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Evaluation of indigenous methanotroph isolates on greenhouse gases mitigation in lowland rice fields of Cauvery Delta Zone, Tamil Nadu

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ABSTRACT

Global warming is a serious problem caused by the greenhouse gases. Though methane emission from rice fields is also responsible for the earth warming, its cultivation cannot be prohibited since rice is a major food crop for Asian countries. Methanogens are anaerobic organisms that produces methane, whereas, methanotrophs are microaerophilic organism that uses methane as a carbon source.Hence,a liquid microbial consortium was developed using two indigenous methanotroph isolates from Keelamaruthuvakudi and Aduthurai. A field experiment was conducted to study the effect of liquid microbial consortium on greenhouse gases mitigation in low land rice (ADT 49) fields. The results indicated that application of 75% recommended dose of NPK fertilizers +azophos (2 kg/ha)+methanotrophs (500 ml/ha)reduced the greenhouse gases emission @ methane (4.00 mg/ m^2/h), N_2O (9.86µ mol/ m^2/s) and CO_2 (0.066µg N/m^2/h) when compared to methanotroph uninoculated control.

Key words: *CH_4, N_2O, CO_2, mitigation, low land rice, methanotrophs*

INTRODUCTION

The primary greenhouse gases emission in the earth's atmosphere are carbon dioxide, methane, nitrous oxide and ozone. Rice field contributes 8% of the methane emission. World population projection indicates that the demand of rice would increase by 50% and for that additional 700 metric tonnes of rice will have to be produced, which would increase as much as 20 % methane in atmosphere during next 10 years.

Methanogens are microorganisms that produce methane as a metabolic by-product in anoxic conditions. Wetland rice ecosystem is an ideal anaerobic environment due to water stagnation, which favours the methanogen growth and methane production (Smith et al., 2014). They are common in wetland, where they are responsible for marsh gas and in the digestive tracts of animals such as ruminants and humans where they are responsible for methane content of belching in ruminants and flatulence in humans (Lengeler, 1999).

Apart from this, another group of microorganisms named as a methanotrophs uses methane as a carbon source. Methanotrophs are ubiquitous and present in freshwater marine and terrestrial environments (Bull et al., 2000). As Espiritu et al. (1997) mentioned that the population fluctuation of methanotrophs in rice rhizosphere is likely to be closely affected by supply of O_2 namely reduced or oxidative condition at each stage of rice cultivation.

In limnic sediments, the oxic surface layer forms an effective barrier and methane oxidizing bacteria may consume up to 95% of the methane diffusing upwards (Kuivila et al., 1989). In soil cores flooded rice fields, about 81% anoxically emitted methane was oxidized (Conrad and Rothfuss, 1991).

Under aerobic conditions, methanotrophs combine oxygen and methane to form formaldehyde, which is then incorporated into organic compounds *via.,* the serine pathway or the ribulose monophosphate (RuMP) pathway. Type I methanotrophs are members

of the Gammaproteobacteria and use RuMP pathway to assimilate carbon. On the other hand, type II methanotrophs are members of the Alphaproteobacteria and utilize the serine pathway of carbon assimilation. Intermediates of methane oxidation, such as methanol, formaldehyde and formate have been detected in methanotrophic cultures and they may even reach inhibitory concentrations (Agrawal and Lim, 1984; Costa et al., 2001)

Watanabe et al. (1997) reported that methanotrophs whose populations were 10^6-10^5/g drwtin rice roots and 10^4 -10^3 / g drwt in basal portion of rice stems inhabited two Japanese rice cultivars indicating the possibility of CH_4 oxidation in association with wetland rice plants.

Fetzer (1993) and Joulian (1998) reported that *Methanobacterium*species, *Methanobrevibacter arboriphilicus* were isolated from hydrogenotrophic rice field environment.

