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OPTIMIZATION OF CULTIVATION PARAMETERS FOR GROWTH AND PIGMENT PRODUCTION BY Streptomyces spp. ISOLATED FROM MARINE SEDIMENT AND RHIZOSPHERE SOIL

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ABSTRACT: Biological pigments are a better substitute to chemical dyes used in the industries and laboratories. Out of the many species of Streptomyces present ubiquitously in soil, S. coelicolor and S. violaceoruber produce an important red-blue antibiotic actinorhodin and associated compounds like α-, β-, ε-, γ-actinorhodin collectively known as Actinorhodin-Related "Blue Pigments". These pigments have a wide range of applications in scientific, medical and industrial sector. So, it is a beneficial alternative
method to isolate these microorganisms and extract actinorhodin, rather than producing chemical colouring agents which have harmful effects on humans and environment. Several solid media are available to cultivate these species but it is difficult to extract the pigment using solid substrate. There are many liquid media which support the growth of the organism, but since pigment production depends greatly on culture conditions, it is necessary to standardize the various parameters that may affect the product quality and quantity. Therefore, an optimum culture media has to be formulated. The soil samples were collected from fields and coastal areas of Chennai and Streptomyces species were isolated. The cultures were screened for actinorhodin production and biochemical characterization was done. It was mass multiplied in broth culture to extract the pigment. The medium was optimized using a basal medium substituted with various carbon and nitrogen sources. Temperature and pH were standardized by maintaining the cultures in required conditions. The isolated organism was found to be Streptomyces violaceoruber. YMG medium was found suitable for growth and Bottcher-Conn's medium showed higher pigment production. Actinorhodin was extracted using NaOH/HCl system. On optimization, the best carbon sources were found to be glycerol and mannose. The most favourable nitrogen sources were peptone and sodium caesinate. The optimum temperature range was 28-30°C and optimal pH was found to be between 7.6 and 8.0.

Key words: Rhizosphere soil, Pigment production, *Streptomyces spp.*

INTRODUCTION

Microorganisms are an indispensable component of our industries. They have been employed for the production of bread, cheese, beer, antibiotics, vaccines and many other important products. Modern biotechnology rests upon a microbiological foundation. In the earlier times, mostly plant and animal sources were used for extraction of industrially important compounds. But these processes had many drawbacks. It led to the loss of valuable species, were expensive, posed an ethical dilemma and large quantities were difficult to produce. But it poses a great threat in form of hazardous wastes like toxic metal compounds, salts, particulate matter, sewage etc. dumped into our environment.

In the modern times, industries have resorted to using microbes as a source of many valuable and industrially important compounds like antibacterials, antitumorals, antifungals, vitamins, enzymes etc. A latest addition to this list has been pigments. Natural pigments have been used since the beginning of organized society, developed so humans could paint their bodies, clothes, houses, weapons and religious icons.

The colours were obtained from plants, animals, fruits and earth. It includes indigo, cochineal (the bug parasite of the prickly pear cactus leaf), moss, nut shells and leaves, wild flowers, tree bark, and even a sea snail that emits a deep purple ink. Natural dyes are scarce, higher priced, and require a much longer, more complex process to produce. Microbial pigments provide a safer alternative to chemical synthetic dyes and an economical substitute for natural pigments obtained from plants and animals. Unlike chemical dyes, many of which have been found to cause diseases like cancers, allergies, etc., most of the microbial pigments are safe for human use, and some even have antibiotic or anticancer properties. Few are also certified as food grade pigments. They are easy to produce as compared to other natural pigments and are economic as well. A major industrially important genus, Streptomycetes has been exploited to produce a wide range of antibiotics. But many Streptomyces species also produce pigments. However, pigment extraction from Actinomycetes on an industrial level is still not in practice due to many underlying causes like unavailability of protocols suitable for large scale fermentation and down streaming processes. Since the capability of these organisms to produce pigments is not a permanent property but can be greatly increased or completely lost under different conditions of nutrition and cultivation. Therefore, it is very essential to develop the correct combination of various culture conditions to enhance the growth and pigment production. Actinorhodin is a biological pigment produced by Streptomyces coelicolor, S. violaceusruber and S. lividans. It is red-blue in colour based on the pH. It has found application as an antibiotic compound against Gram-positive bacteria, as an indicator compound in laboratory agents due to its property to exhibit different colours in acid and alkali mediums and lastly it can be used in food industry in making beverages, desserts etc, and maybe even in cosmetic industry. The complete scope of this pigment‘s application has not yet been explored.

