*Int.J.Curr.Microbiol.App.Sci* (2018) *7*(2): 1674-1691

*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706* Volume 7 Number 02 (2018)**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2018.702.203

# Isolation and Characterization of Methanol Utilizing Microorganisms from Leguminous Crops

**Umang Ahlawat, Rekha Sansanwal, Priyanka Batra and Leela Wati***

*Department of Microbiology, College of Basic Science and Humanities, CCS HAU Haryana Agricultural University, Hisar, Haryana, India*

**Corresponding author*

**Keywords**

Biofertilizer, Methanol, Nodule, Rhizosphere, Phylloshpere

**Article Info**

***Accepted:***
17 January 2018
***Available Online:***
10 February 2018

**A B S T R A C T**

A total of sixty isolates were obtained from rhizosphere, nodule and phyllosphere of different crops. These isolates were analysed for some of their plant growth promoting traits like IAA production, phosphate solubilisation, siderophore production, HCN production, ACC utilization and ammonia excretion. Apart from these traits all the isolates were also screened for their growth at different temperatures and salt concentrations. Three promising isolates (MP12, PP1 and UP4) were characterized morphologically, biochemically and on the basis of 16S rRNA gene sequencing. Phylogenetic tree of methanol utilizing isolates MP12, PP1 and UP4 was prepared by using neighbour joining of BLAST programme. The isolate MP12 showed 98% similarity with *Methylobacterium oryzae*, isolate PP1 showed 99% similarity with *Ochrobacterium intermedium* and isolate UP4 showed 95% similarity with *Microbacterium ginsengisoli*. All these three isolates were able to produce IAA, siderophore, ACC and other plant growth promoting traits. Therefore, these results suggest the possibility to use these methanol utilizing bacteria as biological fertilizers to increase pulse crop production.

## Introduction

Plants are known to produce a large number of volatile organic compounds (Guenther *et al.*, 1995). The emission of some of these compounds may represent a significant loss of carbon from plants. Methanol is one of these volatile atmospheric carbon compound and its annual emission is estimated to be 150 Tg (Galbally and Kirstine, 2002). Methanol is principally generated from plant pectin and lignin during plant growth and decay (1.5-45.7 µg per g dry weight $h^{-1}$) (Nemecekmarshall *et al.*, 1995). Biological methanol oxidation to carbon dioxide in terrestrial ecosystems has a direct impact on atmospheric chemistry, *i.e.* the lowering of methanol emission and as a consequence lowering of the ozone concentration in the atmosphere (Galbally and Kirstine, 2002). Interestingly, plants typically harbor methylotrophic bacteria which are capable of metabolizing methanol (Corpe and Rheem, 1989; Holland and Polacco, 1994), and these bacteria may be associated with plant surface methanol emission. Methylotrophs are defined as microbes that can utilize C1 compounds as the sole source of carbon and energy. Methylotrophic bacteria

are ubiquitous in most of the plant species, either through their active colonization of plant tissue or their latent resistance. Some methylotrophic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth (Madhaiyan *et al.*, 2006), nitrogen fixation (Sy *et al.*, 2001) and induction of resistance to plant pathogens (Siddiqui, 2006). It has really been proved that leaf surfaces of many temperate plant species are occupied by active methylotrophs, which form about 14–20% of the total microbial population of the phyllosphere (Mizuno *et al.*, 2012). Though, the top most soil layers of forests and grasslands are also properly supplied with oxygen, and belowground parts of plants are likely reservoirs of methanol. They are also found as endophytes and have the capability for biofilm formation, production of quorum-sensing signals, heavy metal and other stress resistance (Zaets and Kozyrovska, 2012). Because of their importance in the rhizosphere and potential for commercial utilization, methylotrophs have gained plenty of attention as bioinoculants for use in agriculture.Therefore, the present investigation was focused on the isolation and characterization of methanol utilizing methylotrophic bacteria from rhizospheric soil, nodules and phyllospere of different leguminous crops *i.e.* mung bean, urd bean, chickpea, field pea and pigeon pea.

## Materials and Methods

### Sample collection

The leaves, rhizosheric soil and nodules of mung bean, urd bean, chickpea, field pea and pigeon pea were used as samples to isolate methanol utilizing bacteria. The fresh samples were collected from fields of Pulses section, Department of Genetics and Plant Breeding, College of Agriculture, CCSHAU, Hisar, Haryana and screened for the presence of methanol utilizing bacteria.

### Isolation of methanol utilizing bacteria

The collected rhizopheric soil was diluted tenfold in series. Methanol utilizing bacteria were isolated by spreading the serial dilutions on ammonium mineral salt (AMS) medium, containing 0.5% (v/v) methanol (Whittenbury *et al.*, 1970). The collected nodules were first surface-sterilized with 70% ethanol, then with 0.1% mercuric chloride and finally washed thoroughly with distilled water. Bacterial isolates were obtained by streaking the crushed root nodules on ammonium mineral salt (AMS) medium. The methanol utilizing bacteria were also isolated by leaf print method (Holland *et al.*, 2002).

### Evaluation of methanol utilizing bacteria for plant growth promoting traits

### Estimation of indole acetic acid (IAA) production

The IAA production was estimated by growing the isolates in 5mL of nutrient broth medium supplemented with 0.2% (w/v) L-Tryptophan at 28 °C for 48 h in shaking incubator.

The supernatants, obtained by centrifugation were assayed with the standard methods described byGlickmann and Dessaux (1995).

### Determination of P solubilizing activity

Phosphate solubilization was detected using Pikovskaya's agar assay (Pikovskaya, 1948). The colonies were incubated at 28°C for 5-7 days on petri plates and the positive colonies developed a halo zone.

### Determination of siderophore production

Siderophore production was evaluated by Chrome azurol S (CAS) Agar assay (Schwyn and Neilands, 1987). The colonies were

incubated on Petri plates at 28°C for 5-7 days and the positive colonies developed orange halo zone around the colonies.

### Utilization of 1-aminocyclo propane-1-carboxylate (ACC)

1-aminocyclo propane-1-carboxylate (ACC) utilization activity was detected on ACC supplemented medium plates (Penrose and Glick, 2003). 5µl of the log phase grown culture of the isolates were spotted on the ACC medium plates and incubated at 28 °C for 3-5 days.

The growth on minimal medium plates supplemented with ammonium sulphate was used as a control.

### Estimation of ammonia production

Forestimation of ammonia production isolates were inoculated into 5 ml peptone medium and incubated for 48 h at 28 °C. After the bacterial growth, Nessler's reagent (0.5 ml) was added to tube in 2:1 ratio and absorbance was measured at 450 nm. Uninoculated medium was used as reference.

### Determination of HCN production

The methanol utilizing isolates were grown on King's B medium broth supplemented with 4.4g/l glycine. Three-millimeter strips of Whatman no.42 filter paper were sterilized and soaked in 2% sodium carbonate and 0.5% picric acid solution (Alstrom and Burns, 1989).

These strips were hanged in the test tube inoculated with methylotrophic bacterial cultures. Tubes were plugged with cotton and incubated at 30º C for 5 days. The change in the color of the strips from yellow to orange-red was observed for hydrogen cyanide production.