There is an increasing interest in methane oxidizing bacteria because of their importance in greenhouse gas consumption and their potential application in bio-remedial degradation of industrial pollutants *e.g.,* trichloroethylene (Hanson and Hanson, 1996). The oxidation of methane to methanol is catalysed by either a soluble or a membrane associated form of methane mono-oxygenase (sMMO and pMMO respectively) (Hanson and Hanson, 1996). One form, the membrane associated or particulate methane monooxygenase (pMMO) is found in most known methanotrophs and is located in the cytoplasmic membrane. Another form the soluble methane monooxygenase (sMMO) is found in some methanotrophs and is located in the cytoplasm. In methanotrophs, that have both forms of MMO, copper (Cu) is known to be a key factor in regulating the expression of the gene encoding both sMMO and pMMO as well as the activity of these enzyme (Takeda, 1976 and Tanaka, 1980).

It is predicted that methanotrophs consume up to 40 Tg CH_4/year and sequester more than 50 % of the methane produced in soils (IPCC 2001; Reeburgh 2003; Reeburgh et al., 1993). The ability of the methanotrophs to lower methane emissions and degrade hazardous organic compounds has been reviewed (Hanson and Hanson, 1996; Jiang et al., 2010; Semrau et al., 2010; Smith and Dalton, 2004; Wendlandt et al., 2010). Hence, the present study was focused to mitigate greenhouse gases emission from rice field using indigenous methanotrophs isolates.

ABBREVIATIONS

RDF: Recommended dose of NPK fertilizers, DAT: Days after transplanting

MATERIALS AND METHODS

Isolation of methanotrophs

Soil samples were collected (5cm depth) from ten different locations (5km apart) of low land paddy fields of the Cauvery delta zone. Representative field composite soil samples were serially diluted to 10^{-3} level in phosphate buffer and plated in the nitrate mineral salt medium (Whittenbury, 1970). The plates were incubated in a McIntosh jar (Fig. 1) upto 7-15 days provided with a bladder containing methane gas.

Methane monooxygenase activity

Methanotrophs colonies were removed from the McIntosh jar for MMO assay. Innerside of the Petriplate lids were sprinkled with naphthalene powder and other half of the plate with cultures were kept inversely to allow the colonies to absorb the naphthalene gas from the lid for 15 min. The naphthaline crystals were removed from the lid and replaced to cover the cultures. Then the colonies were flooded with O-dianisidine solution for 30 min (Wackett and Gibson, 1983).

Fig.1. Fabricated mcIntosh jar.

Co-culture cross streak assay

Four methanotroph isolates were tested for compatibility by co-culture cross streak assay. Two different cultures were perpendicularly streaked in a single plate containing nitrate mineral salt medium. Totally 16 plates were used for four different combination of methanotroph culture.

Mass multiplication of methanotrophs

For mass multiplication of methanotrophs, nitrate mineral salt medium (composition/litre: $NaNO_3$:1.0mM, KSO_4: 1.0mM, $MgSO_4$: 0.15mM, $CaCl_2$: 47.6µM, KH_2PO_4: 3.9mM, Na_2HPO_4: 6.0mM, $ZnSO_4$: 2.0 µM, $MnSO_4$: 1.6 µM, H_3BO_3: 6.0µM, $NaMO_4$: 0.4 µM, $COCl_2$: 0.4 µM, KI: 1.0 µM, $FeSO_4$: 40 µM, Agar : 20g and pH : 7.0) given by Whittenbury et al. (1970), M_2 medium (composition/litre : KH_2PO_4:1.75 mg, KNO_3:1.01 mg, NH_4Cl: 8.02 mg, NaCl: 2.92 mg, $CaCl_2$: 17.2 mg, $MgSO_4$: 9.86 mg, Na_2SiO_3: 2.44mg, $AlCl_3$: 1.33 mg and trace element solution: 0.2%) given by Dedysh et al.(1999) and basal medium (composition/litre : $NaNO_3$: 850mg, K_2SO_4: 170 mg, $MgSO_4$: 37 mg, $CaCl_2$: 7mg , phosphate buffer: 10ml, trace element solution (pH: 4.0): 0.5ml, iron solution:1ml and agar: 20mg) given by DeVay and Schnarthorst (1963) were used. The cultures were inoculated in the broth with and without carbon source supplied with methane gasand incubated upto15d.