MATERIALS AND METHODS

Sample collection

The soil samples were collected in triplicates from different areas of fields nearby VIT and coastal regions of Chennai using sterile containers and were brought to laboratory. The triplicates from each location were pooled together to increase the bacterial flora.

Isolation of Organism

The soil samples from both the locations were serially diluted using sterile water and plated on Kenknight‘s Agar, YMG Agar and Starch Casein Agar medium using 10^{-3}, 10^{-4} and 10^{-5} dilutions by spread plate and pour plate techniques. They were incubated for 5-7 days at 28-30^{o} C. The pure colonies were isolated and again plated on Kenknight‘s Agar and Starch Casein Agar medium by streak plate method. The medium preparation for organisms to be isolated from marine sediment was done using 50% sea water.

Identification of Test Organism

The isolated pure colonies of the organism were cultivated in YMG broth for 7 days. After preparing the stock culture the following tests were conducted for the identification and confirmation of isolated organism.

Morphological Characters

Growth on Standard Agar at 25ºC and 37ºC - Kenknight's Agar medium was sterilized and spread plate technique was performed. The plates were incubated at 25°C and 37°C for 10 days and observed. Colony morphology and aerial mycelium colour - Standard Agar plates streaked and incubated for 10 days at room temperature and morphology was observed.

Gram staining - Smear was prepared on a clean glass slide and it was flooded with crystal violet for 1 min. The stain was poured off and slide was flooded with Gram's iodine for 1 minute and it was poured. It was decolourized by washing the slide briefly with Gram's decolourizer. Slide was washed thoroughly with water to remove the acetone. Slide was then flooded with safranin counterstain for 1 minute and was washed with water and blot dried.

Acid Fast Staining - The smear was flooded with carbol-fuchsin dye, heated, and then decolorized.The smear was then counterstained with methylene blue. Pigment production - Visual observation On Agar surface

Biochemical Tests

Carbohydrate fermentation test : The test organism was inoculated into a nutrient broth containing 1.0% of the sugar to be tested mannitol, glucose, sucrose and lactose) and pH indicator phenol red. The tubes were incubated at 37º C for few days and colour change was observed.

Nitrate reduction test

The organism was inoculated into nitrate broth, containing KNO_3. After incubation, alpha-napthylamine and sulfanilic acid were added and observed for colour change.

Gelatin liquefaction test

Nutrient Gelatin medium was inoculated using the test organism by stabbing with a straight inoculating needle. The tubes were incubated at 20°C for 24 to 48 hours. Gelatin liquefaction was determined by tipping the tubes slightly after incubation.

Milk coagulation test

Tubes containing milk were inoculated with the test organism and incubated at 25°C and 37°C for 10 days and observed for coagulation.

Potato Plug

Raw potato slices were inoculated with the test organism and incubated at room temperature for few days under sterile conditions. After incubation, it was observed for colour change.

Culture preservation and Maintenance

Kenknight's Agar slants were prepared and the pure colonies of the isolated organism were streaked on it. After incubation for 7 days, the cultures were preserved at low temperature until further use.

Broth culture and Pigment Extraction

Mass Multiplication

100 ml of different liquid media − Kuster's Medium, Starch Casein Medium and YMG Medium, were prepared in 250 ml conical flasks and sterilized in autoclave at 121°C under 15 lbs pressure for 15 minutes. A loopful of the sporulated cultures was inoculated in broth. The cultures were incubated for 10 days at room temperature on rotary shaker (200 rpm) and observed for growth and pigment production. The medium best supporting the growth was used for further upscaling.