### Detection of methylotrophs for growth at different temperature

All the isolates were checked for their ability to grow at different temperatures *i.e.*, 30, 35, 40 and 45°C, on nutrient medium. The plates containing 30 ml of the medium were spotted with loopful of methylotrophic isolates. The plates were incubated for 3-4 days at different temperatures. The susceptibility of bacterial isolates to different temperatures was recorded as positive or negative result.

### Detection of methylotrophs for growth at different salt concentration

All the isolates were checked for their ability to grow at different concentrations of NaCl *i.e.*, 1, 2, 3, 4 % (w/v), on nutrient medium containing 20 mM HEPES (N-2-hydroxyethane-sulphonic acid) (Marsudi *et al.,* 1999). The plates containing 30 ml of the medium were spotted with loopful of methylotrophic isolates. The plates were incubated for 3-4 days at 28± $2^0$C in B.O.D. incubator. The susceptibility of bacterial isolates to NaCl was recorded as positive or negative result.

### Evaluation of characteristics relevant to persistence and possible functions

Indole production was tested by inoculating the peptone broth with methylotrophic culture and incubated at 30±$1^0$C for 24 h. Following incubation, 0.2 ml of Kovac's reagent was added in 5 ml of culture medium. A cherry red colour in the alcohol layer indicated a positive reaction.The tubes of Methyl red - Voges-Proskauer (MR-VP) broth were inoculated with methylotrophic cultures and incubated at 30±$1^0$C for 48 h for the Methyl red test. Following incubation, 5-6 drops of methyl red solution were added. A positive reaction was indicated by bright red colour at pH 4.2. Yellow or orange colour indicated a negative

reaction while a weekly positive test was indicated by red-orange colour. For Voges-Proskauer (acetoin production) test, the MR-VP broth was inoculated with the test culture and incubated at $30\pm1^0$C for 48 h. After that 1 ml of 40 percent KOH (plus 1% creatine) and 3 ml of 5 percent solution of α-naphthol in absolute ethanol were added to it. A positive reaction was indicated by the development of pink colour in 10-15 min. which became crimson in 30 min. Citrate utilization was determined by streaking each isolate on Simmons' citrate agar slants and then incubated for 96 h incubated for 96 h at $30\pm1^0$C.

A positive test was shown as blue colour on the streak of growth. Retention of original green colour and no growth on the line of streak indicated a negative reaction. Catalase activity was determined by adding tetramethyl-p phenylene diaminedihydro-chloride to colonies grown on a medium plate. Oxidase production was examined by adding few drops of 3% $H_2O_2$ to the loopful bacterial culture on a glass slide. Presence of starch was the appearance of blue color after the addition of Gram' iodine. Motility was examined by stabbing each isolate into the centre of a semi-solid NA medium plate (0.2% agar) and observing the diffusing colonies for 3 days.

## Isolation and quantification of genomic DNA

Genomic DNA was isolated by modified CTAB method (Ausubel *et al.,* 2001). The genomic DNA was diluted 100 times and quantified by measuring the absorbance at 260 nm and 280 nm. The amount of DNA was estimated using the relationship that O.D. of 1.0 corresponds to 50 µg $ml^{-1}$. The purity of DNA was assessed by measuring A260/A280 ratio; A260: A280 = 1.5 - 1.8 for pure DNA. Purity of DNA was also checked on 0.8% agarose gel and bands were observed.

## Amplification of 16S rDNA sequences (Lukow *et al.,* 2000)

Polymerase chain reaction (PCR) was used to amplify the 16S rDNA of the selected methylotrophic bacterial isolates. The forward primer 5' -AGGAGGTGATCCAGCCGC- 3' and reverse primer 5'-AGAGTTTGATCCTGGCTCAG-3' enable the amplification of 16S rDNA sequences present in methylotrophic bacterial DNA.

The 50µL reaction mixture contained 50 pmol of each primer, 200 mM of each of the fourd NTPs, 1.5 mM $MgCl_2$ and 1.25 U *Taq* polymerase (A Geno Technology, Inc. USA).

The temperature program was as follows: 1min denaturation at 94°C, 1 min annealing at 53°C and 1 min chain extension at 72°C for 30 cycles, followed by a post-run of 1 min at 72°C before denaturation.

## Partial 16S rDNA sequence analysis

The partial sequence of 16S rDNA of selected bacterial isolates were obtained after sequencing (from Xcelris Genomics, Ahemdabad, India) and compared with the sequences already submitted in NCBI (National centre for Biotechnology Information) database (Altschul *et al.,* 1997).

## Results and Discussion

The methnol utilizing bacteria were isolated from the rhizoshperic soil, root nodules and phylloshere of mungbean, urdbean, pea, pigeon pea and chickpea.

A total of sixty isolates were obtained from rhizosphere, nodule and phyllosphere of different crops. Out of 60 isolates, 8 from the rhizospheric soil, 23 from nodule samples and 29 from the phyllosphere of different crops were isolated (Table 1).

## Evaluation of methanol utilizing bacteria for plant growth promoting traits

All 60 isolates were screened for *in vitro* plant growth promotion activities.

## Determination of indole acetic acid (IAA) production and P solubilization activity

The results originated from qualitative and quantitative assay of IAA reflected the ability of 55 isolates out of 60 tested microorganisms to produce indole acetic acid. All the 55 isolates exhibited a pink to red color with a little variation in intensity. In the quantitative measurements, the highest value of IAA production was obtained by MP12 (29.99 µg/ml), followed by UP4 and MP15, as they produced (26.31 µg/ml) and (24.94 µg/ml) respectively (Table 2). For the determination of P solubilization activity all the isolates were spotted on the plates of Pikovskaya's agar medium and incubated at 28ºC for 5 days. P solubilization index (P-S.I.) was calculated as ratio of diameter of solubilization zone and colony. The P-S.I. of methylotrophic isolates varied from 0.60 to 2.96 (Fig. 3). The maximum P-S.I. was recorded in the isolate PP1 (2.96) followed by MP12 (2.31) and UP4 (1.83) (Table 2).

## Determination of siderophore production, HCN production and 1-aminocyclo propane-1-carboxylate (ACC) utilization

Siderophore production by the methanol utilizing bacterial isolates was assessed in solid medium and tested by Chrome AzurolSulfonate (CAS) assay method by spotting all the 60 methylotrophic isolates on the Chrome AzurolSulfonate (CAS) medium plates followed by incubation at 37ºC in BOD for ten days. The ability to synthesize siderophore was restricted to nine isolates that showed orange color on CAS plates (Table 3). The maximum siderophore production was observed in UP4. Utilization of ACC was tested on the basis of growth observed on ACC supplemented medium plates and all the isolates were divided into four categories (Table 3). Out of total sixty isolates thirteen isolates (22%) showed good growth on ACC plates, seventeen isolates (28%) showed moderate growth, twenty-seven isolates showed poor growth (45%) and three isolates (5%) did not show any growth. Sidewise all the bacterial isolates were tested for the production of HCN on King'B medium supplemented with glycine (4.4 g/L). Three-millimeter-wide strips of Whatman No. 42 filter paper were sterilized and soaked in 0.5% picric acid, and 2% sodium carbonate solution. These strips were hanged in the test tube containing KB medium broth inoculated with different bacterial cultures. The change in the color of the strips from yellow to orange red was observed with some isolates after 2 to 3 days of incubation, indicating the production of HCN. Nine bacterial isolates showed HCN production (Table 3). HCN production based on visual observation was maximum in isolate UP4 and CR1. Overall, 15% bacterial isolates showed hydrogen cyanide production.

