Absorbance before extraction in solvent (510nm)	Absorbance (510nm) Aqueous phase	Absorbance (510nm) Non aqueous(solvent) phase
Benzene	2.5	2.490	0
Petroleum ether	2.5	2.5	0
Chloroform	2.5	2.491	0
Ethyl acetate	2.5	2.499	0.518
Butyl acetate	2.5	2.433	0.633
Di ethyl ether	2.5	2.499	0

Fig.1 Moist chamber technique and E-SEM images of SR2

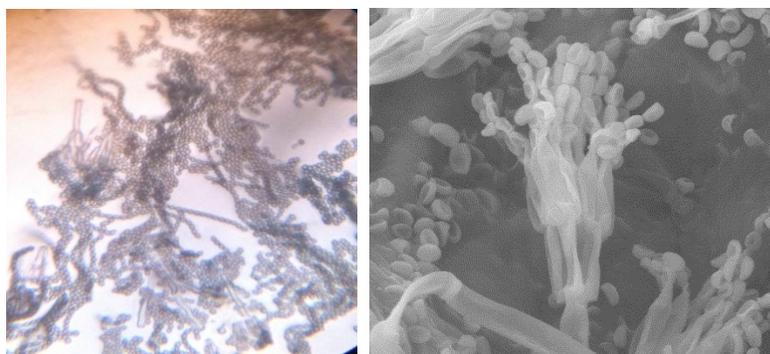


Fig.2 Phylogenetic tree showing the relationship of Query Isolate with *Penicillium purpurogenum* strain CASMB-SEF 7