Alternate Methodology

Sterile cotton mats soaked in Bottcher-Conn's medium were used as a substrate to grow the test organism. The cotton mats were sterilized, placed in petri plates and bathed with broth. The sporulated culture was used as inoculum and the cultures were incubated for 7 days at room temperature.

Pigment Extraction

From broth culture-The broth culture after incubation was adjusted to pH 12 using 2 M NaOH. The culture was shaken vigorously for 25 minutes and centrifuged (4000g, 15 min). The supernatant was collected and adjusted to pH with 2 M HCl. The crude pigment was harvested as sediment. The sediment was suspended in methanol:chloroform (1:1). Crude extract of the pigment was obtained by drying the solvent at room temperature. From cotton mats - The mats were squeezed for obtain pigment solution. The solution was treated in the same way as the broth culture.

Thin Layer Chromatography

The crude extract was loaded using a capillary tube on silica-coated plate on the line drawn around 1.5 cm from one of the plate. The plate was immersed in the solvent just below the line where samples were loaded. The solvents used were benzene-acetic acid (9:1). After the solvent front reaches around half of the plate, the plate is removed and dried. Pigment spots were visualized under normal light. R_f values were calculated and compared with the standard R_f values for Actinorhodin and its analogues.

$$\text{Rf value} = \frac{\text{Distance travelled by pigment}}{\text{Distance travelled by solvent front}}$$

Optimization

Basal Medium

It is a medium consisting of NaCl(0.5gm/l), K_2HPO_4(2.28gm/l), $MgSO_4.7H_2O$(0.5gm/l), $FeSO_4.7H_2O$ (0.01gm/l). It lacks carbon and nitrogen sources which can be easily substituted during optimization

Effect of Carbon Source

Four 100 ml Erlenmeyer flasks, each containing 50 ml of basal medium were prepared. Casein (0.1%) was added as a standard nitrogen source, pH adjusted to 7.5 and sterilized by autoclaving. The carbon sources to be tested (glycerol, mannose, raffinose & xylose) were sterilized separately and add to the basal medium at 1% w/v concentration. 1 ml of the sporulated broth culture was inoculated into the media and was incubated at room temperature for 10-15 days on a rotary shaker at 300 rpm to allow continuous aeration and agitation. After 15 days, the cultures were observed visually for pigment intensity. The biomass was filtered and dry biomass weight was measured after drying it in hot air oven to remove the moisture completely.

Effect of Nitrogen Source

A series of 100 ml Erlenmeyer flasks was prepared with 50 ml basal medium and 1% glucose as a standard carbon source. The pH was adjusted at 7.5 and the nitrogen sources to be tested (Sodium caseinate, Peptone, Tryptone & Asparagine) were added at 1% w/v concentration. The medium was inoculated at incubated at room temperature for 10-15 days on a rotary shaker at 300 rpm. After incubation, pigment intensity was observed and dry biomass weight was measured.

Effect of pH

Nine 100 ml Erlenmeyer flasks with 50 ml Bottcher-Conn's Medium were prepared. The pH of each flask was adjusted to give a range of pH from 7 to 8.6 (7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6) and sterilized. Flasks were incubated at room temperature for 10-15 days on a rotary shaker at 300 rpm. After incubation, pigment intensity was observed and dry biomass weight was calculated.

Effect of Temperature

A series of 100 ml Erlenmeyer flasks containing 50 ml sterilized Bottcher-Conn's Medium were inoculated, as above, and incubated at 25, 28, 30, 33, and 37°C. After 15 days of incubation, pigment intensity and dry biomass weight were observed.

RESULTS AND DISCUSSION

Isolation and Screening

Figure 1 shows the pure culture of Streptomyces spp. isolated from marine sediment (Culture A) obtained from coastal regions of Chennai and Figure 2 shows the Streptomyces spp. isolated from the rhizosphere soil of paddy fields (Culture B) collected from areas around Vellore.