## Estimation of ammonia production

All the methanol utilizing isolates were tested for ammonia excretion. Out of sixty isolates twenty-nine isolates were able to excrete ammonia, which varied from 0.31 to 4.73 µg/ml in liquid medium after 4 days of incubation (Table 4).

## Detection of methylotrophs for growth at different temperature

All the isolates were able to grow on nutrient agar medium plates at 30°C. At $35^0$ C all isolates except MP-9 were able to grow. As the temperature was increased further up to 40 and $45^0$C, a decrease in growth was observed. At 40ºC, forty-three isolates showed good

growth and only 17 isolates were able to grow at 45°C (Table 5).

## Detection of methylotrophs for growth at different salt concentration

All the isolates were tested for salt tolerance in nutrient broth medium supplemented with various (1-5%) concentration of NaCl. All the isolates showed growth on medium containing 1% NaCl. Higher level of NaCl repressed the growth of microbes. Ten isolates showed tolerance against 4% NaCl and only two isolates were able to grow on medium containing 5% NaCl (Table 6).

## Screening of methylotrophic isolates for multiple plant growth promoting traits

Among sixty methylotrophic isolates, three isolates (MP12, UP4 and PP1) were selected based upon the presence of growth promoting traits (Table 7). Methylotrophic isolate (MP12) showed the highest IAA production (29.99 µg/ml) as well as ammonia excretion (4.73 µg/ml) whereas PP1 showed highest phosphate solublization index *i.e.* 2.96. Siderophore production, ACC utilization and HCN production was found maximum in UP4 and all four methylotrophic isolates were able to tolerate high temperature (45°C) as well as salt concentration (4%).

All three multitrait methylotrophic isolates (MP12, UP4 and PP1) showing plant growth promoting (PGP) traits (Table 7) were selected to characterize and identify through various biochemical tests and 16S rDNA sequencing.

## Identification of promising methylotrophs using standard morphological and biochemical tests

Bacterial isolates were grown on nutrient medium slants at 28±2ºC for 24 h. For the identification of bacterial isolates, various morphological and biochemical characteristics of the cultures were studied as per procedure described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Morphological characters studied were colony morphology, pigment production, Gram reaction and bacterial cell morphology. Round gummy whitish colonies were observed in bacterial isolate PP1. Orange and pink colonies were observed in isolate UP4 and MP12 respectively (Table 8). Bacterial isolates MP12 and PP1 were found to be Gram's negative small rods while UP4 was Gram's positive.

Study of biochemical characteristics included indole production, methyl red test, Voges-Proskauer reaction, citrate utilization, oxidase, catalase and motility. MP12 and PP1 developed intense deep purple bluish color after rubbing a loop of bacterial growth on filter paper moistened with Wurster's reagent, indicating that these cultures were oxidase positive.

Bacterial isolate UP4 was found to be positive for VP reaction and developed pink color in MR-VP broth. All three bacterial isolates *i.e.*, MP12, PP1 and UP4 showed effervescence after addition of 3% $H_2O_2$ on the bacterial growth, indicating that these cultures were catalase positive. Two bacterial isolates *i.e.*, MP12 and UP4 utilized the citrate and developed blue color along the streak of growth in Simmon's citrate medium (Table 9).

## Partial 16S rDNA sequencing of promising methylotrophs

Sequencing of the 16S rRNA gene has served as an important tool for identification of bacteria over a number of years. In present study, the DNA of all three promising methylotrophs (MP12, PP1 and UP4) was extracted and approx 1500 bp DNA fragment was amplified from 16S rRNA gene.

**Table.1** List of methanol utilizing bacteria isolated from different crop samples

| Crop | Rhizosphere | Nodule | Phylloshere |
|---|---|---|---|
| **Mungbean** | MR1, MR2 | MN1, MN2, MN3, MN4, MN5, MN6, MN7, MN8, MN9, MN10, MN11, MN12 | MP1, MP2, MP3, MP4, MP5, MP6, MP7, MP8, MP9, MP10, MP11, MP12, MP13, MP14, MP15, MP16 |
| **Urdbean** | UR1 | UN1, UN2, UN3, UN4, UN5, UN6, UN7, UN8, UN9, UN10, UN11 | UP1, UP2, UP3, UP4, UP5, UP6 |
| **Pea** | PR1, PR2 | - | PA1 |
| **Chickpea** | CR1, CR2, CR3 | - | CP1 |
| **Pigeon pea** | - | - | PP1, PP2, PP3, PP4, PP5 |

**Table.2** Indole acetic acid production and phosphate solubilization by methanol utilizing isolates

| Sr. No. | Isolate | IAA (µg/ml) | P-S.I. | Sr. No. | Isolate | IAA (µg/ml) | P-S.I. |
|---|---|---|---|---|---|---|---|
| **1** | MP-1 | 02.84 | - | 31 | MN-2 | - | - |
| **2** | MP-2 | 01.25 | - | 32 | MN-3 | 07.89 | - |
| **3** | MP-3 | 01.68 | - | 33 | MN-4 | 02.75 | - |
| **4** | MP-4 | 04.41 | 0.80 | 34 | MN-5 | 08.55 | - |
| **5** | MP-5 | 03.77 | - | 35 | MN-6 | 04.43 | 1.13 |
| **6** | MP-6 | 01.84 | 1.15 | 36 | MN-7 | 05.66 | - |
| **7** | MP-7 | 07.67 | 1.26 | 37 | MN-8 | 18.48 | 1.36 |
| **8** | MP-8 | 05.30 | - | 38 | MN-9 | 20.09 | - |
| **9** | MP-9 | 04.90 | - | 39 | MN-10 | 08.29 | 1.20 |
| **10** | MP-10 | 16.11 | - | 40 | MN-11 | 15.10 | - |
| **11** | MP-11 | 03.97 | - | 41 | MN-12 | 09.31 | - |
| **12** | MP-12 | 29.99 | 2.31 | 42 | UN-1 | - | - |
| **13** | MP-13 | 10.14 | - | 43 | UN-2 | - | - |
| **14** | MP-14 | 06.90 | 1.60 | 44 | UN-3 | - | - |
| **15** | MP-15 | 24.94 | 1.57 | 45 | UN-4 | 06.54 | - |
| **16** | MP-16 | 15.71 | - | 46 | UN-5 | - | - |
| **17** | UP-1 | 06.48 | - | 47 | UN-6 | 09.10 | - |
| **18** | UP-2 | 11.24 | 1.00 | 48 | UN-7 | 12.42 | - |
| **19** | UP-3 | 07.27 | 1.03 | 49 | UN-8 | 18.98 | - |
| **20** | UP-4 | 26.31 | 1.83 | 50 | UN-9 | 02.63 | 1.33 |
| **21** | UP-5 | 09.04 | 0.60 | 51 | UN-10 | 12.89 | 1.28 |
| **22** | UP-6 | 19.91 | 1.20 | 52 | UN-11 | 15.05 | - |
| **23** | PP-1 | 21.12 | 2.96 | 53 | CR-1 | 10.28 | 1.40 |
| **24** | PP-2 | 11.94 | - | 54 | CR-2 | 10.73 | 1.16 |
| **25** | PP-3 | 13.62 | - | 55 | CR-3 | 14.02 | 1.00 |
| **26** | PP-4 | 04.97 | - | 56 | MR-1 | 08.23 | - |
| **27** | PP-5 | 08.20 | - | 57 | MR-2 | 12.68 | - |
| **28** | CP-1 | 01.09 | - | 58 | UR-1 | 08.52 | 1.35 |
| **29** | PA-1 | 09.77 | 1.50 | 59 | PR-1 | 14.25 | - |
| **30** | MN-1 | 05.85 | 1.42 | 60 | PR-2 | 15.61 | - |