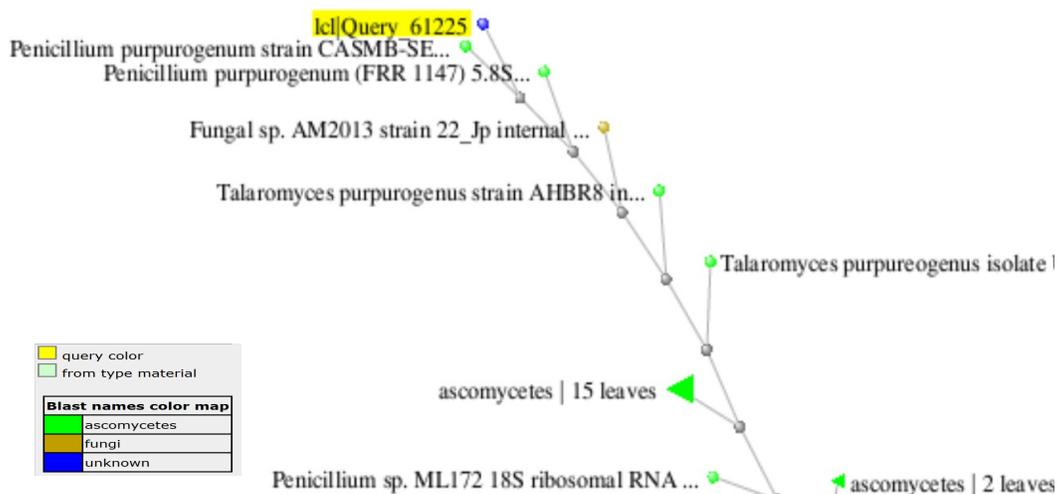


Fig.3 Effect of pH and temperature on the pigment production of *P. pupurogenum*

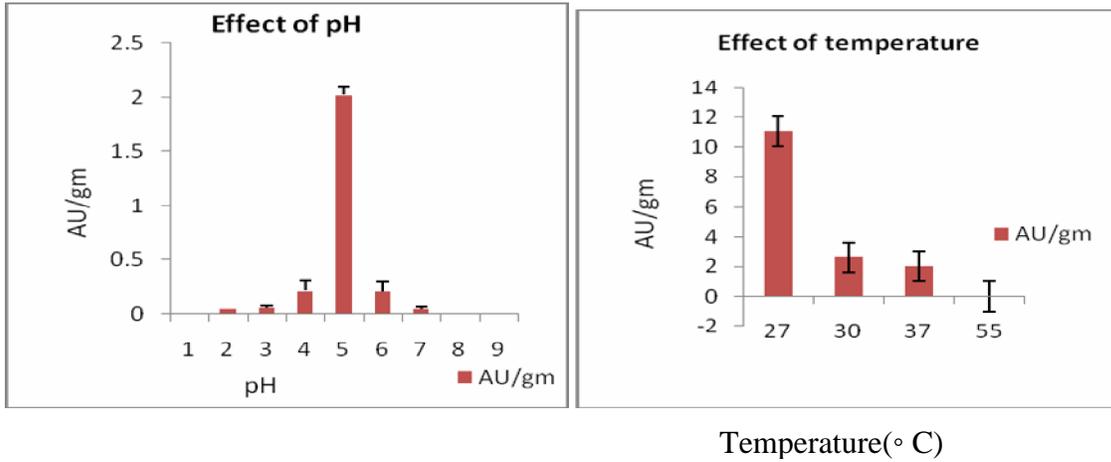


Fig.4 Effect of agitation on the pigment production of *P. pupurogenum*

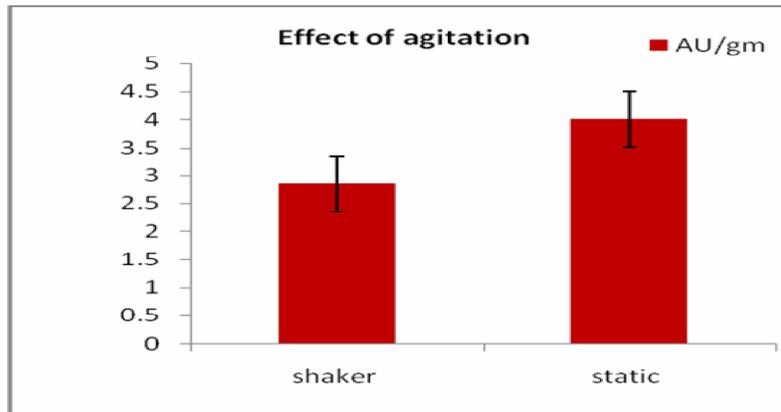


Fig.5 Effect of different sugars on the pigment production of *P. pupurogenum*

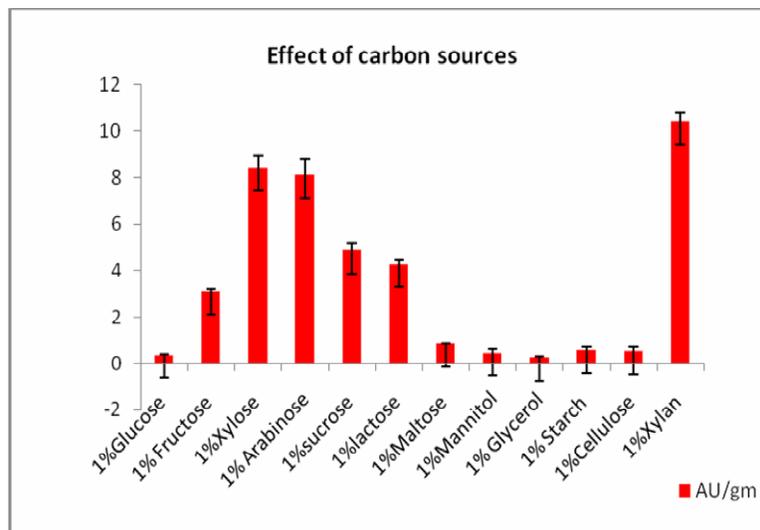


