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Isolation and Characterization of Pigment Producing Actinomycetes from Different Sources.

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ABSTRACT

Now days there is an increasing demands for natural products, people are more concentrating towards the natural product to replace the synthetic product. In this regard pigments are one of the natural products that replace synthetic colors. Natural pigments and synthetic dyes have been extensively used in various fields of everyday life such as food production, textile industries, paper production, agricultural practices and researches, cosmetic, water science and technology. Natural pigments have different activities like anticancerous, antibacterial, antifungal, antioxidant agent hence they are good coloring agent than synthetic dye. Different sources are found which produce pigment. Among them actenomyces are potent pigment producer. In this work attempt is made to isolate pigment producing actenomyces from different sources. The screening of actinomyces, for pigment production is done using starch casein media. As many as five isolates we have got are potent for pigment production. These isolates comprises of different shades red, yellow. black. brownish red, brown. These isolates were checked for their reproducibility in starch casein agar and broth. These isolates were characterized morphological, cultural and biochemical. The results obtained are all isolates are gram positive with long rod shape with an ability to grow in different pH range of 5-9. All isolates were able to degrade starch and produce zone of clearance in iodine solution but they cannot able to degrade skim milk. All isolates shows luxuriant growth till 10% salt (NaCl) concentration and minimal at 15%. These isolates were able to ferment different sugars (glucose, mannose, maltose, lactose and arabinose). These isolates with varying growth of cultural condition holds promise as potential candidates for biocolour production which can be of commercial value.

Keywords: Actinomycetes, food-colour, natural pigments.

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INTRODUCTION

Color is an important characteristic of food. Color of a food substance is important to indicate its freshness and safety that are also indices of good aesthetic and sensorial values (2). The appearance of the food as a sensory attribute is of as much significance as the nutritional aspects. Pigments are the natural compounds which replace synthetic dyes. Pigments are biodegradable and hence eco-friendly in nature. Pigments find applications in variety of industries such as foods, cosmetics, textiles, pharmaceuticals and water treatment. Pigments also offer functional attributes like anticancerous, antifungal, antibiotic and antioxidant. These place natural pigments over advantageous position than the synthetic dyes. Pigments are used in various fermentation products to replace synthetic dyes due to their complex nature and ability to withstand fermentation conditions. Pigments are generally classified as natural pigments and synthetic pigments. Natural pigments of microbial origin come from bacteria, fungus and actinomycetes. Some of the most important natural pigments are carotenoids, flavonoids, tetrapyrroles and some xanthophylls as astaxanthin, melanin, violacein and riboflavin. Thus, the food industry has become an increasingly new vista for the use of biopigments. It can also help to overcome the growing public apprehension over the adverse health effects of addition of synthetic colors in food products. Nowadays several fermentative food grade pigments like Monalin from *Monascus*, astaxanthin from *Xanthophyllomyces dendrorhous*, Arpink Red from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and carotene from *Blakeslea trisporatrispora* are considered harmless and are approved by regulatory agencies for their usage in foods.

Actinomycetes are a group of bacteria which possess many important and interesting features. They are of considerable value as producers of natural pigments, antibiotics and other therapeutically useful compounds. Actinomycetes are recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (2). The bio colorants identified by their chemical name can be synthesized easily by cheaper biotechnological sources particularly by various microorganisms. They exhibit a range of life cycles which are unique among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem.

MATERIAL AND METHODS

Saline solution (0.85% NaCl):

Dissolve 0.85gm of Sodium hydroxide in hundred milli liter of distilled water.

Starch casein agar (Hi Media Laboratories):

Dissolve Casein powder (1.0gram), Starch (10.0 gram), Sea water (37.0 gram), Agar powder (15.0gram) in one liter of distilled water to prepare starch casein agar.

Luria Bertani agar (Hi Media):

Dissolve Casein enzyme hydrolysate (10.0 gram), Yeast extract (5.0), Sodium Chloride (10.0), Agar powder (15.0 gram) in one liter of distilled water.

Basic media composition for carbon source degradation

Peptone (1.0 grams), Beef extract (0.5 grams), Sugar(0.5grams) and phenol red indicator(0.02ml). Dissolve these ingredients in one liter of distilled water.

Composition of Skim milk agar (Hi Media Laboratories):

It contains casein (5.0 grams), Yeast extract(2.5grams), Glucose(1.0 grams) and skim milk (100ml) dissolve in one liter distilled water and adjust pH to 8.0.