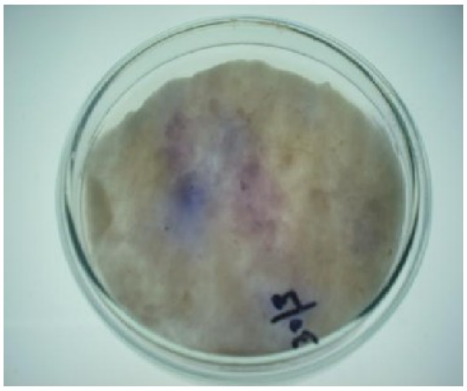

Figure 1: Culture A
(Non Pigmented Culture)

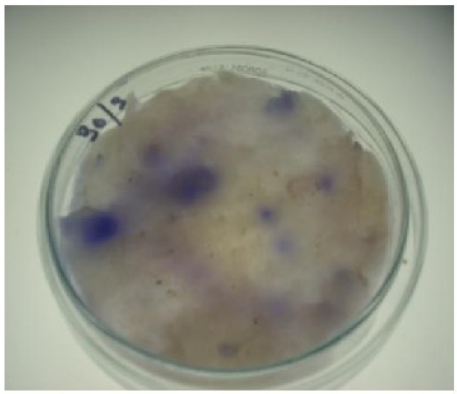

Figure 2: Culture B
(Pigmented Culture - S. violaceoruber)

Primary observations confirmed both the cultures as Streptomyces on the basis of - Colony Morphology - Chalky, tough, leathery.

Gram Staining - Positive. Acid Fast Staining - Negative.

Arrangement of cells - Filamentous Screening for pigment production-Culture A - Negative.

Culture B- Positive : Culture B was found to be Streptomyces violaceoruber after biochemical characterization. It was streaked on Kenknight's Agar and the culture was preserved in slants and maintained at 4°C for further use.

Results of morphological, physiological and biochemical tests for s. Violaceoruber

Here the results of morphological and biochemical tests have been recorded in Figure 3,4,5,6,7,8,9.

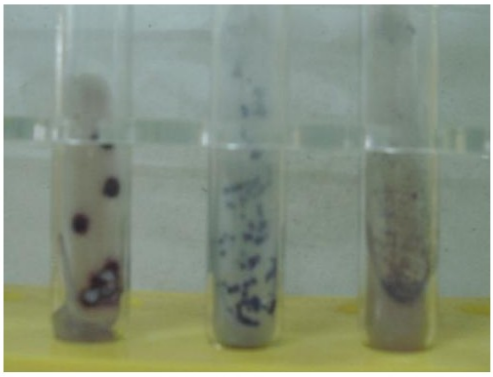

Figure 3: Slants (Streptomyces violaceoruber)

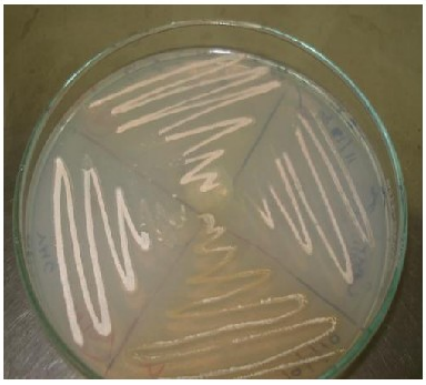

Figure 4: Pigment production by S. violaceoruber in Bottcher-Conn's Medium after 7 days

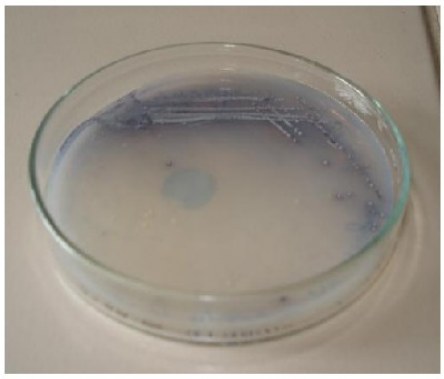

Figure 5: Pigment production by S. violaceoruber in Bottcher-Conn's Medium after 15 days