Fig.6 Effect of inorganic and organic Nitrogen sources on pigment production

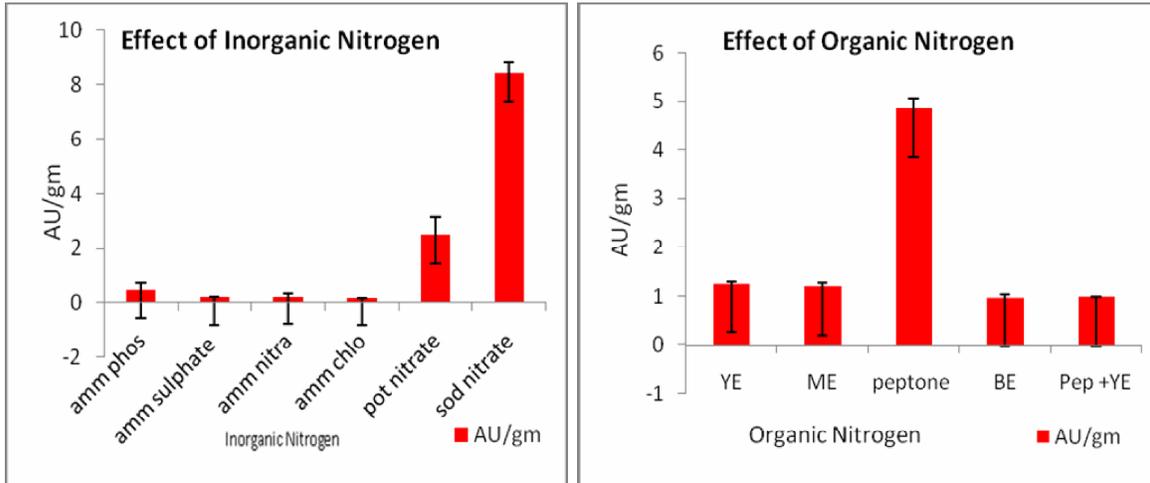


Fig.7 Effect of salts on pigment production

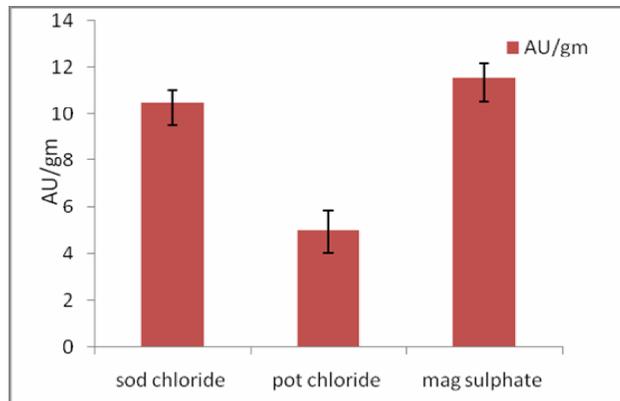


Fig.8 Comparison of pigment extraction in different solvents

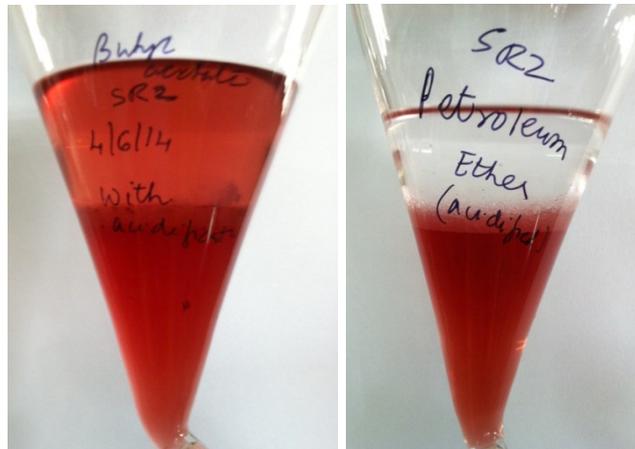


Fig.9 Visible spectra of red pigment from SR2

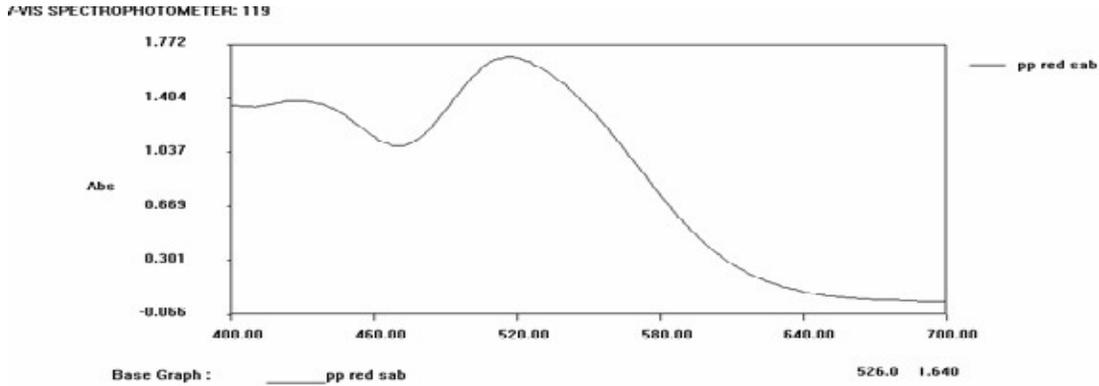