**Table.3** Siderophore production, HCN production and ACC utilization by methanol utilizing isolates

| Sr. No. | Isolate | Siderophore production | HCN production | ACC utilization | Sr. No. | Isolate | Siderophore production | HCN production | ACC utilization |
|---|---|---|---|---|---|---|---|---|---|
| **1** | MP-1 | - | - | ++ | 31 | MN-2 | - | - | + |
| **2** | MP-2 | - | - | ++ | 32 | MN-3 | - | - | + |
| **3** | MP-3 | - | - | - | 33 | MN-4 | - | - | +++ |
| **4** | MP-4 | - | - | +++ | 34 | MN-5 | - | - | + |
| **5** | MP-5 | - | - | + | 35 | MN-6 | - | - | + |
| **6** | MP-6 | + | ++ | +++ | 36 | MN-7 | - | - | + |
| **7** | MP-7 | - | - | +++ | 37 | MN-8 | - | - | + |
| **8** | MP-8 | - | - | - | 38 | MN-9 | - | - | + |
| **9** | MP-9 | - | - | ++ | 39 | MN-10 | + | ++ | +++ |
| **10** | MP-10 | - | - | ++ | 40 | MN-11 | - | - | ++ |
| **11** | MP-11 | - | - | - | 41 | MN-12 | - | - | + |
| **12** | MP-12 | ++ | + | ++ | 42 | UN-1 | - | - | ++ |
| **13** | MP-13 | - | - | ++ | 43 | UN-2 | - | - | ++ |
| **14** | MP-14 | - | - | +++ | 44 | UN-3 | - | - | + |
| **15** | MP-15 | + | + | ++ | 45 | UN-4 | - | - | + |
| **16** | MP-16 | - | - | +++ | 46 | UN-5 | - | - | + |
| **17** | UP-1 | - | - | +++ | 47 | UN-6 | - | - | + |
| **18** | UP-2 | - | - | ++ | 48 | UN-7 | - | - | + |
| **19** | UP-3 | - | - | ++ | 49 | UN-8 | - | - | + |
| **20** | UP-4 | +++ | +++ | +++ | 50 | UN-9 | - | - | + |
| **21** | UP-5 | - | - | ++ | 51 | UN-10 | - | - | +++ |
| **22** | UP-6 | + | - | +++ | 52 | UN-11 | + | - | +++ |
| **23** | PP-1 | ++ | ++ | + | 53 | CR-1 | - | +++ | + |
| **24** | PP-2 | - | - | ++ | 54 | CR-2 | - | - | + |
| **25** | PP-3 | - | - | + | 55 | CR-3 | - | - | + |
| **26** | PP-4 | - | - | + | 56 | MR-1 | - | - | ++ |
| **27** | PP-5 | - | - | + | 57 | MR-2 | + | - | ++ |
| **28** | CP-1 | - | - | ++ | 58 | UR-1 | - | + | + |
| **29** | PA-1 | - | + | +++ | 59 | PR-1 | - | - | + |
| **30** | MN-1 | - | - | + | 60 | PR-2 | - | - | + |

Good (+++), Moderate (++), Poor (+), None (-)

**Table.4** Ammonia excretion by methanol utilizing bacteria

| Sr. No. | Isolate | Ammonia excretion (µg/ml) | Sr. No. | Isolate | Ammonia excretion (µg/ml) |
|---|---|---|---|---|---|
| 1 | MP-1 | 2.93 | 31 | MN-2 | - |
| 2 | MP-2 | 2.63 | 32 | MN-3 | - |
| 3 | MP-3 | 3.71 | 33 | MN-4 | - |
| 4 | MP-4 | 1.11 | 34 | MN-5 | - |
| 5 | MP-5 | 3.26 | 35 | MN-6 | - |
| 6 | MP-6 | 0.31 | 36 | MN-7 | - |
| 7 | MP-7 | 3.47 | 37 | MN-8 | - |
| 8 | MP-8 | 3.19 | 38 | MN-9 | - |
| 9 | MP-9 | 3.76 | 39 | MN-10 | - |
| 10 | MP-10 | 3.65 | 40 | MN-11 | - |
| 11 | MP-11 | 1.09 | 41 | MN-12 | - |
| 12 | MP-12 | 4.73 | 42 | UN-1 | - |
| 13 | MP-13 | 0.85 | 43 | UN-2 | - |
| 14 | MP-14 | - | 44 | UN-3 | - |
| 15 | MP-15 | - | 45 | UN-4 | - |
| 16 | MP-16 | - | 46 | UN-5 | - |
| 17 | UP-1 | - | 47 | UN-6 | - |
| 18 | UP-2 | 2.07 | 48 | UN-7 | - |
| 19 | UP-3 | - | 49 | UN-8 | - |
| 20 | UP-4 | 4.59 | 50 | UN-9 | - |
| 21 | UP-5 | 2.13 | 51 | UN-10 | - |
| 22 | UP-6 | 3.08 | 52 | UN-11 | - |
| 23 | PP-1 | 4.17 | 53 | CR-1 | 2.12 |
| 24 | PP-2 | 2.53 | 54 | CR-2 | 2.77 |
| 25 | PP-3 | 2.21 | 55 | CR-3 | 2.29 |
| 26 | PP-4 | - | 56 | MR-1 | 4.25 |
| 27 | PP-5 | - | 57 | MR-2 | 2.89 |
| 28 | CP-1 | 3.70 | 58 | UR-1 | - |
| 29 | PA-1 | 3.34 | 59 | PR-1 | 3.43 |
| 30 | MN-1 | - | 60 | PR-2 | 3.69 |

