Isolation And Characterization Of Methylotrophic Bacteria From Western Ghats

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Abstract

A Pink Pigmented Facultative Methylotrophic (PPFM) bacterium was isolated from the rhizosphere region of Western Ghats by serial dilution method on Hyphomicrobium medium and Minimal Salts Agar supplemented with 2% methanol. Their distinctive pink pigmentation is due to carotenoids, 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 optimum pH for growth and pigment production was 7.5 at a temperature of 27˚ C with 1% methanol. The PPFM bacterium was mass multiplied at these optimum conditions, samples were extracted, analyzed using UV-visible Spectrophotometer, subjected to FAME analysis by Gas Chromatography.

Keywords: PPFM, Hyphomicrobium medium, carotenoids, *Methylobacterium*, FAME analysis

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Introduction

Soils typically contain 10^9 to 10^{10} microorganisms per gram (dry weight), which might represent more than a million bacterial species. In each managed and natural ecosystems, plants will move with a large vary of bacterium, which could pose unhealthful, neutral, or useful effects towards their hosts. It's been proved that majority of microsymbionts include flora and acknowledged diverse group of bacterium colonizing the rhizosphere. A set of the rhizosphere microflora may enter and proliferate at intervals plants as endophytes [2], [4]. However, small fraction of these microbes has been cultivated and characterized which provided only a glimpse of their potential physiological capacity and their influence towards soil ecosystems. It's been long known that some non-photosynthetic micro-organisms can grow on organic C, relying on compounds as their sole source of energy and carbon. The means whereby such organisms grow has been the subject of speculation [5], [7].

Endophytes may derive from different sources of organization and these styles of microorganisms are principally isolated from endorhiza, stems and leaves. Few studies have conjointly incontestable that a part of the endophytic microflora will colonize plant generative organs. However, very little data makes it difficult, to know whether or not specific microorganism taxa square measure ready to colonize plant generative organs [1],[2]. Pink Pigmented Facultative Methylotrophic (PPFM) bacteria are gram negative rod shaped bacteria belonging to genus *Methylobacterium*, are aerobic bacteria that utilize one-carbon compounds, such as methane, methanol, and methylated compounds containing sulfur, as sources of carbon and energy [3], [9]. Reports suggest that Methylotrophic bacteria are found in a variety of habitats such as leaf, polluted water, air, soil, drinking water, vehicular soot and rice [6], [10].

In recent years, *Methylobacterium* received most of the attention due to its application in the field of industrial, agriculture and bioremediation. Even so, our lack of understanding of the ecology of bacterium colonizing generative organs of plants growing under natural conditions remains restricted [3]. Moreover, if endophytic bacteria occur within plant generative organs, niches of organization ought to be conjointly incontestable by microscopic analysis to any proof the presence of those endophytes in these plant components [2]. The widespread occurrence of carotenoid in non-phototrophic bacteria like PPFM 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.

Methylobacterium species, which have the capacity to fix nitrogen, have previously been isolated from citrus, Scotch pine, and crotalaria, showing the capacity of members of this bacterial genus to colonize the plant habitat [1]. The formation of fatty acid methyl esters (FAME) by simple thermal degradation may open new gateways not only in aspects to bioaugmentation and food processing but also in microbial profiling. In addition, fatty acid methyl ester can also serve as a raw material of bio-diesel which is a new environment-friendly energy source.

Materials and methods

Sample collection

The method that was used to carry out the study was random sampling which involved the transfer and culturing in an appropriate culture medium under optimum conditions for growth in the laboratory. Twelve samples from rhizosphere of local trees were collected for isolation and quantification of viable aerobic microorganisms, all from Western Ghats, Coimbatore, Tamil Nadu. Samples were examined within four hours of collection. If immediate bacteriological examinations were not performed, samples were stored at 4^0C until they were examined.

Sterilization and media preparation

Glassware such as petridishes, conical flasks, test tubes, etc., and Hyphomicrobium medium and Minimal Salts agar were prepared and sterilized by autoclaving at 121˚C for 15 minutes. The medium was later cooled to 50˚C and supplemented with filter sterilized methanol as carbon source. To isolate the PPFM bacteria from rhizosphere region, cyclohexamide (20µg/ml) was added to the medium to prevent fungal contamination.

