A comprehensive Study of Biological activity of Natural pigment and its Applications

Ramendra Singh Parmar*[1] and Charu Singh[2]
[1]ITM University, Gwalior
[2]K.R.G. PG Girls College, Gwalior

ABSTRACT

Actinomycetes, a large group of filamentous bacteria account for 70-80% of secondary metabolites available commercially. The present investigation was undertaken with an aim to identify and characterize pigment from actinomycetes. Actinomycetes were isolated from rhizosphere soil samples collected from different regions of Madhya Pradesh state. Out of 85 actinomycetes, only 7 actinomycetes showed pigment production and based on diffusible pigment production ability one actinomycete ARITM02 was selected. The extraction of pigment was done by solvent extraction method using methanol and purified by TLC and column chromatography. The results suggested that no enzymatic activity was shown by pigment. The pigment showed excellent results to dye various materials. The conclusion of study suggested that this novel pigment could be a versatile natural, safe and multipurpose.

Keywords: Pigment; Antioxidant

Introduction

Colours have been widely used in many industries such as textiles, food, painting, cosmetic and pharmaceuticals and they play an important role in providing an attractive look to the product. As a food colorant, they used as an additive in food industries and play a significant role due to safety and serious environmental problems caused by artificial and synthetic pigments research has been focused on research of new natural pigments to use in food and pharma industries (Lu *et al.*, 2009).

Microorganisms and plants both are two major sources which can produce natural pigments. The pigment production from microorganisms is very advantages because they can produce pigments on a cheap medium and they have fast growth. The growth of microorganisms is free from weather conditions and can be produced with different shades of colours. It is an emerging field of research which demonstrate its advantages for various industries (Mohanasrinivasan *et al*, 2013).

There are several organisms which produce many varieties of intracellular and extracellular pigments including melanin with different biological functions. Pigments were primarily used as a colouring

agent in various industries, from the past decade. Researchers have focused the usage of natural pigments for various industries including pharmaceutical for antitoxic and antioxidant agents (Prakash *et al*., 2001).

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^{o}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).

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 readymade 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 *(Rf)* value was calculated according to the following equation from the chromatogram **(**Willson and Walker, 2010).

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

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 R*f* 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.

Enzymatic and non enzymatic activity of selected isolate

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; KH_2PO_4, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 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 mints, than 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.

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; KH_2PO_4, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 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.

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; KH_2PO_4, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 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.

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 (7µg/ml) for bacteria and nystatin (10µg/ml) used for fungi (Pandey *et. al.*, 2011)

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.

Textile Materials

A textile material (cotton) which was commercially available selected for the experiment. Material was cut into equal size of 2 cm^2. 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.

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.

Rubber Products

Rubber sheet was purchased and melted at 50^0C and pigment dissolved in methanol was applied at 40 ^{0}C. A control was taken untreated with pigment.

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 cm^2. 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.

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.

Extraction and purification of pigment

The pigment was extracted by using different solvents. The methanol was found suitable solvent for extraction of pigment. The crude pigment compound was screened for number of components by Thin-Layer Chromatography (TLC) plate. The Methanol:acetone:water: (4:4:2) was found best solvent system for TLC. A dark red single spot along with very light spot was found after spray of ninhydrin (Figure 4.14). The R*f* value was found 0.76. The pigment obtained was stored in an ampoule at 4°C.

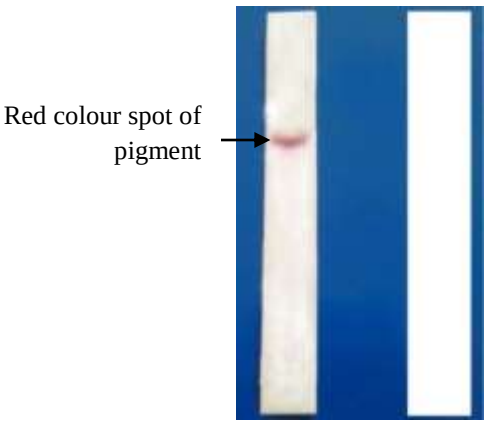

Fig. 4.14 Chromatogram of pigment.