Collection of soil samples:

Soil samples from different locations were collected. Moist soil was collected in a sterile container and stored in cool place under shade.

Collection of water samples:

High salt concentration in the sea induces stress on bacteria which in turn induces the production of pigments by bacteria and actinomycetes. . Water sample from different locations in the sea are collected.

Serial dilution:

1g of soil sample was dissolved in 100 ml of 0.85% of saline to make 10⁻¹ dilution. Each tube contains 9ml of sterile 0.85% saline solution was prepared and serial dilutions till 10⁻⁵ (5) were prepared.

Isolation of Actinomycetes:

Starch casein (SC) agar was used for the isolation of actinomycetes (HiMedia) from soil and water sources. The medium contains starch and casein as carbon and protein sources respectively. The media was sterilized and pre-pored plates were made ready. Appropriate dilutions of samples were spread plated and incubated at 28 °C for 7-14 days (5). After incubation, the actinomycetes colonies were isolated, purified and sub-cultured on SC agar plates and stored for further assay.

Isolation of pigment producing Bacteria:

Luria broth (LB) was used for the isolation of pigment producing bacteria from the soil and water sources. The media provides optimum nutrition for the growth of bacteria and induces pigment production. The media was sterilized and pre-pored plates were made ready. Appropriate dilutions of samples were spread plated and incubated at 37 °C for 24-48 hours (5). After incubation, the bacterial colonies were purified and sub-cultured on LB agar plates and stored for further assay.

Identification of Actinomycetes:

The actinomycetes strains were identified on the basis of phenotypic appearance. The color of the colony was observed, selected and purified. Observations were made by basic tests, which includes, aerial mass color, reverse side pigment, melanoid pigments and spore chain morphology.

Morphological characterization:**Aerial mass color:**

Chromogenicity of the aerial mycelium is considered to be an important character for classifications of Actinomycetes. The colors of the mature sporulating aerial mycelium are range from white, grey, red, green, blue and violet(5). When the aerial mass color falls between two color series, both the colors were recorded. In these cases where aerial mass color of a strain showed intermediate tints, both the color series were noted (6)

Reverse side pigments:

Isolated strains were divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony. If the colony show color on both side (+) it is called dual side pigment production. If colony produce pigment only on one side then it is single side pigment production (-) (5).

Melanoid Pigments:

The grouping was made on the production of melanoid pigments (i.e. greenish brown, black or distinct brown pigment modified by other colors) on the media. These strains were grouped as melanoid pigment producers (+) and not producers (-) (6). For the melanoid pigment observation the inoculated plates were kept under incubated for 4- 5 days. The strains which showed cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color were recorded as positive (+) for melanin pigment production. Total absence of diffusible pigment, were recorded as negative (-) for melanin pigment production (5).

Species affiliation- Physiological and Biochemical characteristics:

Assimilation of carbon sources:

The ability of actinomycetes to utilize different sugars as energy source and growth was studied by using different sugars (5). Different sugars (D-glucose, L-arabinose, Sucrose, D-fructose and lactose) were added into the basal medium in 1% concentration and growth was recorded after incubation of 4-7 days.

Sodium Chloride tolerance:

Different concentrations of Sodium Chloride (0, 5, 10, 15, 20, 25 and 35%) solution were added to the Starch casein agar medium to check the Sodium chloride tolerance test. Isolates were inoculated into Starch casein broth with different concentration of Sodium Chloride and incubated at 37°C for 7-15 days. The presence or absence of growth was recorded from 7th day onwards (5).

Degradation of cellulose:

1% Carboxy methyl cellulose (CMC) was added to the starch casein media. The plates were inoculated and incubated for 7-15 days. Control plate was used as standard to check the growth of actinomycetes after 7-15 days for cellulose degradation activity which could be visually observed.

Hydrogen-sulphide production:

The inoculated Starch casein agar slants were incubated for 7 days for the test. Observations on the presence of the characteristic greenish-brown, brown, bluish-black or black color of the substrate, indicative of H₂S production were recorded on 7th day. The inoculated tubes were compared with uninoculated controls.

Gelatin liquefaction:

Due to the absence of tryptophan, Gelatin is called as incomplete proteins its value in identifying bacterial species is well established. Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Below the temperature of 25 °C, gelatin maintains its gel properties and exist as solid at temperatures above 25 °C, gelatin is liquid(5). Liquefaction is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyze this protein to amino acids. Once this degradation occurs, even very low temperatures of four degree Celsius will not restore the gel characteristics. Gelatin deep tubes were used to demonstrate the hydrolytic activity of gelatinase. The medium consists of nutrient supplemented with 12% gelatin which serves as the substrate for the activity of gelatinase (5). Gelatin liquefaction is studied by sub-culture the strain on gelatin containing medium and inoculated them at 25°C. Observations were made after 7 days. The extent of liquefaction was recorded after keeping the tubes in cold conditions (5-10 °C) for an hour. Cultures that remained liquefied were indicative of slow Gelatin hydrolysis.