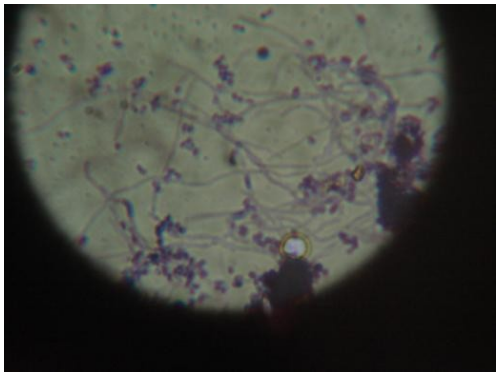

Figure 6: Gram's staining - Microscopic view of Gram positive, Rod shaped filamentous bacteria (S. violaceoruber)

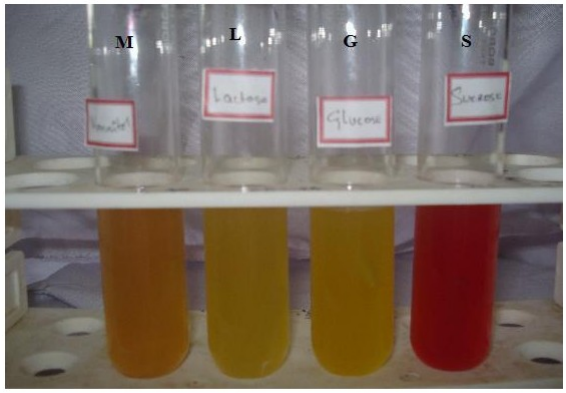

Figure 7: Gelatin Liquefaction Test

Figure 8: Milk Coagulation Test

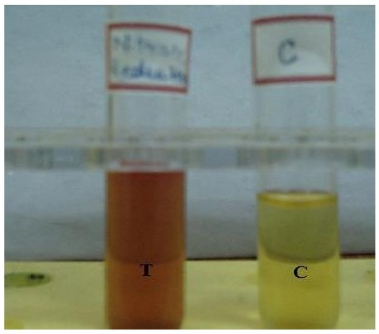

Figure 9: Nitrate Reduction Test

Pigment Extraction

The cotton soaked in Bottcher-Conn's media was squeezed to get a bluish colour solution. The medium was treated with NaOH to obtain blue coloured crude extract and centrifuged to obtain a clear blue solution. When treated with conc. HCl, a red precipitate was formed. The precipitate was decanted and treated with NaOH again to get a crude blue extract. After extraction with methanol: chloroform (1:1) vials of Actinorhodin related ―Blue Pigments were obtained.

All figures are shown in Figure 10,11,12,13.

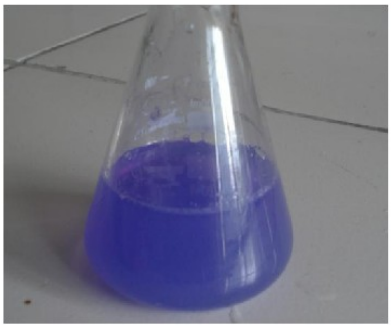

Figure 10: Bottcher-Conn's Medium treated with NaOH before centrifugation

Figure 11: Blue supernatant obtained after centrifugation

Figure12: Red precipitate formed after adding conc. HCl

Figure 13: Crude Extract (Actinorhodin Related Blue Pigments)

Thin Layer Chromatography

The spots were analyzed visually under visible light. The Rf values of Spot A (Blue) was calculated to be 0.272 which is comparable to the Rf value of γ-Actinorhodin and the Rf value of Spot B (Red) was determined as 0.519 which is analogous to the Rf value of Actinorhodin when TLC is performed using benzene and acetic acid in the ratio of 9:1. (**Figure 14)**

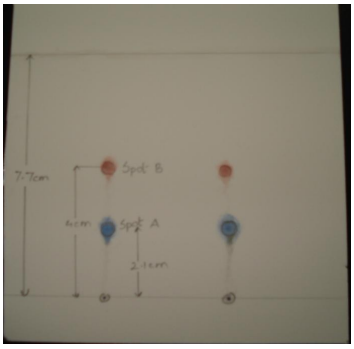

Figure 14: TLC Plate showing Spot A (Blue) and Spot B (Red)

