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Research Article

STANDARDIZATION OF CULTIVATION PARAMETERS FOR THE EXTRACTION OF CAROTENOID FROM PINK PIGMENTED FACULTATIVE METHYLOTROPHIC (PPFM) BACTERIA

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ABSTRACT

A Pink pigmented facultative Methylotrophic bacterium was isolated from the phyllosphere region of cotton by leaf imprinting method on Ammonium mineral salt agar supplemented with 0.5% of methanol and cyclohexamide. Their distinctive pink pigmentation is due to carotenoid, which render them to be tolerant to extreme light condition and radiation. The isolate was morphologically and biochemically characterized and identified as a member of the genus *Methylobacterium.* The parameters such as temperature, pH, and carbon source were optimized for the extraction of pigment from PPFM bacteria. The optimum pH for growth and pigment production was around 7.5 at temperature of 25˚ C with 0.5% of methanol. The PPFM bacterium was mass multiplied at these optimum conditions, pigment was extracted, and the sample was analyzed by UV-visible Spectrophotometer.

Key words: Methylotrophic bacterium, Optmization of growth parameters, PPFM, Morphological and Biochemical characterization

INTRODUCTION

Carotenoid is an organic pigment that is found widely in plants and microorganisms. They are essential to plants for photosynthesis, and protection against destructive photo oxidation (Goodwin, 1984). In microbial system their distribution is limited to some fungal organisms, which have potential economic importance. In the bacterial world carotenoid may occur in certain group in connection with photosynthesis or in relation to some unusual ecological features. Carotenoids are generally not manufactured by animal, although one species of aphid is known to have acquired the genes for carotenoid synthesis from fungi, by mechanism of horizontal gene transfer. Humans are not capable of synthesizing carotenoid and must obtain through diet. Most of the carotenoid are C40 tetraterpenoids. Structurally they are in the form of a polyene chain which may be terminated by cyclic end-groups (rings) and may be complemented with oxygen-containing functional groups. There are over 600 known carotenoid and are split into two classes as hydrocarbon carotenes (alpha-carotene, beta-carotene and lycopene) and Xanthophylls (lutein, astaxanthin, canthaxanthin and zeaxanthin). Carotenoid are highly colored (red, orange and yellow) and their color is directly linked to their structure. The double carbon-carbon bonds interact with each other in a process called conjugation, which allows electrons to move freely across the molecule. As the number of double bond increases, the wavelength of the absorbed light increases, giving the compound increasingly red appearance.

Carotenoids are used as natural food colorants or feed additives in aquaculture (Benemann, 1992; Torrissen, 1995). Carotenoid plays an important role in human health. Several dietary studies have shown that carotenoid combat various types of cancers, macular eye diseases and cardiovascular problem and other diseases because of provitamin A function (Artorg, 1997). Carotenoids are potent biological antioxidant that can absorb the excited energy of singlet oxygen onto the carotenoid chain leading to the degradation of the carotenoid molecules but preventing other molecules or tissues from being damaged (Mortensen *et al*., 1997).

A carotenoid pigments represent a group of most valuable molecules for industrial applications. Although over 600 different structures have been described only a few carotenoids are currently exploited on industrial scale. Currently, commercial carotenoid production is mostly based on extraction from plant tissues or chemical synthesis. However, owing to a growing worldwide market for these compounds microbial production has great potential in terms of both the efficiency of production and the diversity of carotenoid structures. Pigmentation is wide spread among bacteria. In photosynthetic bacteria, carotenoid help in harvesting and transferring light energy to chlorophyll and also protect the photosynthetic apparatus against photo oxidation .The widespread occurrence of carotenoid in non-phototrophic bacteria suggests that their presence is crucial for the viability of these organisms in their natural environment and serve as an important taxonomic marker for the identification of isolates.

