Research in Biotechnology, 5(5): 01-06, 2014 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Growth promotion of crop plants by *Methylobacterium organophilum*: Efficient bio-inoculant and bio-fertilizer isolated from mud

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Nitrogen fixing bacterium was isolated from mud near hot springs located at Unkeshwar in Nanded district, Maharashtra, India. Gram negative, oval rod, motile bacterium using nutrient broth medium from mud samples. Isolate bacterium showed luxuriant growth at 45°C and pH 8. Isolate also showed growth on Azatobacter-Mannitol agar. The isolated species identified as *Methylobacterium organophilum* using morphological and biochemical tests. Isolate capable of growth at 5% salt concentration and alkaline pH. Isolate was used as growth promoting agent. Effect of isolate on germination of seeds; their ability of plant growth promotion was also studied. Remarkable growth of *Vigna radiate* was observed in the presence of isolated thermophilic *Methylobacterium sp.*

Keywords: Legumes, Mung beans, di-nitrogen fixation, hot springs, thermophiles

Methylobacterium organophilum is capable to fix atomospheric nitrogen due to presence of nitrogenase and fixed di-nitrogen made available to plants in soil. This microbe utilizes variety of compounds such as such as methanol and methylamine, and a variety of heterotrophic substrates (Green, 2006). This strain usually contains poly-3-hydroxybutyrate and is catalase and oxidase positive. Member of genus *Methylobacterium* sp. are ubiquitous in nature and found in variety of habitats such as including soil, dust, freshwaters, lake sediments, nodules, air etc. These conditions allow *Methylobacterium* to grow in nitrogen containing as well as in nitrogen free medium (MacLennan et al., 1971; Patt et al., 1976; Anthony, 1982). It has been documented that some nitrogen fixing bacteria capable of growth at elevated temperature (Seldin et al., 1984). The reports are available on thermophilic nitrogen-fixing bacteria (Wickstrom, 1984). Field and laboratory evidence indicates that these heterotrophs are common among the alkaline thermal springs of Yellowstone National Park, Wyoming (USA) and that more than one type of bacterium may be involved (Gordon, 1981; Wickstrom, 1984; Madigan et al., 2008).

Huge amount of chemical fertilizers used for the production of crops results in increased soil salinity. The soil microbes play vital role by mediating nutrient transformation from the soil to plants (Patt et al., 1976). The adverse effect of salinity on soil micro flora could affect growth of plant and ultimately the production of crops will be reduced (Barreto et al., 2011).

In this report, we describe a thermophilic strain isolated from the mud samples nearer to Unkeshwar hot spring.

Based on morphological and biochemical data, this growth-stimulating associated bacterium is identified as *Methylobacterium organophilium*. In addition, we further studied effect of isolated species on growth of crop plants.

Materials and methods

Sample collection, isolation and identification of microorganisms

Mud samples were collected in vicinity of Unkeshwar hot spring (19° 85′ N and 78° 24′ E). Microorganisms were isolated using three media (Nutrient agar, J agar, Glycerol agar). All isolates were studied for their morphological and biochemical characteristics (Aneja, 2003; Atlas, 2010; Pathak and Rekadwad, 2013). Nitrogen fixing ability was examined by growing bacteria on *Azactobacter* agar followed by MacLennan minimal medium (MM) supplemented with 0.5% (vol/vol) methanol (MacLennan et. al., 1971). Selected isolate was identified using Bergeys Manual of Systematic Bacteriology (1984).

Effect of inoculums on seed germination and on growth of plants

Seeds of *Glycine max* and *Vigna radiata* were used to check the effect of isolate. 25 good quality seeds were separately surface sterilized with 95 % alcohol for 30 seconds, followed by 0.1 % sodium hypochlorite for 2–3 min and then washed with sterile distilled water for 5-6 times. Seeds were soaked in inoculum (10^9 CFU/mL) for one hour. Seeds with bioinoculant were placed on moist sterile filter paper in Petri plates. To each Petri plate, 2 ml sterile water was added. The seeds were allowed to germinate in triplicate for 48 h 30°C for 48 hours. For control plate, seeds were soaked in sterile distilled water. The lengths of sprouts were measured in centimeters (cm) after each 24 h. The same inoculum was used to study effect on growth of plants. The seeds were surface sterilized as above. Saline was brought from Tuppa area of Nanded district, Maharashtra (India). The good quality seeds were taken and soaked in inoculum for one hour. After soaking period over ten seeds were sowed (in triplicate) in each pots containing sterile saline soil. Alternate dark and light periods were maintained as per crop plant requirement. For control pot, seeds were soaked in sterile distilled water. Plant growth was measured in centimeters (cm) after each 24 h for seven days (Weller and Cook, 1983).