Optimization of Carbon Source

The organism was able to grow in all the examined carbon sources Chart 1. It produced highest biomass (0.063gm/50 ml) in glycerol supplemented medium whereas highest pigment intensity was observed in glycerol and mannose both. Other carbon sources such raffinose and xylose also favoured growth but pigment intensity was less when compared with glycerol and mannose. (Figure 15)

Figure 15: Optimization of Carbon Sources

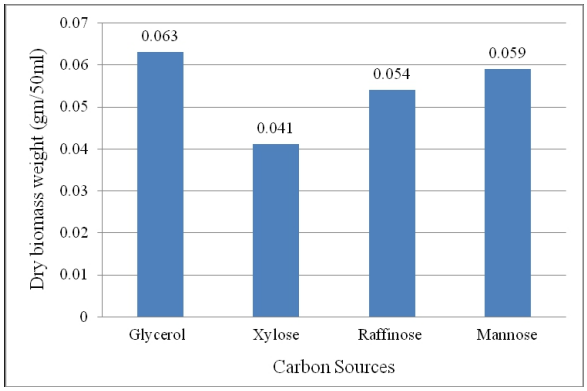

Chart 1: Amount of Dry Biomass Weight produced using different Carbon Sources

Optimization of Nitrogen Source

Of all the tested nitrogen sources, Sodium caseinate and peptone were found to be the best nitrogen sources for pigment production Chart 2. Sodium caseinate provided highest biomass yield (0.058gm/50 ml). Tryptone and Asparagine also supported good growth but did not show significant effect on pigment production (Figure 16).

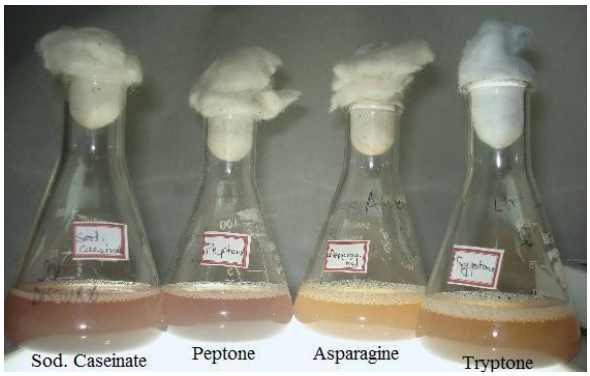

Figure 16: Optimization of Nitrogen Sources

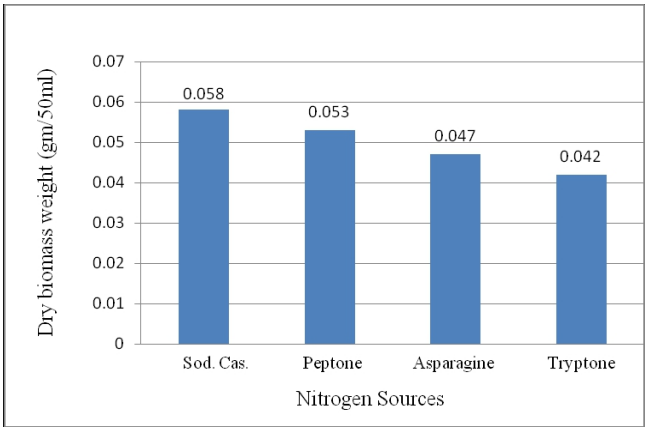

Chart 2: Amount of Dry Biomass Weight produced using different Nitrogen Sources

Effect of pH

The pH range that favoured highest growth of the organism and pigment production was established to be 7.6 – 8 Chart 3. The culture showed growth in pH ranging from 7 to 8.6 but pH below 7.6 and above 8 did not have positive effect on pigment production. Highest biomass yield was observed at pH 8.0 (0.063gm/50 ml) Figure 17.