Pink pigmented facultative methylotrophic (PPFM) bacteria belong to genus *Methylobacterium*. They are gram negative rod shaped bacteria and able to grow on one-carbon compounds (e.g. Methanol, Methylamine) as well as on a wide range of multicarbon substrates as sole source of carbon and energy (Green, 1992).They are widely distributed in nature. They are common leaf epiphyte representing an important bacterial population (Corpe, 1985). The most common niche for synergism between *Methylobacterium* and plant is the phyllosphere, where they utilize methanol evolved from leaves as the sole source of carbon and energy (Trotsenko *et al* ., 2001) Their distinctive pink pigmentation is due to carotenoid, which render them to be tolerant to extreme light condition and radiation. In recent years, due to their different applications in the fields of industrial, agriculture, and bioremediation, *Methylobacterium* attracted the much attention.

Materials and Methods

Sample collection

Malvaceae are distributed widely in tropical and temperate region. The leaves of Malvaceae, *Hibiscus rosa-sinensis* (Rose mallow), Gossypium (Cotton), *okra* (Lady's Finger) were used as sample to isolate Pink Pigmented Facultative Methylotrophic (PPFM) bacteria. Fresh leaf samples were collected from Thorapadi region of Vellore and screened for the presence of PPFM bacteria.

Sterilization and Media preparation

The glassware such as petridishes, conical flask, test tubes, etc., was sterilized. The Ammonium Mineral Salt agar was prepared and sterilized by autoclaving at 121˚C for 15 minutes. The medium was then cooled to 50˚C, and supplemented with 0.5% of filter sterilized methanol as a carbon source. To isolate the PPFM bacteria from phyllosphere region cyclohexamide (20µg/ml) was added to the medium to avoid the fungal contamination.

Isolation of ppfm bacteria

Leaf samples were washed with sterile water to remove the dirt and soil. The dorsal surface of leaf was laid on the surface of the medium, after making an impression leaf was lifted carefully and discarded. The inoculated plates were incubated at 25˚C for 7 days. A single

pink colony was picked and quadrant streak was done to isolate the individual colonies. The bacterial colonies were examined for further characterization and identification.

CHARACTERIZATION OF PPFM BACTERIA

Morphological characterization

Colony morphology aid in identifying and classifying organism into taxonomic groups The colony morphology such as size, shape, color and margin was recorded. The tests were evaluated by Gram's staining method, Sudan black b staining and Motility test.

Biochemical characterization

Indole Test

The biochemical characteristics were shown by Indole test. Some bacteria possess the enzyme tryptophanase, which acts upon the tryptophan present in the peptone of the culture medium, and convert it into Indole, Skatol and Indole Acetic acid. This indole reacts with the aldehyde in Kovac's reagent to form a red colored product. So organism was grown in tryptophan rich medium and tested for the presence of indole.

Methyl Red Test

Organisms ferment glucose via pyruvate to produce mixed acid such as acetic, lactic, succinic and formic acids, ethanol and carbon dioxide. As a result final pH of the medium drops to less than 4.5, this can be detected by the pH indicator Methyl red.

Voges Proskauer Test

Some organism produces non-acidic or neutral end products, such as acetyl methylcarbinol (acetoin), from the organic acids that result from glucose metabolism. In the presence of atmospheric oxygen and 40% potassium hydroxide solution, acetoin is converted to diacetyl and α -naphthol serves as a catalyst. As a result a pink complex is formed imparting rose color to the medium.

Citrate utilization Test

Some microorganism produces an enzyme citrate permease which acts on citrate and produces oxaloacetic acid and acetate .These products are enzymatically converted into pyruvic acid and carbon dioxide. Carbon dioxide combines with sodium and water present in the medium to form sodium carbonate, an alkaline product. The presence of sodium carbonate changes the bromothymol blue indicator incorporated into the medium from green to Prussian blue.

Urease Test

Urea is a diamide of carbonic acid. Urease the enzyme possessed by bacterium hydrolyses urea and release ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonates which is alkaline that causes the phenol red to change its color from yellow to deep pink, indicating the presence of Urease activity.