**Table.5** Growth of methylotrophic isolates at different temperatures

| S. No. | Isolate | Temperature (°C) | | | | S. No. | Isolate | Temperature (°C) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 35 | 40 | 45 | | | 30 | 35 | 40 | 45 |
| 1 | MP-1 | + | + | + | - | 31 | MN-2 | + | + | + | - |
| 2 | MP-2 | + | + | + | - | 32 | MN-3 | + | + | + | - |
| 3 | MP-3 | + | + | + | - | 33 | MN-4 | + | + | + | - |
| 4 | MP-4 | + | + | - | - | 34 | MN-5 | + | + | + | - |
| 5 | MP-5 | + | + | + | - | 35 | MN-6 | + | + | + | - |
| 6 | MP-6 | + | + | - | - | 36 | MN-7 | + | + | + | + |
| 7 | MP-7 | + | + | - | - | 37 | MN-8 | + | + | + | + |
| 8 | MP-8 | + | + | - | - | 38 | MN-9 | + | + | + | - |
| 9 | MP-9 | + | - | - | - | 39 | MN-10 | + | + | + | + |
| 10 | MP-10 | + | + | - | - | 40 | MN-11 | + | + | - | - |
| 11 | MP-11 | + | + | - | - | 41 | MN-12 | + | + | + | + |
| 12 | MP-12 | + | + | + | + | 42 | UN-1 | + | + | + | - |
| 13 | MP-13 | + | + | - | - | 43 | UN-2 | + | + | + | - |
| 14 | MP-14 | + | + | - | - | 44 | UN-3 | + | + | + | - |
| 15 | MP-15 | + | + | + | + | 45 | UN-4 | + | + | + | - |
| 16 | MP-16 | + | + | + | - | 46 | UN-5 | + | + | + | - |
| 17 | UP-1 | + | + | + | - | 47 | UN-6 | + | + | + | - |
| 18 | UP-2 | + | + | - | - | 48 | UN-7 | + | + | + | - |
| 19 | UP-3 | + | + | + | - | 49 | UN-8 | + | + | + | - |
| 20 | UP-4 | + | + | + | + | 50 | UN-9 | + | + | + | - |
| 21 | UP-5 | + | + | + | + | 51 | UN-10 | + | + | + | + |
| 22 | UP-6 | + | + | + | + | 52 | UN-11 | + | + | + | + |
| 23 | PP-1 | + | + | + | + | 53 | CR-1 | + | + | + | + |
| 24 | PP-2 | + | + | + | - | 54 | CR-2 | + | + | + | + |
| 25 | PP-3 | + | + | - | - | 55 | CR-3 | + | + | + | + |
| 26 | PP-4 | + | + | - | - | 56 | MR-1 | + | + | - | - |
| 27 | PP-5 | + | + | + | + | 57 | MR-2 | + | + | + | + |
| 28 | CP-1 | + | + | - | - | 58 | UR-1 | + | + | + | - |
| 29 | PA-1 | + | + | + | - | 59 | PR-1 | + | + | - | - |
| 30 | MN-1 | + | + | + | - | 60 | PR-2 | + | + | - | - |

**Table.6** Growth of methylotrophic isolates at different salt concentrations

| Sr. No. | Isolate | NaCl concentration (%) | | | | | Sr. No. | Isolate | NaCl concentration (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | | | 1 | 2 | 3 | 4 | 5 |
| 1 | MP-1 | + | + | + | - | - | 31 | MN-2 | + | + | - | - | - |
| 2 | MP-2 | + | + | + | + | - | 32 | MN-3 | + | + | - | - | - |
| 3 | MP-3 | + | + | + | + | - | 33 | MN-4 | + | + | - | - | - |
| 4 | MP-4 | + | + | + | - | - | 34 | MN-5 | + | + | - | - | - |
| 5 | MP-5 | + | + | + | + | - | 35 | MN-6 | + | - | - | - | - |
| 6 | MP-6 | + | + | + | + | - | 36 | MN-7 | + | + | - | - | - |
| 7 | MP-7 | + | + | + | - | - | 37 | MN-8 | + | + | - | - | - |
| 8 | MP-8 | + | + | + | - | - | 38 | MN-9 | + | + | - | - | - |
| 9 | MP-9 | + | + | - | - | - | 39 | MN-10 | + | + | - | - | - |
| 10 | MP-10 | + | + | - | - | - | 40 | MN-11 | + | + | - | - | - |
| 11 | MP-11 | + | - | - | - | - | 41 | MN-12 | + | + | - | - | - |
| 12 | MP-12 | + | + | + | + | + | 42 | UN-1 | + | + | - | - | - |
| 13 | MP-13 | + | - | - | - | - | 43 | UN-2 | + | + | - | - | - |
| 14 | MP-14 | + | + | + | - | - | 44 | UN-3 | + | + | - | - | - |
| 15 | MP-15 | + | + | + | + | - | 45 | UN-4 | + | + | - | - | - |
| 16 | MP-16 | + | + | - | - | - | 46 | UN-5 | + | + | - | - | - |
| 17 | UP-1 | + | + | - | - | - | 47 | UN-6 | + | + | - | - | - |
| 18 | UP-2 | + | + | + | - | - | 48 | UN-7 | + | + | - | - | - |
| 19 | UP-3 | + | + | - | - | - | 49 | UN-8 | + | + | - | - | - |
| 20 | UP-4 | + | + | + | + | - | 50 | UN-9 | + | + | - | - | - |
| 21 | UP-5 | + | + | + | - | - | 51 | UN-10 | + | + | - | - | - |
| 22 | UP-6 | + | + | + | - | - | 52 | UN-11 | + | + | + | + | - |
| 23 | PP-1 | + | + | + | + | + | 53 | CR-1 | + | + | + | + | - |
| 24 | PP-2 | + | + | - | - | - | 54 | CR-2 | + | + | - | - | - |
| 25 | PP-3 | + | + | - | - | - | 55 | CR-3 | + | + | - | - | - |
| 26 | PP-4 | + | + | - | - | - | 56 | MR-1 | + | + | + | - | - |
| 27 | PP-5 | + | - | - | - | - | 57 | MR-2 | + | + | - | - | - |
| 28 | CP-1 | + | - | - | - | - | 58 | UR-1 | + | - | - | - | - |
| 29 | PA-1 | + | + | + | - | - | 59 | PR-1 | + | + | + | - | - |
| 30 | MN-1 | + | + | - | - | - | 60 | PR-2 | + | + | + | - | - |

**Table.7** Multiple plant growth promoting traits of methylotrophic isolates

| Sr. No | Isolate | Temperature tolerance (upto 45°C) | Salt tolerance (upto 4%) | ACC utilization | IAA production (µg/ml) | Ammonia excretion (µg/ml) | Siderophore production | HCN production | P-solublization index |
|---|---|---|---|---|---|---|---|---|---|
| 1 | **MP-12** | + | + | ++ | 29.99 | 4.73 | ++ | + | 2.31 |
| 2 | **UP-4** | + | + | +++ | 26.31 | 4.59 | +++ | +++ | 1.83 |
| 3 | **PP-1** | + | + | + | 21.12 | 4.17 | ++ | ++ | 2.96 |

**Table.8** Morphological characteristics of promising methanol utilizing isolates

| S. No. | Isolates | Colony morphology | Pigment | Gram reaction | Morphology |
|---|---|---|---|---|---|
| 1 | MP12 | Dark pink, smooth, rounded | Pink | -ve | Small rods |
| 2 | PP1 | White, gummy, rounded | - | -ve | Small rods |
| 3 | UP4 | Orange, smooth, rounded | Orange | +ve | Small rods |

**Table.9** Biochemical characteristics of promising methanol utilizing isolates

| Biochemical Characters | MP 12 | PP1 | UP4 |
|---|---|---|---|
| Indole production | - | - | - |
| Methyl red test | - | - | - |
| Vogesproskaure | - | - | + |
| Citrate utilization | + | - | + |
| Catalase action | + | + | + |
| Oxidase test | + | + | - |
| Motility | + | + | + |