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Enumeration, isolation, characterization and identification of viable aerobic bacteria

All isolates were identified by standard techniques described by Cowan and Steel in the manual of identification of medical bacteria. Colonial appearance of the organisms on the media, morphological characteristics such as size, form and odour, specific biochemical test e.g. catalase, oxidase, indole production, urease activity and methyl red were performed for the identification of the organisms. Enumeration was performed after the plates were incubated at room temperature for 72 hours.

FAME analysis

The PPFM bacterium was mass multiplied in Hyphomicrobium broth, pH 7.5 with 1% of methanol and incubated 27˚C for 7 days. The cell pellets were extracted using 25ml of the broth culture it was resuspended in 5ml of methanol at 65˚C and vortexed for 1 minute and centrifuged at 10000 rpm for 5 minutes. The bottom organic layer was extracted and stored in clean tubes. The organic layer was extracted again using 1ml of methanol and the extract was stored overnight at -20˚C. The sample was centrifuged and the supernatant was evaporated to dryness and redissolved in 0.5ml of methanol. They were then subjected to transesterification in boiling hot water bath for 10 minutes. The visible absorption spectrum of sample was measured and analyzed using Gas Chromatography.

Results

Isolation and characterization of endophytic *Methylobacterium*

Microscopic examination of the isolate showed gram negative rod shaped bacteria that occurred singly or in rosettes**.** They are motile and showed poly β-hydroxyl butyrate granule which appeared black and vegetative cells appeared pink when stained with Sudan black B. Biochemical analyses of the bacterial isolate revealed that they are

positive for Catalase, Oxidase, Urease and negative for Indole and MR. Based on the morphological and biochemical property, the isolate was identified as PPFM bacteria belonging to the genus *Methylobacterium.*

Optimization of pH, temperature and carbon source

Temperature (^{o}C)	22	27	32	37
Growth of PPFM (CFU/ml)	$6*10^{6}$	$8*10^{7}$	$4*10^{6}$	$5*10^{5}$

Table 1: Optimization of Temperature for cultivation of PPFM

pH	6	6.5	7	7.5
Growth of PPFM (CFU/ml)	No Growth	$6*10^{6}$	$5*10^{7}$	$13*10^{7}$
Pigmentation	-	+	++	+++

Table 2: Optimization of pH for cultivation of PPFM

Methanol Concentration (%)	0.5	1	1.5	2
Pigmentation	-	+++	++	+

Table 3: Optimization of methanol concentration for cultivation of PPFM

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Gas Chromatogram of volatile compounds after transesterification

The combination of phenotypic traits and cellular methyl ester composition enables the endophytic isolates described here to be characterized as *Methylobacterium* sp. Since every microorganism has specific microbial fingerprinting such as FAME profile, its stability and heritability was justified.