Development of liquid microbial consortium

The best grown cultures were selected after mass multiplication and mixed with one another before inoculated into the field experiment

Table 1. The physico-chemical and microbiological populations of the experimental field soil.

Soil properties	Results (Mean ± SE)
pH	7.8± 0.09
EC (dSm^{-1})	0.04± 0.15
Organic carbon (%)	0.50 ± 0.01
Available N (%)	162± 2.88
Available P (%)	35± 0.57
Available K (%)	250± 1.45
Total bacteria (cfu x 10^5 /g drwt of soil)[a]	46 ± 1.15
Fungi (cfu x 10^3 /g drwt of soil)b	2 ± 0.16
Diazotrophs (cfu x 10^4 /g drwt of soil)[c]	21 ± 1.15

[a]Total bacteria were enumerated by serial dilution plating method on soil extract agar medium (James, 1958).

[b]Total culturable fungi were enumerated by serial dilution plating method as described by Parkinson et al. (1971)

[c]Total diazotrophs were enumerated by the procedure as described by Rennie (1981).

Field experiment

Field experiments were carried out during thaladi season of 2016-2017 and 2017-2018 at Tamil Nadu Rice Research Institute, Aduthurai to assess the effect of developed microbial consortium on greenhouse gases emission mitigation under irrigated low land rice ecosystem. The physico-chemical and microbiological populations of the experimental field soil are presented in Table 1. Experiments were carried out in a randomized block design with three replications with a plot size of 40 sq.m. each. Fertilizers were applied at 150:50: 50 kg NPK / ha. When microbes were included as treatment, 75% (112.5:37.5:37.5 kg/ha) and 50% (75: 25:25 kg/ ha) of recommended NPK fertilizers were applied. Methanotroph cultures (109 cells/ml) were inoculated @500ml/ha and fertilizers were applied 7dafter microbial inoculation. Microbial inoculation was done at 10 and 20DAT. Two gas samplings at 10 and 20d after sowing were made (IAEA, 1992, IGAC, 1994, Parkin and Venterea, 2010 and deklein and Harvey, 2012) from the rice field through manual closed chamber method. The rhizosphere soil samples were collected to assess methanotrophic population and MMO activity. The plant and soil samples were collected to measure plant growth parameters and soil NPK content. The oxidation reduction potential of the soil samples were enumerated by using ORP meter (range: 1999-1999mv, YK (23 RP), Model No: A121798) made in Taiwan. The root and shoot length were measured and the mean values were expressed as cm/plant. The plant samples were collected and dried in a hot air oven at 60°C to a constant weight and mean plant drwt was expressed as g/plant. The available N, P and Kcontent was estimated by employing alkaline permanganate method (Subbian and Asija, 1956), Olsen's method (Olsen et al., 1954) and flame photometer (Standford and English, 1949) respectively.

Enumeration of methanotrophs

One g of soil (wet weight) was inoculated in the flask added with basal medium (Dedysh et al., 1999) and sealed with rubber cock tightly supplemented with 5 % methane gas and enriched for 15 days. After enrichment bacterial growth was purified on nitrate mineral salt

agar medium (Whittenbury, 1970) incubated in anaerobic jar containing methane gas.

Soluble methane monooxygenase activity

One g of soil sample was transferred to 1ml aliquots in 10 ml screw -cap tube and 1ml of pre-filtered saturated naphthalene solution was added to each tube. The samples were prepared in triplicate keeping sterile medium control as blank. The reaction mixtures were incubated at 200 rpm on incubator shaker at 25°C for 1 to 3 h. After incubation, 100µl of freshly prepared 4.21mM tetrazotized-o-dianisidine solution was added to each tube and the intensity of coloured diazo-dye complex was immediately monitored by recording the A525 by spectrophotometry. The intensity of diazo-dye formation is proportional to the naphthol concentration (1naphthol and 2 naphthol). The specific activity of sMMO was expressed as nm of naphthol formed/mg of cell protein/m (Anthony, 1971).