**Fig.1** Phylogenetic tree of isolate MP12 by using neighbor joining method of blast programme

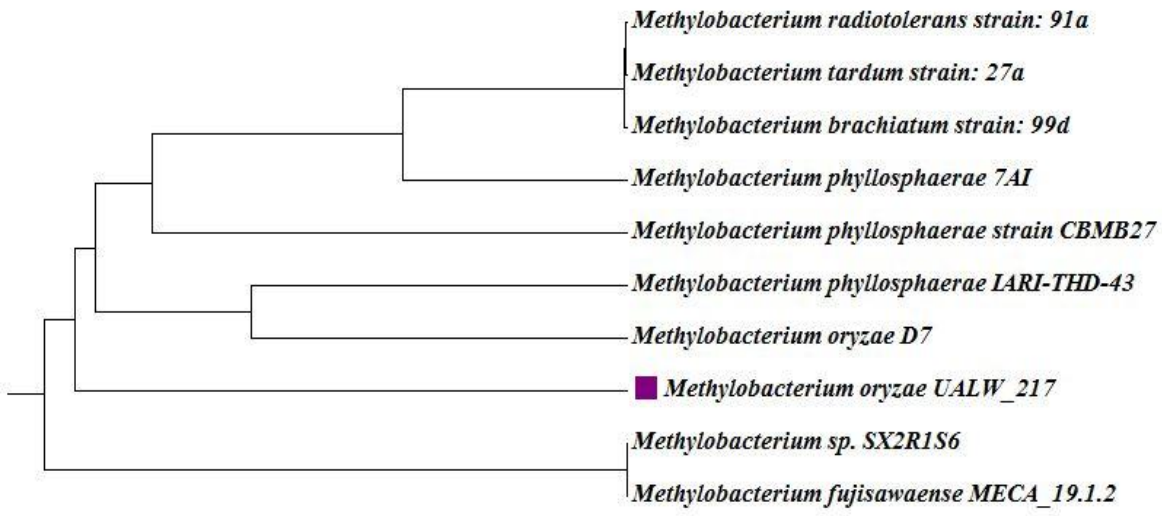


**Fig.2** Phylogenetic tree of isolate PP1 by using neighbor joining method of blast programme

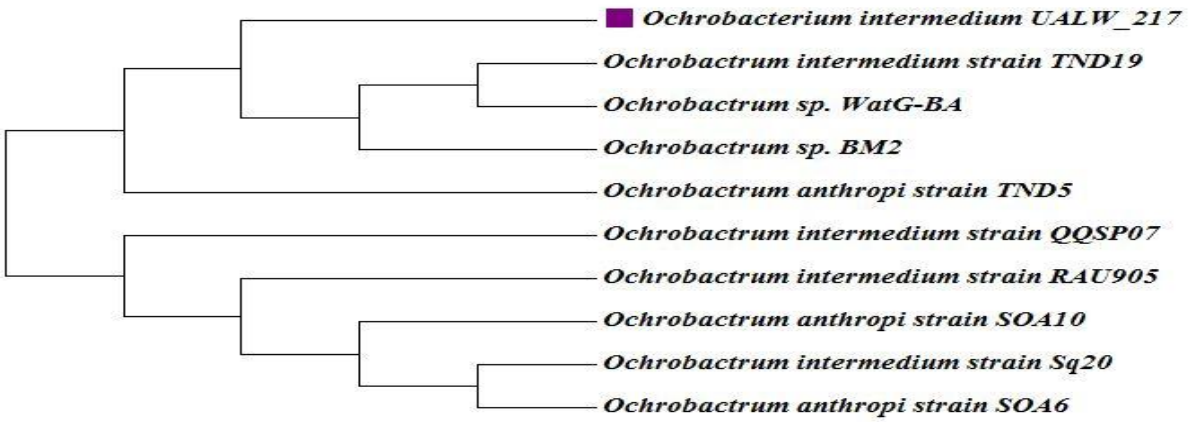
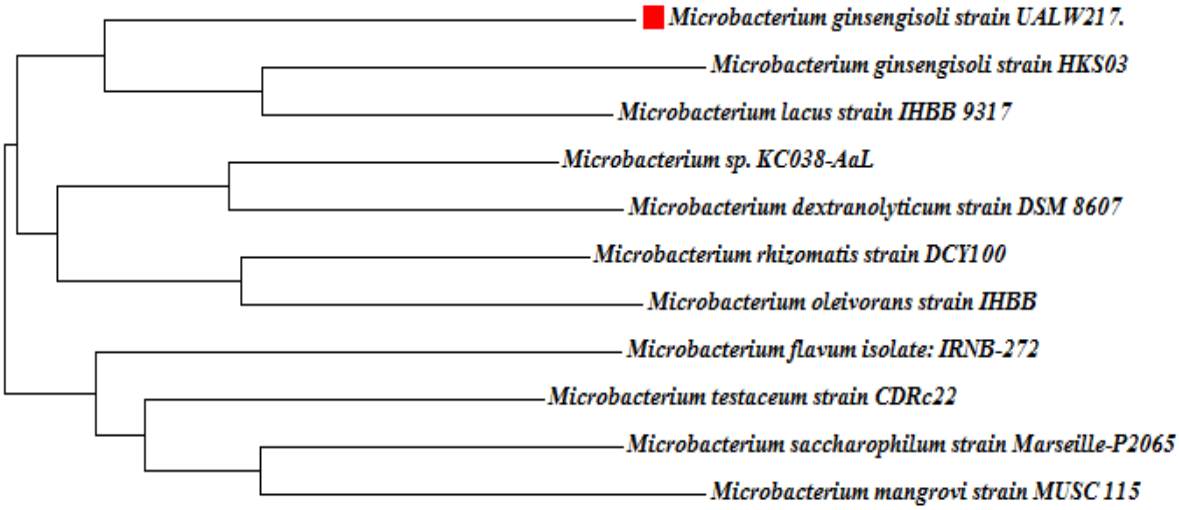



**Fig.3** Phylogenetic tree of isolate UP4 by using neighbor joining method of blast programme




After amplification the fragment got sequenced after purification from Xcelris Genomics, Ahemdabad, India. The sequences were aligned with NCBI database using BLAST programme. Phylogenetic tree of methylotrophs MP12, PP1 and UP4 was prepared by using neighbour joining of BLAST programme. The methylotroph MP12 showed 98% similarity with *Methylobacterium oryzae* (Fig. 1), isolate PP1 showed 99% similarity with *Ochrobacterium intermedium* (Fig. 2) and isolate UP4 showed 95% similarity with *Microbacterium ginsengisoli* (Fig. 3).