Catalase Test

During aerobic respiration, microorganism produces hydrogen peroxide. Accumulation of these substances results in the death of organism. Some organisms possess the enzyme Catalase which splits the hydrogen peroxide to water and oxygen. This was visualized by the evolution of the air bubbles when drop of the culture was introduced into hydrogen peroxide.

Oxidase Test

Some organisms possess the enzyme oxidase that form the part of electron transport system during the aerobic respiration. Oxidase reagent is colorless in its reduced state and dark purple in its oxidized state. The enzyme oxidizes the reagent N tetra methyl paraphenylene diamine dihydrochloride to the colored product indophenols.

Optimization of cultivation parameters

A loopful of culture was inoculated into the sterile AMS broth with 0.5% of methanol. It was incubated at 25˚C for 48 to 72 hours. 2% of the log phase culture was used as an inoculum for the pigment production. Optimization was performed by varying the single parameter while others are kept constant.

Temperature

Log phase culture was inoculated in AMS broth with 0.5% of methanol and incubated at different temperatures vis., 25˚C, 28˚C, 33˚C, 37˚C for 7 days. After incubation flask was observed for the presence of intense pigmentation and growth was detected by serial dilution agar plate method .The temperature at which more growth and pigmentation observed was chosen and maintained in the following studies.

pH

Log phase culture was inoculated in AMS broth with 0.5% of methanol with various initial pH vis., 6, 6.5, 7, 7.5. The flask was incubated at 25˚C for 7 days. The pH at which more growth and pigmentation observed was chosen and maintained.

Carbon Source

The isolate was cultivated in the presence of different carbon source vis., methanol, dichloromethane, formaldehyde, glucose and fructose (0.5%) in AMS broth. The pH of the broth was adjusted to 7.5. The log phase culture was added to each of the broth and was incubated at 25˚C for 7 days.

Pigment Extraction and Analysis by UV Spectrophotometer

The PPFM bacterium was mass multiplied in AMS broth, pH 7.5 with 0.5% of methanol and incubated 25˚C for 7 days. The cell pellets was obtained from 100ml of the broth culture it was resuspended in 1 ml of methanol at 65˚C and vortexed for 1 minute. A 0.4ml of water and 0.3ml of chloroform was added and sample was vortexed for 2 minutes. The bottom organic layer containing the carotenoid was extracted into clean tubes. 1ml of methanol and 0.4ml of water was added and organic layer was extracted again. 2ml of acetone was added to the extract in order to remove the phospholipids and glycolipid and the sample was placed at -20˚C overnight. The sample was centrifuged and the supernatant was evaporated to dryness and redissolved in 0.1ml of chloroform. The visible absorption spectrum of sample was measured.

RESULTS AND DISCUSSION

Isolation and characterization of ppfm bacteria

Cotton, lady's finger, hibiscus leaves of Malvaceae family were collected from Thorapadi region of Vellore and screened for the presence of PPFM bacteria by leaf imprinting method on AMS media supplemented with 0.5% of methanol and cyclohexamide (**Fig 1).** Among the leaf samples used, a pink colony of Methylobacterium was obtained from cotton leaf, which indicates its colonization and colony was purified by quadrant streak inoculation (**Fig 2)**

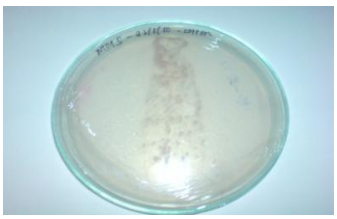

Fig 1: PPFM Bacteria isolated from cotton leaf

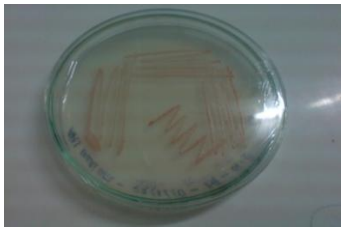

Fig 2: Quadrant streak inoculation

Microscopic examination of the isolate showed gram negative rod shaped bacteria that occurred singly or in rosettes (**Fig 3).** They are motile and showed poly β hydroxyl butyrate granule which appeared black and vegetative cells appeared pink when stained with Sudan black B (**Fig 4).**