CH_4 flux measurements

Plant mediated CH_4 emission flux from the experimental plots was measured by closed chamber method of Adhya et al. (1994). Samplings for CH_4 flux measurements were made at 09:00-10:00 h and 15:00-16:00 h and the average of morning and evening fluxes were used as the flux value for the day. For measuring CH_4 emission, eighteen rice hills were covered with a locally-fabricated transparent acrylic sheet chamber (59.3 cm length, 59.3 cm width and 87.8 cm height). A battery-operated fan was fixed for air circulation (to avoid plant suffocation) to mix the air inside the chamber and draw the air samples into air-sampling bags (Tedlar®). The air samples from the sampling bags were analyzed for CH_4. Each chamber was placed in the soil surface in such a way that the chamber is inserted into the 4-5 cm soil, 10 min prior to each sampling for equilibration to reduce the disturbance to the sampling site (Fig. 2.)

CH_4 Estimation

CH_4 was estimated in a Shimadzu GC-2014 gas chromatograph equipped with FID. The gas samples were introduced into the analyzer by filling the fixed loop (1.0 ml) on the sampling valve. Samples were injected into the column system by starting the analyzer which automatically activates the valve and back flush

Fig. 2. Methane gas extraction from the field by base chamber method.

the samples according to the time programmed.The retention time of CH_4 was between 4 to 4.17 min. The GC was calibrated before and after each set of measurements using 1 ppm, 2.3 ppm and 5 ppm CH_4 (Chemtron® science laboratories Pvt. Ltd., Mumbai) as primary standard curve which was linear over the used concentration ranges. The minimum detectable limit for CH_4 was 1 ppm. CH_4 flux was determined by peak area and CH_4 flux was expressed as mg/m_2/dusing the equation given by Lantin et al. (1995).

RESULTS AND DISCUSSION

Isolation, confirmation and compatibility of methanotrophs

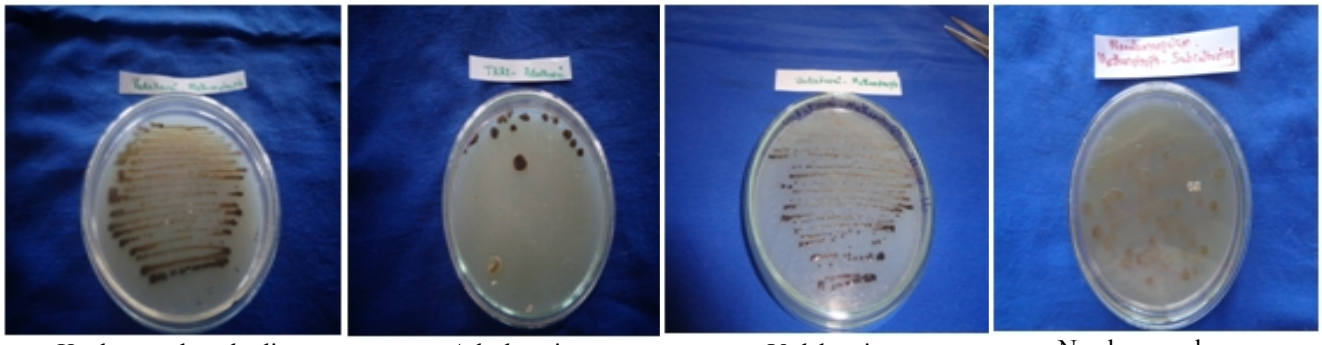
Promising methanotroph cultures (Fig. 3) were isolated from low land rice field of Cauvery delta zone. For isolation of methanotrophs,continuous supply of methane gas is essential. The rate of growth depends upon concentration of CH_4 gas supply. *Invitro* assay on methane monooxygenase activity revealed that positive colonies were turned into reddish brown colour (Fig. 4). Among the ten isolates, isolates from Aduthurai, Needamangalam, Keelamaruthuvakudi and Vadagarai were positive for MMO activity (Fig. 3).