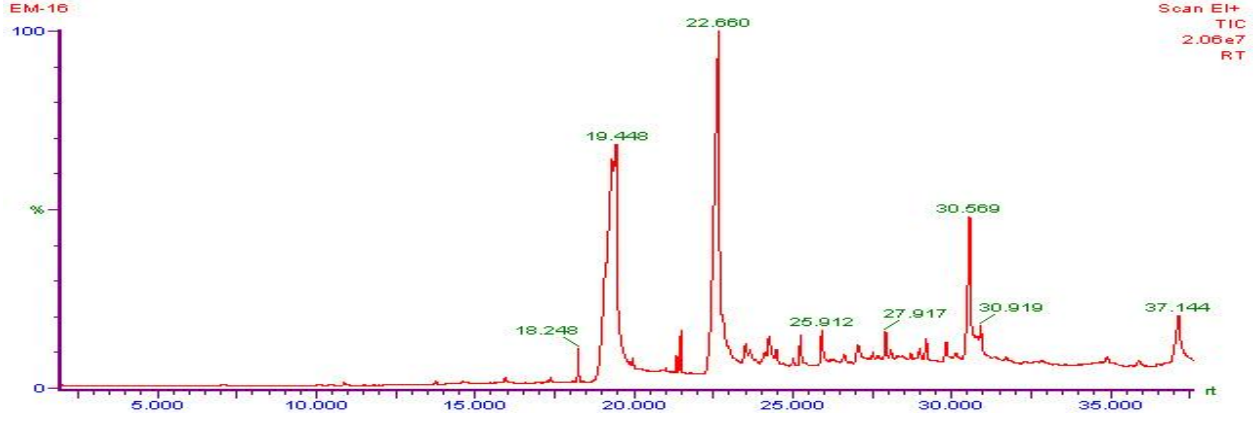

Figure 2: Chromatogram of volatile compounds after transesterification by methanol

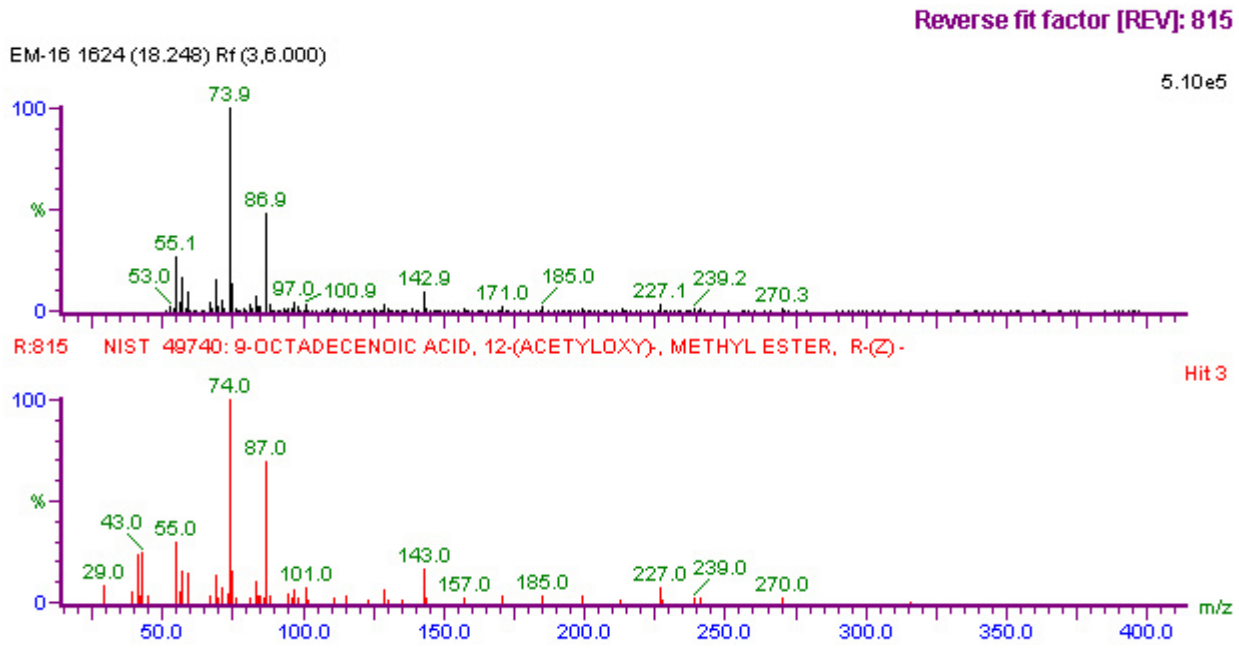

Figure 3: Chromatogram showing the detection of FAME from PPFM

Discussion

Modern day biogeochemical processes, particularly the global carbon cycle are solely vested on the Methylotrophic organisms which have also increased the interest of biotechnological scientists all over the world. There was an escalated progress in the fields of bioremediation, genetics, genomics and biochemistry and also physiology of Methylotrophic bacteria illustrates that methylotrophy is much more widespread and its endeavor into nature was even bigger than assumed [8]. The description of the Methylotrophic bacterial diversity within the Western Ghats contributes to our understanding of these microorganisms in this ecosystem. This highly adapted bacterial species is a potential source of biotechnological resources for future investigations, such as the search for bacteria that can promote bioremediation of a polluted environment and its resulting degradation products can be recycled into energy source.