Statistical analysis

Values are the mean of triplicate observations with standard deviations indicated in parenthesis.

Results and discussion

Mud sample from hot spring moiety was used to isolate thermophilic, salt tolerant, nitrogen-fixing bacteria. We have isolated 13 bacteria, from these only one isolates were found to grow very well on nitrogen-free medium at high temperature indicating that they could fix nitrogen for survival and growth. Positive nitrogen fixation test taken by using Nessler's reagent. The isolate was gram negative, motile and having cell size 1.5x1.0 µm. It is oxidase and catalase positive. Isolate utilizes glucose, inositol, lactose, maltose, mannitol, sorbitol, sucrose, galactose and cellobiose as carbon source. Isolate showed growth at 2% and 5% salt concentration (Table 1). On the basis of morphological and biochemical characteristic, isolate was identified as *Methylobacterium organophilum* using Bergey's Manual of Systematic Bacteriology. Isolate tolerated temperature up to 60°C, and could not grow below 30°C (Figure 1). It also has showed optimum growth at pH 8.0 (Figure 2), however tolerated a wide pH range of 4.0-10.0.

Effect on seed germination and on growth of plants

Enhanced seed germination was recorded in experimental plate in case of *Vigna radiata*. The pot experiments were continued with *Vigna radiata* seeds. The isolated *Methylobacterium organophilum*

species showed remarkable effect on seed germination (Table 2, Photo 1-2) in addition with magnificent growth of *Vigna radiata* (Table 3, Photo 3-6). Hence, isolated strains that grew on nitrogen-free isolation plates, the ability to fix di-nitrogen with legumes suggesting a higher efficiency of the di-nitrogen fixation (Sy et al., 2001).

Di-nitrogen Fixation by *Methylobacterium* and its inoculants in association with leguemes

Methylobaterium played significant role in enhanced growth of legumes. Results indicates that *Methylobacterium* strain have converted atmospheric molecular nitrogen into nitrogenous compounds like NH_3. Similar types of results were recorded by various research groups worldwide. Sy et al., (2001) studied di-nitrogen fixing ability of *Methylobacterium* strains on the roots of leguminous plants. They have reported that *Methylobacterium* strains are specifically associated with some particular legumes. Jourand et al., (2004) isolated and identified 72 non-pigmented *Methylobacterium* strains that specifically induce nitrogen-fixing root nodules on the legume species. Munusamy and Ji (2013) patented nitrogen fixing bacterial inoculants comprises *Methylo-bacterium* for improvement of crop productivity and also reported *Methylobacterium* reduces atmospheric nitrogen (N_2) to ammonia (NH_3).

Table 1. Morphological and biochemical characteristics of *Methylobacterium organophilum*

Characteristic/Test	Results	Characteristic/Test	Results
Size	1.5x1.0	Mannitol	+
Shape	Oval Rod	Resorcinol	-
Gram staining	-	Ribose	-
Arrangement	Single, Pair	Sorbitol	+
Endospore	Absent	Sucrose	+
Motility	Motile	Xylose	-
Colour of colony	Pinkish white	Galactose	+
Colony size (mm)	2	Fructose	-
Form of colony	Circular	Cellobiose	+
Margin	Entire	Biotin	-
Elevation	Raised	Arabinose	-
Density	Opaque	Oxidase	+
Growth in Broth Medium	Sediment	Catalase test	+
Glucose	+	Salt tolerance	
Inositol	+	02%	+
Lactose	+	05%	+
Maltose	+	10%	-