Compatibility studies by co-culture cross streak assay revealed that all the four cultures (Aduthurai, Vadakarai, Keelamaruthuvakudi and Needamangalam) were compatible with each other (Fig. 5.)

The difficultly in growth of methanotroph was supported by many authors. Escoffier et al. (1997),

Table 2. Mass multiplication of methanotrophs in different culture media.

Medium	Keelamaruthuvakudi	Aduthurai	Vadakarai	Needamangalam
M_2medium	-	-	-	-
M_2medium+ glucose	-	-	-	-
Nitrate mineral salt medium	++	++	-	-
Nitrate mineral salt medium + glucose	++++	++++	++	++
Basal broth + methanol + glucose	++++	++++	++	++



Keelamaruthuvakudi Aduthurai Vadakarai Needamangalam

Fig 3. Methanotroph isolates.

Whittenbury (1970) and Hanson et al. (1992) reported that the isolation of indigenous methanotrophs can be rather problematic due to their slow growth rates and growth of other non-methane utilising bacteria during cultivation. Furthermore, the majority of them do not express colony morphology or pigmentation suitable for selection. In the absence of methane or plating of a soil sample without methane oxidation potential none or very few colonies were formed. Enrichment of the culture was performed in a continuously stirred reactor under a continuous flow of methane in air at room temperature (21- 23°C). It has been reported that methanotrophs in soil bio-filters under microaerophilic conditions oxidize methane more rapidly than when under aerophilic conditions (Stein and Hettiaratchi, 2001)

Mass multiplication and development of liquid microbial consortium

It was very difficult to grow the methanotroph cultures. Different media were tried. Even CH_4 gas was supplied, the methanotrophs growth was happened only after addition of carbon source (glucose and methanol) to the medium. Among the different growth media tried, nitrate mineral salt medium + glucose and basal broth+ methanol+ glucose resulted higher growth rate than the other (Fig. 6 and Table 2). Among the cultures, isolates from Keelamaruthuvakudi and Aduthurai exhibited maximum growth and MMO activity than Vadakari and Needamangalam. Some of the published results

Table 3. Effect of methanotroph inoculation on CH_4, CO_2 and N_2O emission of soil cropped with rice (ADT 49) under irrigated ecosystem.

Treatments	CH_3(mg/m^2/h)		CO_2(µmol/m^2/s)		N_2O(µgN/m^2/h)	
	20 DAT	10 DAT	20 DAT	10 DAT	20 DAT	10 DAT
T_1 -Un inoculated control	16.67	18.80	40.23	56.30	0.480	0.510
T_2 -100% RDF of NPK	11.92	12.07	37.59	52.78	0.241	0.256
T_3 -75% RDF of NPK + azophos	9.57	10.35	31.48	37.59	0.213	0.226
T_4-75% RDF of NPK + methanotrophs	5.75	6.76	17.76	25.56	0.065	0.129
T_5 -75% RDF of NPK+ azophos+ methanotrophs	4.00	4.24	9.86	11.88	0.088	0.066
T_6 -azophos + methanotroph	6.82	8.33	15.68	22.26	0.129	0.152
T_7-methanotrophs alone	6.17	9.04	15.56	20.08	0.220	0.188
SEd	0.54	0.62	1.53	2.09	0.013	0.015
CD (P =0.05)	1.19	1.34	3.34	4.57	0.028	0.032

Table 4. Effect of methanotroph inoculation on soil methanotrophs population, methane monooxygenase activity and redox potential of the soil cropped with rice (ADT 49) under irrigated ecosystem.