Enzymatic and non enzymatic activity of selected isolate ARITM02

The results indicate that all enzyme assays was found negative for selected isolate ARITM02. In all enzymatic assays no zone of inhibition was found in Petri plates so it is concluded that the test enzyme production ability was not present in selected isolate ARITM02.

Applications of pigment

Results clearly indicate that pigment produced by isolate ARITM02 can be effectively used to dye different types of materials like textile material, paper, plastic and rubber. The wash performance suggests that dyed material can be washed at 50^0C (Figure 4.24).

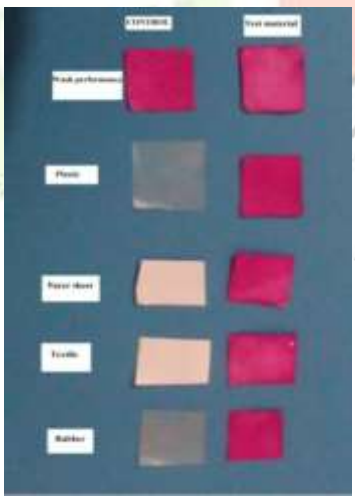

Fig. 4.24 Effect of pigment on various dyed material and wash performance of pigment.

Discussion

Rhizosphere soil is a key source and treasure of microbes with great diversity. Among the different groups of microbes, actinomycetes in soil play important role in biological activities like nutritional sources,

antagonism, degradation of various organic matters, fixation of nitrogen and enhancement of growth promoting substances.

These abilities of soil have enhanced the interest towards isolation of actinomycetes from soil. Actinomycetes have been the source of antibiotics, natural pigment producing ability and other non-antibiotic molecules of pharmaceutical interest such as antioxidant ability of pigment. In this study the pigment producing actinomycetes was isolates, identified and characterized along with its biological applications with industrial utilization.

Natural pigments, isolated from microorganisms are useful alternative over chemically synthesized colours. In recent research on natural pigment many researchers showed applications of natural pigment in textile industries as a natural colorant, in plastic industries to generate different shades of colours in plastic products, in paper industries and to colour plastics. The lab scale showed that natural pigment, isolated from actinomycete ARITM02 has excellent ability to colour above materials.

The results of study are supported by the results found by Krishna *et. al.*, (2008), which also observed good results after implying natural red pigment on different material like plastic, papers, textile samples and rubber.

Venil *et al.*, (2013), also showed results which are identical with present studies. In the study, the natural pigment was isolated from bacteria was used to colour textile and cotton material. They also checked the application of pigment in food, pharmaceutical industries.

Krishna *et al.*, (2013), reported different applications of pigment. They applied various methods on different textile materials like paper, plastic, rubber and textile materials. The present research fully supports the results and found similar results.

References

Lu Y., Wang L., Xue L., Zhang C., Xing X. H., Lou Z., Z., Li Y., Zhang G., Bi J., Su Z., "Production of violet pigment by a newly isolated psychrotrophic bacterium from a glacier in Xinjiang, China", Biochem. Eng. J. 43., 135–141, (2009).

Mohanashrinivasan V., Sriramkalyan P., Ipsita N., Subhadradevi C., Selvaranjan E., Suganthi V., Jemimah, N.S., "Fermentative production of extracellular pigment from *Streptomyces coelicolor"*, Res. J. Biotech., 8(4)., 1-8, (2013).

Prakash F., Rigelhof J., Miller E.,"Medallion laboratories analytical progress: Antioxidant activity, Medallion Laboratories", In J. De Vries, 19(2).,1-6, (2001).

Venil C.K., Zakaria Z.A., Ahmad W.A.,"*Bacterial pigments and their applications*", *Process* Biochem., 48*(7).,* 1065–1079, *(*2013).

Krishna J.G., Basheer S.M., Beena P.S., Chandrasekaran M., "Marine bacteria as source of pigment for application as dye in textile industry", Proc. Internatl. Conf. Biodiv. Conserv. Mgt., 743–4, (2008).