As methanol is plant-derived in terrestrial ecosystems, plant surfaces, such as leaves, can be reflected as favoured habitats of aerobic methanol utilizing bacteria. The methanol utilizing bacteria also known as

methylobacteria are physiologically and functionally an interesting group of bacteria. They are frequently reported from plant material and proved to be associated with more than 70 plant species (Corpe, 1985; Holland and Polacco, 1994) which makes them interesting and potential agents for affecting plant growth. They are known to produce auxins (Doronina *et al.*, 2002) and cytokinins (Koenig *et al.*, 2002) that stimulate cell division or plant elongation and lower the accumulation of ethylene in plant roots by the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Lidstrom and Chistoserdova, 2002; Trotsenko *et al.*, 2001). Due to their plant growth promoting and disease control attributes, methylotrophic bacteria can be applied in the form of bioinoculants in agriculture as supplements to promote plant growth and health. The present study was conducted to isolate, characterize and to identify the promising methanol utilizing bacteria for various growth promoting traits. Pulse crop samples *i.e*. rhizospheric soil, root nodules and leaves were collected from CCSHAU Hisar farms. In total, 60 methylotrophic bacteria were isolated from different samples using ammonium mineral salt medium (AMS) containing 0.5% methanol as the sole source of carbon. Selected methylotrophic bacteria with plant growth promoting traits were identified on the basis of 16S rRNA gene sequencing. Screening of all (60) methylotrophic bacterial isolates for plant growth promotion characters such as IAA, phosphate solubilisation, HCN production, siderophore production and ACC utilization showed that 3 methylotrophic isolates exhibit all of these growth promoting activities which may directly or indirectly help the leguminous crop plant growth.

In vitro screening for characteristics commonly associated with plant growth promotion revealed that 55 isolates out of 60 were able to produce IAA in a range of 1.09-29.99 µg/ml, indicating a substantial variability among methanol utilizing bacterial isolates for IAA production. Indole acetic acid (IAA) is an important physiologically active auxin and supports the production of longer roots with an increased number of root hairs and root laterals which are required for nutrient uptake (Datta and Basu, 2000). *Methylobacterium* strains have been reported to produce indole acetic acid, which promote both cell division as well as cell elongation (Schauer and Kutschera, 2011).

The beneficial effect of plant growth promoting bacteria in maintaining adequate levels of mineral nutrients especially the P in crop production had been previously reported (Rodriguez and Fraga, 1999). In our study, P-solubilization activity of methanol utilizing bacterial isolates on solid medium was restricted to 38% of the isolates only and their P-solubilization index (P-S.I.) varied from 0.60 to 2.96.

Ability to convert the insoluble phosphates (both organic and inorganic) to a form available to the plants, like orthophosphate, is an important feature for a plant growth promoting bacteria for increasing plant yields. Kumari (2011) reported four different strains of facultative methylotrophs that solubilized tricalcium phosphate in Pikovskaya's agar medium and solubilization index ranged from 1.28 to 1.85.Siderophore production is an another important character as they are biological molecules produced by several bacteria having application in agriculture to improve soil fertility and also act as a biocontrol agent. The primary function of these molecules is to chelate the ferric iron [Fe(III)] and thereby make it accessible for microbial and plant cells and hence improving the plant growth. In the present study only 15% methanol utilizing bacteria were able to produce siderophore as tested in solid

medium. Ventorino *et al.,* (2014) reported siderophore production by *M. populi*VP2, which was able to influence plant growth by binding Fe3+ and made the iron less available for other microorganisms in the rhizosphere, such as plant pathogens.

The results of qualitative test of HCN production showed that low percent of isolated bacteria were capable of producing HCN (about 15%). Till now there is no report on HCN producing methanol utilizing bacteria. The presence of ACC deaminase has been studied in various plant growth promoting bacteria (Penrose and Glick, 2001), which may be involved in modulating the associated ethylene signaling pathway. Our study demonstrated that 95% of the total isolates can utilize ACC, a precursor of ethylene, which implies that these isolates can reduce the ethylene levels. ACC deaminase producing microorganisms are known to be beneficial to the plants because the high levels of ethylene inhibit the growth of the plant (Lugtenberg and Kamilova, 2009).

Similarly, accumulation of ammonia as a result of nitrogen fixation can prohibit both synthesis as well as activity of nitrogenase enzyme complex in the cell, resulting in the nitrogen fixation to stop (Colnaghi *et al.*, 1997). Some differences of nitrogen fixing regulation mechanism found in several nitrogen fixing bacteria strains, in which ammonia produced by nitrogen fixation can be excreted via a simple diffusion mechanism (Kleiner, 1982). Hence these methanol utilizing bacterial isolates were characterized for the ability to excrete ammonia and approximately 48% of the total bacterial isolates were able to excrete ammonia. Apart from these plant growth promoting traits temperature and salt tolerance activity in the isolates can also help in growth promotion of plants (Doronina *et al.,* 2003; Egamberdieva *et al.,* 2015). In the present study, 17 isolates were able to grow up to 45°C and only 10 isolates were able to tolerate high NaCl concentration up to 4%.

Among sixty methylotrophic isolates, three isolates (MP12, UP4 and PP1) were selected based upon the presence of growth promoting traits. All three multitraitmethylotrophic isolates (MP12, UP4 and PP1) showing plant growth promoting (PGP) traits were selected to identify through 16S rDNA sequencing. The methylotroph MP12 showed 98% similarity with *Methylobacterium oryzae*, isolate PP1 showed 97% similarity with *Ochrobactrum intermedium* and isolate UP4 showed 96% similarity with *Microbacterium ginsengisoli.* Madhaiyan *et al.,* (2012) employed comparative 16S rRNA gene sequence based phylogenetic analysis to identify a novel plant associated obligate methylotrophic bacterium from the rhizospheric soil of field grown red pepper from India and designated strain as *M. rhizosphaerae* sp. Nov. Ca-68T. Similarly, Tambekar *et al.,* (2014) isolated a methylotrophic bacteria from Lonar Lake which was characterized by cultural, morphological, biochemical tests and identified by 16S rRNA gene sequencing as *Ochrobactrum oryzae*.

The search for beneficial bacteria is important for the development of new and efficient inoculants for agriculture. The nodules, rhizosphere and phyllosphere of different leguminous crops in CCSHAU farms contain high diversity of microorganisms. A total of 3 (MP12, PP1 and UP4) methanol utilizing bacteria, out of 60 isolates on the basis of different plant growth promoting traits were selected for identification by morphological, biochemical tests and further confirmed by 16SrRNA gene sequencing. MP12, PP1 and UP4 were identified as *Methylobacterium oryzae, Ochrobacterium intermedium* and *Microbacterium ginsengisoli* respectively

during this study. All these three isolates were able to produce different plant growth promoting traits as well as they can survive under high temperature and salt concentrations. In perspective, these methanol utilizing bacteria will be assessed to promote plant growth and development.

## References


Alstrom, S. and Burns, R.G. (1989) Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biology and Fertility of Soils*, 7, 232-238.

Altschul, S.F., Madden, T.L. and Schaffer, A.A. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389–3402.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.M., Coen, D.M., Varki, A. and Chanda, V.B. (eds.) (2001) *Current Protocols in Molecular Biology*. John Wiley, New York.

Colnaghi, R., Green, A., Luhong, H., Rudnick, P. and Kennedy, C. (1997) Strategies for increased ammonia production in free living or plant associated bacteria. *Plant and Soil*, 194, 145-154.

Corpe, W.A. and Rheem, S. (1989) Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiology and Ecology*, 62, 243–250.