Treatments	Methanotroph ($x10^4$ cfu/g)			MMOs activity (nmol/min/mg protein)	O/R value (-Eh)
	30 DAT	60 DAT	90 DAT		
T_1 -un inoculated control	39	22	8	13	-250
T_2 -100% RDF of NPK	46	25	6	4	-211
T_3 -75% RDF of NPK + azophos	53	28	9	6	-180
T_4-75% RDF of NPK + methanotrophs	54	29	11	23	-040
T_5 -75% RDF of NPK+ azophos + methanotrophs	62	33	14	27	-038
T_6 -azophos + methanotroph	57	21	16	17	-035
T_7-methanotrophs alone	45	19	5	20	-031
SEd	3.17	1.6	0.7	1.15	8.28
CD (P = 0.05)	6.92	3.6	1.5	3.20	18.24

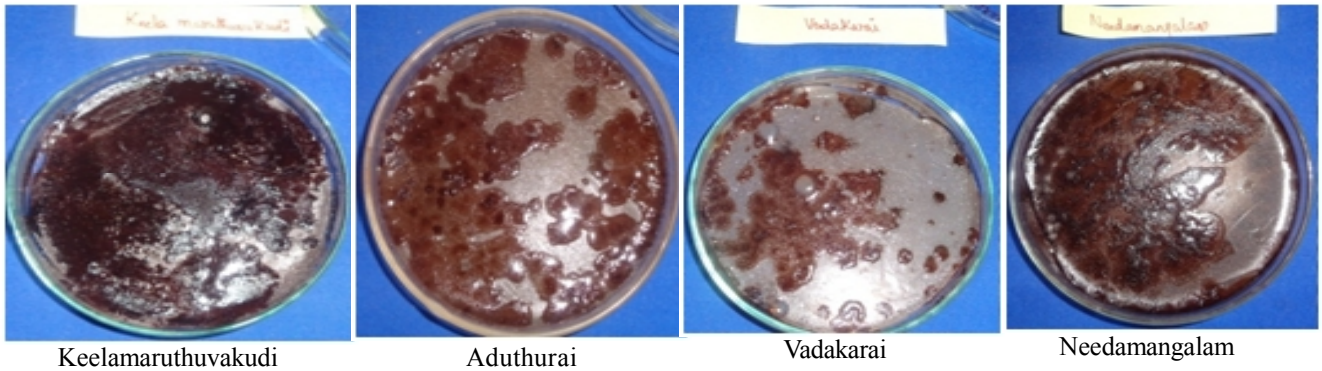
Table 5. Effect of methanotrophs inoculation on plant growth and yield of rice (ADT 49) under irrigated ecosystem.

Treatments	Root length (cm/plant)			Shoot length (cm/plant)			Dry weight (g/plant)			Yield (kg/ha)
	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT	30 DAT	60 DAT	90 DAT	
T_1-un inoculatedcontrol	15.3	16.0	10.0	76.2	78.0	70.0	22.0	24.0	15.0	5333
T_2-100% RDF of NPK	15.0	16.0	9.5	77.0	79.0	72.0	29.5	32.0	22.0	5500
T_3-75% RDF of NPK + azophos	15.5	16.0	10.0	75.5	76.0	73.0	33.5	35.0	37.0	6500
T_4-75% RDF of NPK + methanotrophs	16.0	17.0	10.0	78.8	79.0	74.0	34.5	36.0	28.0	6500
T_5-75% RDF of NPK+ azophos + methanotrophs	16.3	18.0	10.0	78.5	79.0	74.0	36.5	38.0	30.0	6700
T_6-azophos+ methanotroph	16.8	17.0	10.0	80.0	82.0	77.0	22.5	24.0	26.0	5500
T_7- methanotrophs alone	17.0	18.0	10.0	81.0	83.0	78.0	30.0	33.0	28.0	6660
SEd	1.00	1.024	0.60	4.72	4.79	4.47	1.91	2.02	1.69	377
CD (P =0.05%)	2.11	2.23	1.31	10.30	10.4	9.74	4.15	4.39	3.69	822

reported that methanotorphs yields for growth on methane vary widely (19-70% substrate carbon incorporated into cell material), even when studies have used the same or similar organisms (Whittenburyet al., 1970; Harwood and Pirt, 1972; Stanley, 1977; Linton and Vokes, 1978). Large populations of methanotrophs can be found in soils, surface layers of sediments and natural waters, primarily just above the anaerobic layer (Higgins et al., 1980; Anthony, 1982; Mancinelli, 1995; Holmes et al., 1996).