The endophytes colonize the inner tissues of their plant hosts without causing disease, and they can establish mutualistic associations with the hosts, promoting a better adaptation of the host plants to the environment through mechanisms including the immobilization of heavy metals that are toxic to the plants. Since the introduction of gas chromatographic analysis of cellular fatty acid methyl ester (FAME), this technique has been used frequently in various taxonomic studies. FAME analysis has become established in many laboratories involved in microbial taxonomy and diagnostics [11]. On the basis of the above results, the specific FAME profile of *Methylobacterium* can be used as a taxonomic tool for microbial source tracking in the future. Despite such progress, system-level description of the Methylotrophic metabolism is currently lacking, and much remains to understand regarding the network-scale organization and properties of methylotrophy, and how the Methylotrophic capacity emerges from this organization, especially in facultative organisms.

References

1. Araújo, W.L., Marcon, J., Maccheroniv Jr, W., van Elsas, J.D., van Vuurde, J.W.L and Azevedo, J.L. 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in Citrus plants. *Applied Environmental Microbiology*. 68(10):4906-14.

2. Compant, S., Mitter, B., Colli-Mull, J.G., Gangl, H. and Sessitsch, A. 2011. Endophytes of grapevine flowers, berries and seeds: Identification of cultivable bacteria, comparison with other plant parts and visualization of niches of colonization. *Microb Ecol*. 62:188-197.

3. Doronina, N.V., Braus-Stromeyer, S.A., Leisinger, T. and Trotsenko, Y.A. 1995. Isolation and characterization of a newly facultative Methylotrophic bacterium: Description of *Methylorhabdus multivorans*, gen. nov., sp. nov. *Systematic and Applied Microbiology*. 18(1):92-98.

4. Dourado, M.N., Ferreira, A., Araujo, W.L., Azevedo, J.L. and Lacava, P.T. 2012. The diversity of endophytic Methylotrophic bacteria in an oil-contaminated and an oil-free mangrove ecosystem and their tolerance to heavy metals. *Biotechnology Research International*. 2012: Article 759865.

5. Eichorst, S.A., Breznak, J.A. and Schmidt, T.M. 2007. Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum *Acidobacteria*. *Appl. Environ. Microbiol*. 73(8):2708-2717.

6. Laxmi, J., Palanichamy, V., Reddy, N.N., Rajsekaran, C., Kumari, D.N.V. and Mitra, B. 2012. Standardization of cultivation parameters for the extraction of carotenoid from Pink Pigmented Facultative Methylotrophic (PPFM) bacteria. *Asian J Pharm Clin Res*. 5(2):52-57.

7. Peel, D. and Quayle, J.R. 1961. Microbial growth on C_1 compounds. 1. Isolation and characterization of *Pseudomonas* AM 1. *Biochem. J*. 81:465-469.

8. Peyraud, R., Schneider, K., Kiefer, P., Massou, S., Vorholt, J.A and Portais, J-C. 2011. Diversity of endophytic bacterial populations and their interaction. *BMC Systems Biology.* 5(189):1-22.

9. Tawfeek, K.A., Al-Fassi, F.A. and Ramadan, E.M. 1989. Selection of Methylotrophic microorganisms for the formation of single cell protein. *K.A.U.: Sci*. 1:25-38.

10. Waturangi, D.E., Nicholas, C.D., Susanto, C.O. and Suhartono, M.T. 2011. Isolation and identification of Methylotrophic bacteria producing Methanol dehydrogenase from Human feet and mouth. *HAYATI J Biosci*. 18(1):11-15.

11. Yousef, L.F., Wojno, M., Dick, W.A. and Dick, R.P. 2012. Lipid profiling of the soybean pathogen *Phytophthora sojae* using Fatty Acid Methyl Esters (FAMEs). *Fungal Biol*. 116:613-619.

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