ANTIMICROBIAL ACTIVITY AND CYTOTOXICITY TESTING OF BLUE PIGMENT BY *STREPTOMYCETES* SPECIES

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Summary

Streptomycetes sp was isolated from lakh soil. This strain effectively produced pigment by submerged fermentation. It found to produce blue pigment with antibiotic activity. The extraction of crude pigment with ethyl acetate yielded bioactive crude extract that displayed activity against a panel of clinical pathogens tested. The crude pigment was assayed by TLC with the solvent system. R_f value calculated as 0.51. Antimicrobial activity of the partially purified pigment from Thin layer chromatography was determined using the bioautography method. The pigment showed minimum of 8 mm inhibition against *Proteus mirabilis* and a maximum of 23 mm inhibition against *S. aureus* in the well diffusion method. The cells appear after the inoculation of the pigment and incubation of HEP_2 and RD cell line as normal cells it shows there is no toxicity to the cells and there is no morphological change in the cells. It showed no cytopathic effect (CPE) while inoculated in mice and they were observed for 14 days. The characteristics of the pigment can be exploited in the food industries and Textile industries.

Keywords: *Streptomycetes sp,* blue pigment, Cytotoxicity, cell line.

Introduction

Microbial secondary metabolites are important sources of natural compounds. As one type of versatile microorganisms, the *Streptomycetes* are potent producers of secondary metabolites. So far, approximately 10,000 antibiotics have been identified, and almost half of them are generated by *Streptomycetes* [1,2]. A variety of bioactivities are associated with secondary metabolites generated by *Streptomycetes*, including antibacterial, antifungal, antitumor, and enzyme inhibitory activities. Soil is the most common habitat for *Streptomycetes* [3]. They have provided more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds [4]. Streptomycetes represent an important source of biologically active compounds [5].

Streptomycetes sp., is a kind of Actinomycetes which can synthesize blue pigment. This is one of the edible color are widely used in food industry now-a-days. With the recent understanding of the harmfulness of synthetic food colours, natural pigments are being increasingly emphasised.

The synthetic colors are hardly nutrients, and to some extent they are potentially carcinogenic. In order to compensate this naturally synthesized colors are widely used which have the natural character, safety and use as additives. These natural pigments are soluble, have stability and have safety measures these important indices make to use in application in food industry.

Streptomycetes sp. produced pigment can inhibit most gram positive bacteria such as *Staphylococcus aureus* at a relatively high concentration [6] but it has, no effect on Gram negative bacteria. The aim of the present work was the production of blue pigment from *Streptomycetes* spp. The blue pigment which showed antimicrobial activity. Cytotoxicity testing of blue pigment from *Streptomycetes* sp., were checked.

Materials and methods

Strain, Media and Fermentation conditions

S. coelicolor 10 was screened from soil and identified [7]. The spore suspension was spread onto slant medium agar plates (20 g mannitol; 20 g soy bean flour; 20 g agar and 1 liter distilled water at pH 7.5) and incubated for 7-10 days at 30˚C to allow for sporulation. To the fermentation medium (20 g mannitol; 20 g soy bean flour; 0.5 g NaCl; 2.28 g K_2HPO_4; 0.5 g $MgSo_4.7H_20$ and 0.01g $FeSo_4.7H_2O$ in 1 l of distilled water at pH7.5) the washed mycelium culture was incubated for 7 days at 30˚C, pH 7.2-7.5, with aeration at 3 L/min agitation at 300rpm [7].

Refinement of the blue pigment from the fermentation broth

Fermentation broth was adjusted to pH 12 with NaOH and centrifuged at 4,000x g for 15 minutes. The supernatant was collected and adjusted to pH 2 with 2M HCl. After centrifuging the amaranth sediment, the crude blue pigment, was harvested and dried in a vaccum. A yield of about 3 g blue pigment /L of fermentation broth were taken for refinement of the pigment [8].