Effect of methanotroph inoculation on GHG emission and methanotroph population

The result of the field experiment revealed that application of 75% RDF of NPK+azophos +methanotrophs showed better performance on greenhouse gaseous emission reduction *viz.,* CH_4 (4mg/m^2/h), CO_2 (9.86 µmol/m^2/s) and N_2O (0.088 µgN/m^2/



Keelamaruthuvakudi Aduthurai Vadakarai Needamangalam

Fig. 4. Methane Monooxidase activity of the methanotroph isolates.

Table 6. Effect of methanotrophs inoculation on available N, P and K content of the soil cropped with rice (ADT 49) at 90 DAT under irrigated ecosystem.

Treatments	N (kg/ha)	P (kg/ha)	K (kg/ha)
T_1- Uninoculated control	177	145	180
T_2 -100% RDF of NPK	385	237	287
T_3 -75% RDF of NPK + azophos	317	180	237
T_4-75% RDF of NPK + methanotrophs	324	187	262
T_5-75% RDF of NPK+ azophos + methanotrophs	332	200	225
T_6 -azophos + methanotroph	280	135	200
T_7 -methanotrophs alone	260	125	185
SEd	19.01	10.99	14.21
CD(P : 0.05)	41.42	23.95	30.97

h) and maximum methanotroph (62 x10^4 cfu/g) population. The results were furnished inTable 3 and 4. This was supported by the finding of Conrad and Rothfuss (1991) who reported methane oxidation is an important process which reduced the CH_4 flux from wetland rice fields. Methanotrophs typically oxidize 70-90% of the CH_4 diffusing upward through the oxic soil surface layer in most soils. In addition to the recognized role of methanotrophic bacteria in the carbon and nitrogen cycles, there is also a growing number of reports about their metabolic activities in the transformation of multi-carbon compounds (Colby et al., 1977; Dalton, 1980; Higgins et al., 1980; Green and Dalton, 1986; Fox and Lipscomb, 1990; Alvarez-Cohen et al., 1992; Anderson and McCarty, 1997). Transformations are sometimes simple oxidation reactions analogous to that of transforming methane to methanol, while in some cases further oxidation of hydroxylated intermediates yielding aldehydes and carboxylic acids also takes place by methanotrophs.

Effect of methanotroph inoculation on MMO activity and redox (O/R) value

The result of the experiment revealed that the application of 75% RDF of NPK + azophos + methanotrophs exhibited maximum MMO activity (27nmol/min/mgprotein) followed by 75% RDF of NPK + methanotrophs (23 nmol/min/mg of protein). Among the treatment, application of methanotroph alone exhibited reduced O/R value (-0.31Eh) followed by methanotrophs + azophos (-35 Eh) and uninoculated control (- 250 Eh). The results are given in Table 4.Methane monooxygenase activity is directly proportional to the methanotroph population. O/R value is directly related to the anaerobic condition. This indicates the inoculation of methanotrophs reduces the anaerobic conditions. Oldenhuis et al. (1989) reported that the methanotrophs containing sMMO have a broad