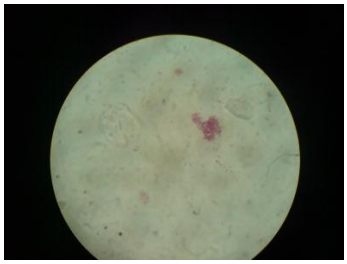

Fig 3 Gram's staining

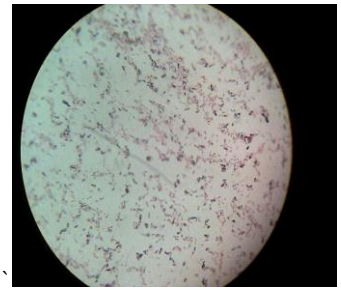

Fig 4 Sudan Black B staining

Biochemical analyses (**Fig 5)** of the bacterial isolate revealed that they are positive for Catalase, Oxidase, Citrate, and Urease and negative for Indole, MR and VP. Based on the morphological, biochemical a property, the isolate was identified as PPFM bacteria belonging to the genus *Methylobacterium.*

Indole Test | Methyl Red Test | Voges Proskauer Test

Citrate Test | Urease Test

Catalase Test | Oxidase Test

Fig 5: Biochemical Characterization of PPFM bacteria

Optimization of cultivation parameters
The following aspects have been monitored (Table 1)

Table 1: Optimization of cultivation parameters

PARAMETERS	GROWTH (CFU/ml)	PIGMENTATION
TEMPERATURE		
25˚C	9x 10^7	+ + +
28˚C	65 x 10^6	+ +
33˚C	34 x 10^6	+
37˚ C	TFTC	-
pH		
6	NG	-
6.5	1x10^7	+
7	5x10^7	++
7.5	8x10^7	+ + +
CARBON (0.5%)		
Methanol	88x10^6	+ + +
Formaldehyde	NG	-
Dichloromethane	NG	-
Glucose	NG	-
Fructose	NG	-

(+) low pigment intensity; (++) moderate pigment intensity; (+++) high pigment intensity

(-) no pigmentation; (NG) No growth

Temperature

Temperature usually plays an important role in cell synthesis. To study the effect of temperature on pigmentation and growth, *Methylobacterium* was cultivated at different temperatures. Among the different temperatures, the isolate showed more pigmentation and growth at 25˚C, moderate pigmentation (**Fig 6)** and growth (**Fig 7)** at 28 and 33 ˚C and no pigmentation and less growth at 37˚C. It indicates that increase in temperature tends to decrease the pigmentation and growth. The optimum temperature was found to be 25˚C and same was maintained in the following studiespH.

The influence of initial pH on growth and pigment production was studied (**Fig 8).** The bacterial isolate was cultivated at different pH at 25˚C. After incubation it was checked visually for the pigmentation and growth by serial dilution agar plate technique. No growth and pigmentation at pH 6, then they increased with increasing pH. Maximum growth and pigmentation was obtained at pH 7.5 **(Fig 9).** This indicates that neutral and alkaline pH (7.5)

support more growth and pigmentation than acidic pH of 6, 6.5 and hence pH 7.5 was maintained in the following studies.