Hydrolysis of starch:

Starch is a high molecular-weight, branching polymer composed of glucose molecule linked together by glycosidic bonds. The degradation of this macromolecule requires the presence of the extracellular enzyme amylase for its hydrolysis into shorter polysaccharides, namely dextrin, and ultimately into maltose molecules.

The hydrolysis of this disaccharide, which is catalyzed by maltase, yields low molecular weight, soluble glucose molecules that can be transported into the cell and used for energy production through the process of glycolysis. The medium was composed of nutrient agar supplemented with 1% starch, which serves as the starch substrate (5). For this test; cultures were grown for 5-7 days on nutrient agar media. The development of zone of clearance around the cultures, when the plates were flooded with Lugol’s iodine solution was recorded as the hydrolysis of starch.

Protease activity:

The skimmed milk was inoculated and incubated at thirty seven degree Celsius. The extent of coagulation was recorded on the 7th and 10th days of incubation.

Growth in different pH:

The cultures were inoculated into different tubes with varying pH (5, 7 and 9). The growth of each strain was recorded after incubation at 37 °C for 7-10 days.

RESULTS AND DISCUSSION

Different soil and water samples from various locations were collected and the isolation was carried out. Various steps involved in the experiments were given in Fig 1 to 5.

Five isolates of Actinomycetes with different shades (Black, Red, Yellow, Pink Red, Brown –Red) were obtained upon screening by Starch-Casein media. Table 1 shows the details of the total counts of isolates from LB and SC media.

Table 1: Results of bacterial colony count on SC agar and LB agar

Sample	Number of isolates (log10 colony forming unit/ml)		Number of isolates showing pigments	Shade
	LB Agar	SC Agar		
Garden soil	6.02	7.41	5	Black Red Yellow Pink –Red Brown –Red
Garden soil	7.03	8.06	0	No color
Garden soil	6.08	8.054	0	No color
Sea soil	TNT	0	0	No color
Mid sea soil	TNT	7	0	No color
Sea water	TNT	9	0	No color
Mid sea water	TNT	5	0	No color

TNT- Too numerous to count

The actinomycetes isolates were studied for morphological and bio-chemical characteristics and the results are as shown in Table 2.

The ability of these isolates to metabolize different carbon sources and grow in varying levels of salt was studied and the results are as shown in Table 3 and Table 4 respectively.

Further, these isolates were subjected for their ability to grow in different pH as shown in Table 5.

Table 2: Morphological and Biochemical Characterization of Cultures

Cultures	Color on Spore chain	Reverse side pigment	Melanoid pigment production	Cellulose degradation	H ₂ S production	Gelatin liquefaction	Protease activity	Starch hydrolysis
Culture 1	White	+	-	+	Red color (-)	Solid(-)	-	+
Culture 2	Grey	+	+	+	Black color (+)	Solid(-)	-	+
Culture 3	White	+	-	+		Liquid (+)	-	+
Culture 4	White	+	-	+	Yellow color (-)	Liquid (+)	-	+
Culture 5	White	+	-	+	Brownish red color (-)	Solid(-)	-	+

Table3: Carbon Source Utilization

Carbon source	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
Glucose	+	+	+	+	+
Maltose	+	-	+	+	+
Mannose	+	+	+	+	+
Lactose	-	+	+	+	+
Arabinose	+	+	-	+	-

+ indicates these isolates ferment the sugars - indicates these isolates cannot ferment the sugars

Table 4: Sodium Chloride Tolerance:

Sodium Chloride %	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
5%	+++	+++	++	+	+
15%	+++	++	+	+	+
25%	++	-	-	-	-
35%	-	-	-	-	-

+++ indicates good growth ++ indicates moderate growth + indicates less growth - indicates no growth

Table 5: Ability to grow in different pH:

PH	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
5.0	++	++	++	++	++
7.0	++	++	++	++	++
9.0	++	++	++	++	++