Pigment measurement and its residual ratio

The maximum absorption wavelength of pigment solution is pH dependent and pigment content in the solution can be measured by optical density (OD). OD values are determined by using 588 nm and pH 9. The pigment residual ratio (%) is determined as the ratio of OD value after treating to that before treating with cells.

TLC Assay

Silica coated glass plates containing a UV- fluorescent indicator were used with benzene, ethyl acetate as solvent. Pigment spots were visualized under normal and UV light. R_f values for blue pigment was 0.51.

Antimicrobial activity by using agar well diffusion method

Muller Hinton agar plates were prepared well was made by using well puncture in uniform diameter and lawn culture of the test organisms were made and producer supernatant (10µl, 20 µl, 30 µl) were poured into each well and kept at 4˚C for 10-12 hrs to allow the pigment to diffuse into the agar. The plates were then incubated at 37˚C for 24 hours and zone of inhibition were measured in mm diameter. The organisms used were *Staphylococcus aureus, Streptococcus pyogens, Corynebacterium diptheriae, Streptococcus pneumoniae, Escherichia coli, Salmonella typhi, Proteus mirabilis.*

Propagation and counting of RD and HEP_2 cells

Growth medium from the cell culture flask was made to wash so that there will be a confluent cell layer with PBS (without Ca and Mg components).Add TPVG solution to the monolayer for dispensing it evenly using disposable pipette leaving just few drops to moisture the cells. Check visually for complete detachment of cells, cells counting was done by using Haemocytometer, dilute 100µl of cell suspension and dilute with 200µl of trypan blue. Count only the viable by omitting unstained cells. Calculation was done by using the formulae,

$$C_1 = n/8*d*10000 = cells/ml$$

where, $d = C_1/C_2$ (ie)., initial cell concentration * working cell concentration.

Preparation of cell culture tube and inoculation of blue pigment

Propagation cells are used for preparation of cell culture tubes. Observe the tubes under inverted microscope after 48 hours of incubation (ie)., after trypsinization for the tubes showing cell sheath tubes showing about 80% confluence are RD cells and HEP_2 cells for each specimen to be inoculated, and one as control. Inoculations of the crude extracellular blue pigment incubate at 36˚C±5˚C. Before incubation replace growth medium with 1 ml maintenance medium. Care should be taken that both cell lines must be inoculated at same time.

Results

Solubility and coloring of blue pigment

The blue pigment was soluble in organic solvents such as acetone, ethanol, and methanol and in water by changing pH. The pigment become red in acidic conditions and was blue at low pH. Its property of changing color with the change in pH value will help in food processing and also to have other applications.

This change in pH can be determined by the maximum absorption wave length (λ_{max}), of the pigment. As the pH value increases, the absorption spectrum also increases. But in there organic solvents there are no such type of change (Table 1).