Corpe, W.A. (1985) A method for detecting methylotrophic bacteria on solid-surfaces. *Journal of Microbiological Methodology*, 3(3-4), 215-221.

Datta, C. and Basu, P. (2000) lndole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub *Cajanus cojan*. *Microbiological* Research, 155, 123 – 127.

Doronina, N.V., Ivanova, E.G., Trotsenko, Y.A. (2002) New evidence for the ability of methylobacteria and methanotrophs to synthesize auxins. *Microbiology*, 71, 116–118.

Doronina, N.V., Tsyregma, D., Darmaeva and Trotsenko, Y.A. (2003) *Methylophaga alcalica* sp. nov., a novel alkaliphilic and moderately halophilic, obligatelymethylotrophic bacterium from an East Mongolian saline soda lake. *International Journal of Systematic and Evolutionary Microbiology*, 53, 223–229.

Egamberdieva, D., Wirth, S., Alqarawi, A.A., and Abd-Allah, E.F. (2015) Salt tolerant *Methylobacterium mesophilicum* showed viable colonization abilities in the plant rhizosphere. *Saudi Journal of Biological Sciences*, *22*(5), 585–590.

Galbally, I.E. and Kirstine, W. (2002) The production of methanol by flowering plants and the global cycle of methanol. *Journal of Atmospheric Chemistry*, 43, 195–229.

Glickmann, E. and Dessaux, Y. (1995) A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied Environmental Microbiology*, 61, 793–796.

Guenther, A., Hewitt, C.N., Erickson, D., Fall, R., Geron, C., Graedel, T., Harley, P., Klinger, L., Lerdau, M., McKay, W.A., Pierce, P., Scholes, B., Steinbrecher, R., Tallamraju, R., Taylor, J. and Zimmerman, P. (1995) A global model of natural volatile organic compound emissions. *Journal of Geophysical Research*, 100, 8873-8892.

Holland, M.A. and Polacco, J.C. (1994) PPFMs and other covert contaminants: is there more to plant physiology than just plant. *Annual Review of Plant Physiology and Plant Molecular*

*Biology*, 45, 197-209.

Holland, M.A., Long, R.L.G. and Polacco, J.C. (2002) *Methylobacterium spp.*: Phylloplane bacteria involved in cross-talk with the plant host?. APS Press: St. Paul, MN, USA.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Stanley, J.T. and Williams, S.T. (1994) Bergey's Manual of Determinative Bacteriology. 9th ed., Baltimore, Williams and Wilkins. 787 pp.

Kleiner, D. (1982) Ammonium (methylammonium) transport by *Klebsiella pneumonia*. *Journal of Biochemistry and Biophysics*, 688, 702-708.

Koenig, R.L., Morris, R.O. and Polacco, J.C. (2002). tRNA is the source of low-level trans-zeatin production in Methylobacterium spp. *Journal of Bacteriology*, 184, 1832–1842.

Kumari, A. (2011) Molecular characterization of mineral phosphate solubilization in *Serratia marcescens* and *Methylobacterium* sp. M.Sc. (Agri.) Thesis, University of Agricultural Sciences, Dharwad (India).

Lidstrom, M.E. and Chistoserdova, L. (2002) Plants in the pink: cytokinin production by *Methylobacterium*. *Journal of Bacteriology*, 184, 1818-1824.

Lugtenberg, B. and Kamilova, F. (2009) Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, 63, 541–556.

Lukow, T., Dunfield, P.F. and Liesack, W. (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiology Ecology*, 32(3), 241–247.

Madhaiyan, M., Poonguzhali, S., Ryu, J.H. and Sa, T.M. (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. *Planta*, 224, 268–278.

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Pragatheswari, D., Lee, K.C. and Lee, J.S. (2012) *Methylobacillus rhizosphaerae* sp. nov., a novel plant-associated methylotrophic bacterium isolated from rhizosphere of red pepper. *Antonie van Leeuwenhoek*, 32, 212-214.

Marsudi, N.D.S., Glenn, A.R. and Dilworth, M.J. (1999) Identification and characterization of fast and slow growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. *Soil Biology and Biochemistry*, 31, 1229-1238.

Mizuno, M., Yurimoto, H., Yoshida, N., Iguchi, H. and Sakai, Y. (2012) Distribution of pink-pigmented facultative methylotrophs on leaves of vegetables. *Biosciences Biotechnology and Biochemistry*, 76, 578–580.

Nemecekmarshall, M., Macdonald, R.C., Franzen, F.J., Wojciechowski, C.L. and Fall, R. (1995) Methanol emission from leaves: Enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development. *Plant Physiology*, 108, 1359–1368.

Penrose, D.M. and Glick, B.R. (2001) Levels of ACC and related compounds in exudate and extracts of canola seeds treated with ACC deaminase-containing plant growth-promoting bacteria. *Canadian Journal of Microbiology*, 47, 368–372.

Penrose, D.M. and Glick, B.R. (2003) Methods for isolating and characterizing ACC deaminase containing plant growth-promoting rhizobacteria. *Plant Physiology*, 118, 10–5.

Pikovskaya, R.I. (1948) Mobilization of

phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya,* 17, 363-370.

Rodriguez, H. and Fraga, R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, 17, 319–339.

Schauer, S. and Kutschera, U. (2011) A novel growth-promoting microbe, *Methylobacterium funariae* sp. *nov*., isolated from the leaf surface of a common moss. *Plant Signal Behaviour*, 6, 510–515.

Schwyn, B. and Neilands, J. B. (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry*, 160(1), 47–56.

Siddiqui, Z.A. (2006) PGPR: Prospective bio-control agents of plant pathogens. In: Z.A. Siddiqui(ed). PGPR: Biocontrol and Biocontrol, Springer, Netherlands, pp. 112–142.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C. and Dreyfus, B. (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legume. *Journal of Bacteriology*, 183, 214–220.

Tambekar, D.H., Rajgire, A.V. and Gaikwad, J.N. (2014) Bioremediation of C1 Compounds from Methylotrophic Bacteria isolated from Lonar lake. *International Journal of Advances in Pharmacy, Biology and Chemistry*, 3(3), 612-616.

Trotsenko, Y.A., Ivanova, E.G. and Doronina, N.V. (2001) Aerobic methylotroph bacteria as phytosymbionts. *Microbiology*, 70,725–736.

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R. and Pepe, O. (2014) *Methylobacterium populi* VP2: Plant growth promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. *The Scientific World Journal*, 1, 1-12.

Whittenbury, R., Davies, S. L. and Wilkinson, J. F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology*, 61, 205–218.

Zaets, I. and Kozyrovska, N. (2012) Heavy metal resistance in plants: A putative role of endophytic bacteria. (eds Zaidi A., Wani P.A., Khan, M.S.) Toxicity of Heavy Metals to Legumes and Bioremediation. Vienna: Springer. 203–217.

**How to cite this article:**

Umang Ahlawat, Rekha Sansanwal, Priyanka Batra and Leela Wati. 2018. Isolation and Characterization of Methanol Utilizing Microorganisms from Leguminous Crops. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 1674-1691. doi: https://doi.org/10.20546/ijcmas.2018.702.203