++ indicates good growth -- indicates no growth

All the cultures have the some properties they show various properties if the culture is able to melanin pigment in the broth it produce black color if not black color is not produced. Most commonly and widely known pigment is melanin pigment it is found in bacteria, fungi and also in human. Hence in this we isolates five different colored isolates and among them only one isolate is black color hence it is melanin pigment producer. Cultures obtained are subjected ferment different sugars hence they are capable of ferment all the sugars they can metabolize these sugars for their growth hence we can add any of the sugar as the growth of these organism to get pigment from them in large scale. For the production and activation of some enzymes precursors are needed these salts act as catalyst for the activation and metabolism of various enzymes. Hence sodium chloride will play important role in growth of the organism. Cellulase is an enzyme which degrades cellulose hence clear zone was observed around the culture using carboxy methyl cellulose as a substrate. Gelatin is gelling agent it hardens media in the low temperature and at room temperature it converts into liquid form. Hence the nutrient broth was supplemented with gelatin and inoculated with culture these actinomycetes degrade this gelatin and remain liquid in cold condition. Hence in this culture 3&4 degrade gelatin and those tubes remain liquid in the cold conditions.

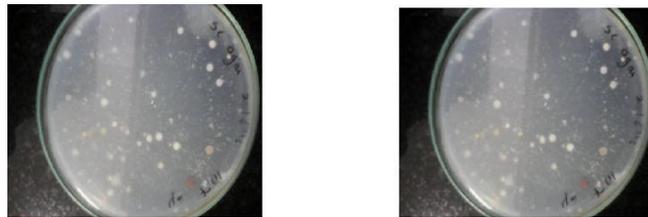


Figure 1: Actinomyceteus colonies on SC Agar plates

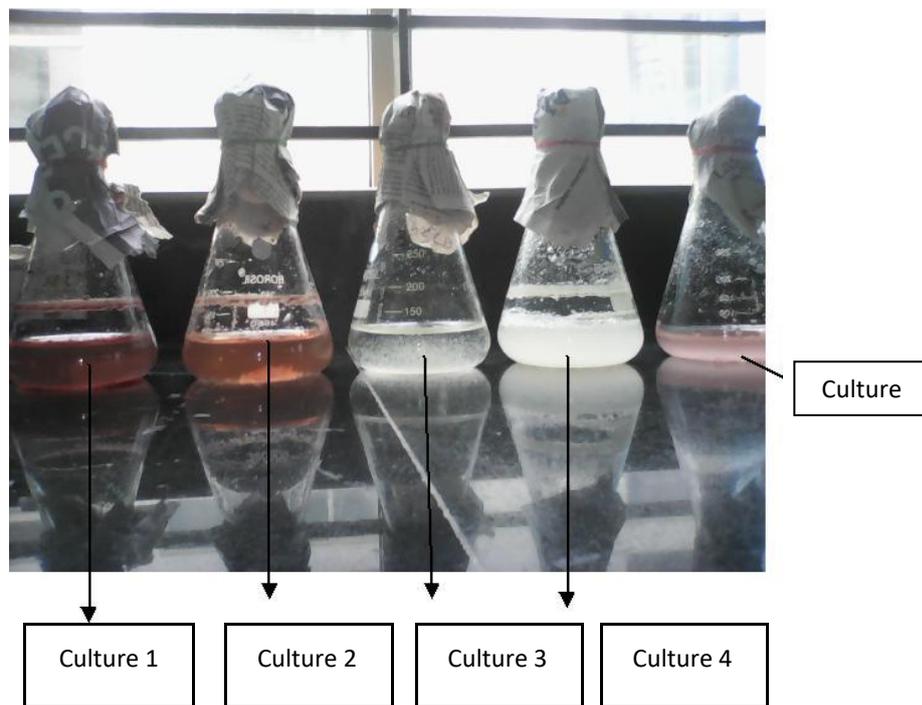


Figure 2: Pigment Production on SC Broth



Figure 3: Fermentation of different sugars



Figure 4: Hydrogen Sulphide production



Figure 5: Gelatin Liquefaction

Some have isolated organism from marine soil which is rich source of microbes they have got eleven actinomycetes colony among them one is black colored other are not colored but we have got five different colored colonies of actinomycetes from garden soil hence this is also a good source for isolation of pigment producing actinomycetes. In the present work among five isolates two cultures show degradation of gelatin where as in other work five cultures show liquidation of gelatin among eleven isolates(5). All eleven cultures shows antimicrobial activity(5) where as in this work only one culture showing antimicrobial activity.

Hence we can isolate various potent actinomycetes from garden soil as well as from marine soil and can be used for various purpose.

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