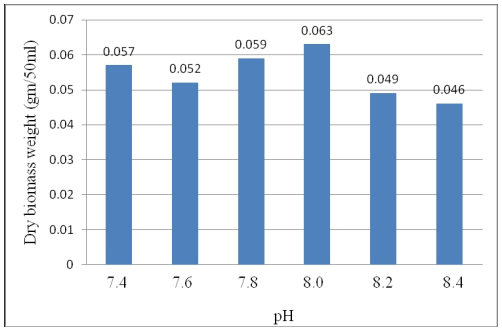

Chart 3: Amount of Dry Biomass Weight produced at different pH conditions

Figure 17: Effect of pH on Growth and Pigment production

Effect on Temperature

The culture showed a narrow range of incubation temperature for relatively good growth and pigment production Chart 4. Highest growth (0.063gm/50 ml) as well as pigment intensity was observed at 30°C Figure 18.

Figure 18: Effect of Temperature on Growth and Pigment production

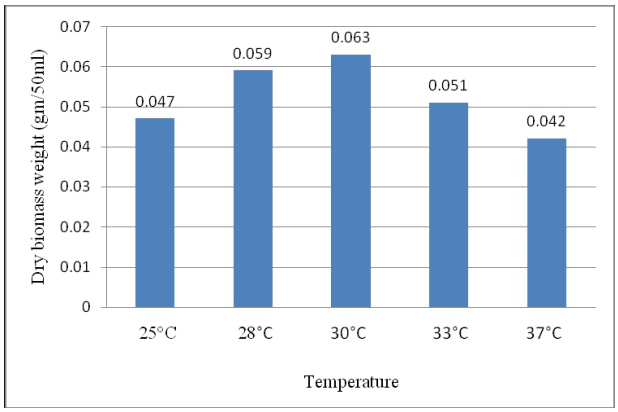

Chart 4: Amount of Dry Biomass Weight produced at different Temperature

Optimization Parameters

Streptomyces species have always been exploited for their ability to produce antibiotic and over the past few decades, many methodologies have been devised to commercially extract and purify these compounds. But, there are many other industrially important compounds produced by this genus that have not yet been isolated and studied.

In this study, importance of one such compound Actinorhodin has been reflected and isolation and extraction protocols have been standardized. Streptomyces species showed better growth and pigment production on solid media as compared to broth cultures and it is very difficult to extract pigment from agar surface. To overcome this drawback, a different method was used. A sterile cotton mat soaked in liquid medium was used as a substrate to anchor the organisms.

It was found that the pigment production and growth was positively influenced by a wide variety of carbon and nitrogen sources, although only a very narrow pH and temperature range seemed effective. Due to the lack of a standard pigment sample, it was difficult to measure the actual amount of the crude extract. Therefore, visual observation of the pigment intensity served as a means to estimate the pigment production.

Since the pigment is not highly soluble in most of the solvents, an alternative method of extraction is required to obtain high pigment yield. This is the major drawback in the entire process. Apart from this, inexpensive and readily available sources like corn steep liquor and molasses etc need to be developed into fermentation media for up-scale production of Actinorhodin.

CONCLUSION

Two Actinomycetes species were isolated from soil samples collected from various places and geographical areas. Out of these, only one culture screened positive for pigment production. The culture was found to be Streptomyces violaceoruber and it produced blue-red benzoisochromanequinone pigments called Actinorhodin related ―Blue Pigments. These pigments belong to Anthraquinones group of polycyclic aromatic hydrocarbons. The organism was mass multiplied in various complex media and it was concluded that YMG media supported good growth whereas Bottcher-Conn‘s medium produced highest pigment intensity. Crude extract of Actinorhodin and accessory pigments was obtained by treating the broth culture with NaOH followed by HCl. The pigment was dissolved in organic solvents methanol and chloroform in 1:1 ratio.

Experiments were conducted to optimize various culture parameters which yielded the following results. Carbon Source - Glycerol and mannose both supported pigment production whereas glycerol yielded highest biomass. Nitrogen Source - Sodium caseinate and peptone were found to be the best for pigment production and Sodium caseinate provided highest biomass yield. pH - Most favourable range for growth and pigment production was established to be 7.6 - 8. Temperature - Best growth and pigment intensity were observed at 28°C and 30°C.

Further work can be carried out to formulate a fermentation media for Actinorhodin production on a large scale by using economical and easily available sources. Since Actinorhodin has a wide range of applications, and a majority of them have not been explored yet. Hence, it is justified to work towards finding a commercial manufacturing process for its production.

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