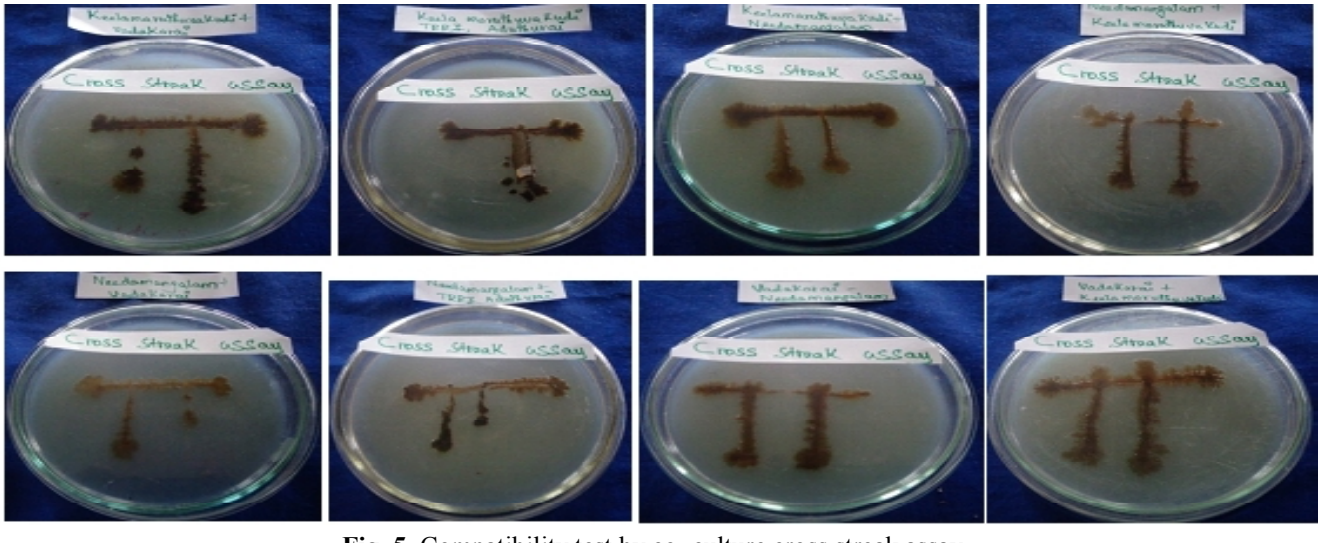


Fig. 5. Compatibility test by co -culture cross streak assay.

Fig 6. Mass multiplication of methanotrophs.

substrate specificity and are of special interest in degradation of halogenated hydrocarbons in land fill soils

Effect of methanotrophs inoculation on plant growth and yield

The result of the experiment revealed that application of 75% RDF of NPK + azophos + methanotrophs produced maximum root length (16.3 cm/plant), shoot length (78.5 cm/plant), dry weight (36.5 cm/plant) and yield (6700 kg/ha) than 100% RDF of NPK as indicated in the Table 5. Some of the following findings supported that the methanotroph inoculation increases the plant growth. Singh et al. (2010) demonstrates that FYM and pyrite application can enhance the methanotrophs population in saline paddy soil. Long term organic regimes can affect both the abundance and the composition of methanotrophs. Thus the pyrite crop residues and other organic amendments could be important strategies for enhancing the rice crop productivity, abundance and community composition of methanotroph in the alkaline paddy soils. Methanotrophic bacteria exerted a positive effect on the formation of the morphogenic callus of wheat (Kopertekh et al., 1995).

Effect of methanotrophs inoculation on soil N, P and K content

The results revealed that application of 100% RDF of NPK was found to have maximum soil available N (385 kg/ha) P (237 kg/ha) and K (287 kg/ha) content followed by 75% RDF of NPK + azophos + methanotrophs (Table 6). Methanotroph has direct relationship with N content. Nitrogen and oxygen are important regulators of methanotrophy. Rudd et al. (1976) found that methane oxidation in a lake is sustained only at low oxygen concentration in the case of nitrogen limitation, whereas it was sustained through out the lake when nitrogen content was high. This was explained by the need for nitrogen fixation by methanotroph when nitrogen is limiting. Whittenbury and Dalton (1981) found that oxygen concentrations below 4% in pure culture were necessary to obtain nitrogen fixation in methanotrophs. Only type II and type X methanotrophs are able to fix nitrogen (Hanson and Hanson, 1996).

CONCLUSION

Invitro growth and multiplication of the methanotrophs is complicated due to the continuous supply of methane. Methanotroph prefers typical microaerophilic condition and above the anaerobic surface. Rice field is the ideal environment for methanotrophic growth. Hence the methanotroph inoculation in low land rice field favors the multiplication of methanotroph. The result of the experiment confirmed that the methanotrophs inoculation drastically reduced the greenhouses gases emission from low land rice.

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