Fig.10 FTIR spectra of SR2 pigment showing the presence of primary and secondary alcohols and amines with double and triple bonds

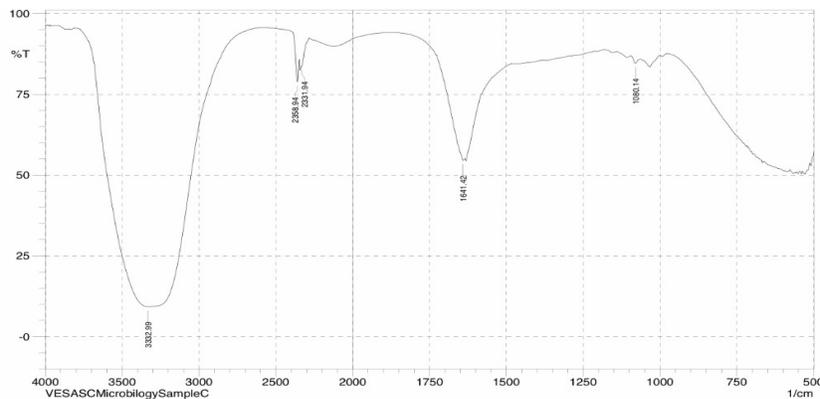
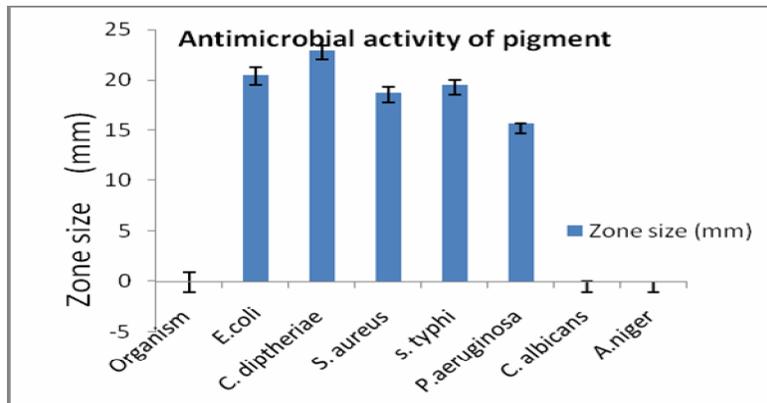


Fig.11 Antimicrobial activity of the butyl acetate extract against gram positive and negative organisms



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