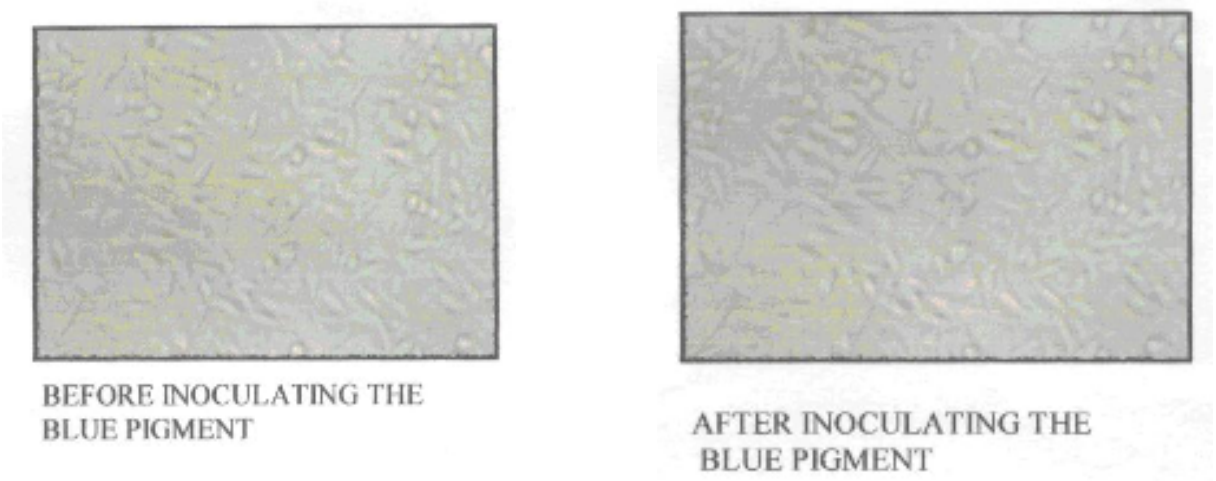
Antimicrobial activity of blue pigment

The organisms *Staphylococcus aureus, Streptococcus pyogens, Corynebacterium diptheriae, Streptococcus pneumoniae, Escherichia coli, Salmonella typhi, Proteus mirabilis* used for the antimicrobial activity showed remarkable zone of inhibition against Gram Positive organisms, especially towards *Staphylococcus aureus* (Table 2). The extracted crude blue pigment shows antimicrobial activity against various Gram positive organisms zones were formed in various diameter while giving various concentrations.

Cytotoxicity testing in RD and HEP_2 cells lines

Cytopathic effect (CPE) not appear. Refractile cells are being attached in the surface of the tube. Performed a blind passage and continued examination for further seven days. Negative cultures examined for 14 days before discarding. No toxicity were observed. So, in both the cell lines HEP_2 and RD cells showed there is no lysing of cells after the addition of the blue pigment (Fig. 1).

Figure 1: HEp2 Cell lines



Mouse acute toxicity trial

Toxicity investigation of a substance falls into three categories in toxicology, acute, short-term and long term toxicity. In the acute toxicity trial nearly 40 mice were divided equally into 4 groups for the different pigment doses of 5000, 10000 and 15000 mg / kg according to Horns method of oral administration twice a day. During a 14 day trail, no mouse died in any dose group. It was verified that the mouse death in the preliminary experiment resulted from taking an overdose pigment once by oral gavages.

From the above results, it is referred that the blue pigment from *Streptomyces coelicolor* is non toxic.

Table 1: Effect of pH value and solvent on color λ_{max} of pigment solution

Solvents or pH	Color	λ_{max}(nm)
Water, pH 3	Red	465
Water, pH 5	Red	475
Water, pH 3	Amaranth	500
Water, pH 3	Blue	565
Water, pH 3	Deep blue	595
Methanol	Red	545
Ethanol	Red	530
Acetone	Red	530

Table 2: Antimicrobial activity against Gram Positive microorganisms by well diffusion method

Organisms	Zone of inhibition in mm (10µl)	Zone of inhibition in mm (20µl)	Zone of inhibition in mm (30µl)
S. aureus	18	21	23
S.pyogens	13	14	18
Corynebacterium diptheriae	14	17	17
S.pneumoniae	12	17	18
E.coli	9	12	13
S.typhi	10	11	11
P.mirabilis	8	9	9

Conclusion

The blue pigment from the fermentation broth of *S. coelicolor* with a yield as high as 2.5g/L. It is soluble in alkaline water and organic solvents except in petroleum ether. The color of the pigment changes if there is a change in pH of water from 3 to 12. The blue pigment depends mainly upon the growth conditions which yields the blue pigment as extracellular product has the anti microbial activity. It also shows no cytotoxicity while inoculated into animal cell lines. It showed no cytopathic effect (CPE) while inoculated in mice and they were observed for 14 days.

The cells appear after the inoculation and incubation of HEP_2 and RD cell line as normal cells it shows there is no toxicity to the cells there is no morphological change in the cells so it can be used as food additive. From, this result we can conclude that the blue pigment produced by *Streptomycetes coelicolor* pigment has no cytotoxic effect, so this pigment can be used as food additive in food industry.

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