Fig 6: Effect of temperature on pigmentation

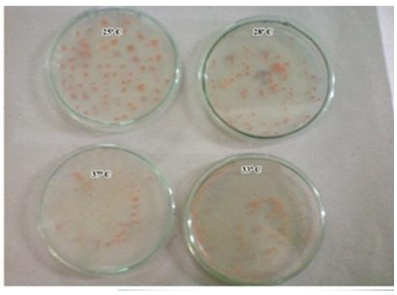

Fig 7: Effect of temperature on growth

Fig: 8 Effect of pH on pigmentation

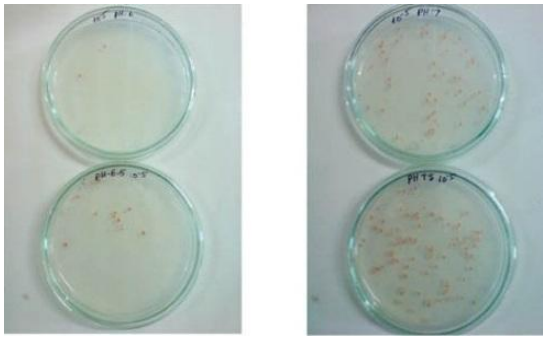

Fig 9: Effect of pH on growth

Carbon source

The influence of carbon source on pigment production (**Fig 10)** and growth (**Fig 11)** was studied by inoculating the log phase culture of bacterial isolate in AMS broth of pH 7.5 with 0.5% of Methanol, Dichloromethane, Formaldehyde, Glucose, and Fructose and incubated at 25˚C for 7days. Although isolate was a Facultative methylotrophic bacterium no carbon source (0.5%) other than methanol supported the growth and pigmentation after 7 days. It indicates that the organism grows well on methanol based medium than the others. Hence the organism was maintained at 0.5% of methanol.

Fig 10: Effect of carbon source on pigmentation

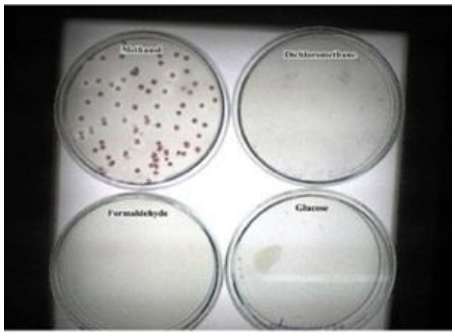

Fig 11: Effect of carbon source on growth

Extraction of pigment

PPFM bacteria was mass multiplied (**Fig 12)** in AMS broth of pH 7.5 with 0.5% of methanol and incubated at 25˚C for seven days. The pigmented was extracted (**Fig 13**) by using the modification of procedure commonly used for the photosynthetic bacteria .The visible absorption spectrum of sample was measured and found to be maximum at 493 nm (**Fig 14)**

Fig 12: Mass multiplication

Fig 13: Extraction of pigment

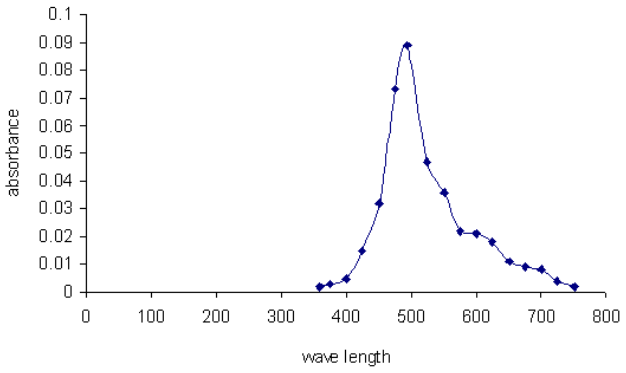

Fig 14: **UV -Visible absorption of extracted pigment.**

CONCLUSION

In the present study, PPFM bacteria a leaf epiphyte was isolated from cotton leaf by leaf imprinting method. Their distinctive pink pigmentation is due to carotenoid, which render them to be tolerant to extreme light conditions and radiation. Based on the morphological and biochemical properties the isolated bacterium was identified as *Methylobacterium* spp. The effects of important parameters such as temperature, pH, and carbon source on growth and pigmentation were studied and optimum range of each parameter was obtained. The optimum condition for growth and pigmentation in AMS broth was found to be 25˚C, pH 7.5, with 0.5% of methanol. The organism was mass multiplied at these optimum conditions and pigment was extracted. The UV-Visible absorption of sample was measured and found to be maximum at 493 nm, which indicate the presence of carotenoid pigment. Further studies are required to analyze the carotenoid sample.

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