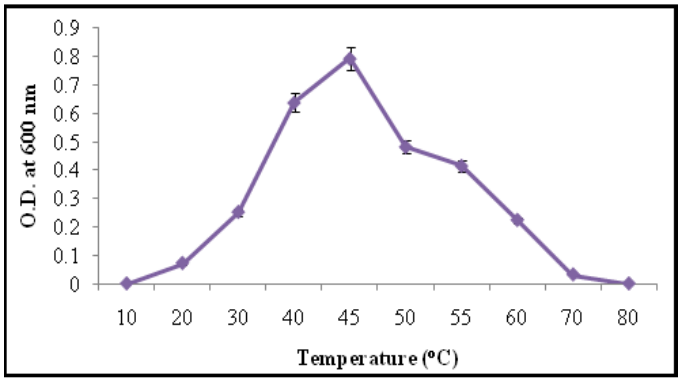

Figure 1. Effect of temperature on the growth of *Methylobacterium organophilum*

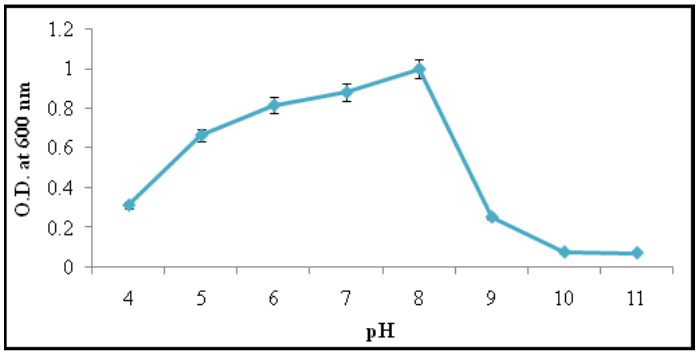

Figure 2. Effect of pH on the growth of *Methylobacterium organophilum*

Table 2 Effect of *Methylobacterium organophilum* on germination of seeds in Petri plates

	24 h		48 h	
	No. of seeds germinated	Average growth in cm	No. of seeds germinated	Average growth in cm
Glycine max				
Control	0	0	9	0.678±0.56
Treatment	4	0.533±0.23	12	1.4±0.19
Vigna radiata				
Control	24	1.61	24	3.7±0.31
Treatment	25	1.67±0.24	25	4.59±0.42

Table 3. Effect of *Methylobacterium organophilum* on growth of *Vigna radiata* seeds in pots

Days	Total ten seeds taken for seed germination under pot study					
	Average growth of germinated plants under pot study in (cm) and number of leaves formed					
	Control		No. of leaves formed	Experimental		No. of leaves formed
Day 1	2	0.3±0.00	0	10	0.5±0.00	0
Day 2	3	2.15±0.52	0	10	3.29±0.22	0
Day 3	4	3.33±0.23	2	10	6.79±0.58	2
Day 4	4	6.07±0.59	2	10	9.13±0.83	2
Day 5	4	7.93±1.02	2	10	10.21±0.21	2 well developed leaves
Day 6	4	9.3±0.91	2	10	13.7±0.86	2 well developed leaves

Photo 1 *Glycine max*: Seed germination assay in Petri plate after 24 hour

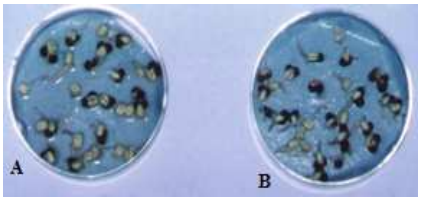

Photo 2 *Vigna radiata*: Seed germination assay in Petri plate after 24 hour

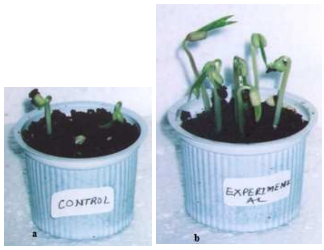

Photo 3 Day 1: Growth of *Vigna radiata* in pots

Photo 4 Day 2: Growth of *Vigna radiata* in pots

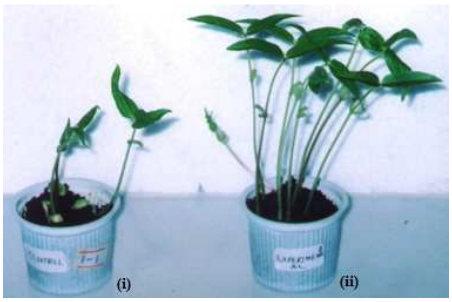

Photo 5 Day 3: Growth of *Vigna radiata* in pots

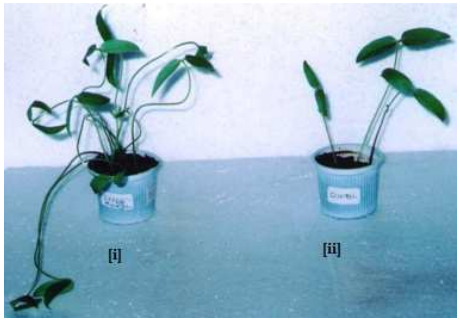

Photo 6 Day 7: Growth of *Vigna radiata* in pots

Conclusion

Isolated *Methylobacterium organophilum* nitrogen-fixing species isolated from hot spring originated mud sample is a thermophile. It may be interesting to microbiologists, agronomists, and microbial ecologists alike, since they fix di-nitrogen efficiently at elevated temperature. During preparation of organic compost, heat was generated. Inclusion of *Methylobacterium organophlium* efficiently fixes atmospheric di-nitrogen into the soil and compost would be available to plants. Thus, this species could be used as efficient bio-inoculant and as a bio-fertilizer.

Acknowledgements

Author is thankful to SRTM University, Nanded for providing necessary facilities required for the research.

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