Exploration of Methane Utilization Potential of Pink Pigmented Facultative Methylotrophs

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Abstract— Pink pigmented facultative methylotrophs were showed slow growth in nitrate minerals salts media, methane served as carbon source and its required prolonged incubation time to get a measurable growth. The naphthalene oxidation assay confirmed the methane utilization by expression of sMMO which was detected as brown coloration in varying intensities. Methane utilization was confirmed by GC analysis also and reference strains utilized methane in the range of 0.3 to 55.28 %; our isolates (MSY200) utilized methane is up to 34.88 %.

Key words: PPFMs, Methane, sMMO, Naphthalene, GC

I. INTRODUCTION

Methane is one of the prominent greenhouse gas (GHG). The contributions of the methane towards the global warming phenomenon are high [1, 2]. The methane emissions have been increasing exponentially with the population explosion. For maintaining a sustainable living environment, methane has to be managed by converting into a green energy source or other beneficial or harmless products.

Methylotrophs are having unique potential to utilize the reduced carbon compounds. Pink pigmented facultative methylotrophs (PPFMs) have received considerable attention for their application in the utilization of environmentally significant carbon compounds devoid of C-C bonds such as methanol, methane etc., which are available in the environment. Methane monoxygenase (MMO) is the key enzyme, which is involved in the pathway of methane catabolism. MMO is further subdivided into soluble (sMMO) and particulate (pMMO). In our study, two different experiments were conducted with the PPFMs isolates to determine their methane utilization potential.

II. MATERIAL AND METHODS

A. Enumeration, Isolation and Selection of PPFMs

The pink pigmented facultative methylotrophs (PPFMs) was enumerated, isolated from the coastal and terrestrial environment using methanol mineral salts media (MMS), methanol (0.5%) as a carbon source [3, 4]. Based on the morphology, strains were selected, purified and preserved in MMS slants. From that, 24 PPFMs were taken for methane utilization research work along with five reference strains (*Methylobacterium* - *M. extorquens* AM1 - (R1), *M. organophilum* - (R2) and methanotrophs - *Methylosinus trichosporium* (OB3b) - (R3), *Methylococcus capsulatus* (Bath) - (R4) and Methylomonas methanica (S1) - (R5)). Strains were provided by Prof. Mary Lidstorm, University of Washington, Seattle, USA).

B. Nitrate Mineral Salts (NMS) Agar Medium

Twenty nine experimental cultures (24 PPFMs and 5 reference strains) were examined for their ability to utilize the methane gas in the incubated atmosphere with the support of NMS medium [5] and methane as a carbon source. The NMS medium was prepared, sterilized and plated. All cultures were streaked on the NMS plates and kept in a plastic bag saturated with 100% of methane atmosphere and incubated at 30ºC for 21 days. At every two to three days of incubation, the plastic bag was replenished with methane. Growth of colonies and pigmentation were recorded at the end of the incubation period.

C. sMMO Activity of PPFMs by Colorimetric Plate Assay

Twenty nine strains which grew in NMS agar were screened for sMMO activity by the following procedure [6]. A few naphthalene crystals were sprinkled on the lid of the plate and the plates were kept in an inverted position which facilitated a saturated atmosphere of naphthalene inside the closed lid, thus an intimate contact between naphthalene and colonies possessed with sMMO activity results in the production of napthol took place. The plates were then opened and lightly sprayed with freshly prepared *o*-dianisidine dye for 2-3sec. The lid was replaced and the plate was stored for 15 min in the presence of the dye. If napthol was produced by the colonies, a purple-red color appears in the area which was in contact with the dye. The color, once formed, remained stable for at least 24 h at room temperature and up to a month at 4°C.

D. Analysis of Methane Utilization by GC

29 cultures were inoculated into the GMS broth (glucose replaced by methanol) and incubated for the inoculum preparation and kept overnight on a shaker at 240 rpm at 30±2ºC for seed culture. The growth and its pigmentation characteristics were recorded.

The NMS media was prepared and 77.5 mL was distributed in serum vials and sterilized at 121°C for 15 min. The vials were sealed with rubber cork and aluminum cap before autoclave. After sterilization, 20 mL of internal atmospheric air in the vials was replaced with 20 mL of methane gas. Then, 5% (v/v) of 29 seed cultures from glucose mineral salts (GMS) broth (2.5mL) was inoculated into each of the vials using sterile syringe, and this was incubated at 30±2ºC on a shaker at 240 rpm to evaluate the methane utilization by the PPFM isolates. The experiment was conducted in triplicates. After three days of the initial incubation period, the head space gas samples were collected at every 24 h and analyzed by GC for methane utilization by individual PPFM isolates.

GC analysis was carried out to monitor the methane utilization by 29 cultures. The methane utilization was quantified by injecting 0.1 mL of head space gas from the serum vials into a GC fitted with Porapak Q column and TCD uses dry nitrogen as the carrier gas (Chemito, India). Injector and Detector temperature were 50 & 100°C respectively. The column, stainless steel packed with Porapak Q (80/100 mesh), was maintained at 50°C. The

carrier gas N_2 flow rate was fixed at 25 mL/min and the retention time of methane was about 0.4 min. Peak areas were quantified with an HP 3396 Series II electronic integrator. The percentage of methane utilization was determined by referring to the standard values obtained with 100% methane gas. All the quantification was performed in triplicates. The percentage of methane was calculated using the formula.

Methane utilization (%) =

$$\frac{\text{Standard area - Sample area}}{\text{Standard area}} \times 100$$

E. Statistics

The data were expressed as the means (±) standard error (SE). All statistical analyses were performed using the SPSS 14.0. Mean differences were established by Duncan. Data were analyzed using ANOVA. In all cases p values < 0.05 were considered statistically significant.

III. Results and Discussion

A. Growth of PPFM in nitrate mineral salts (NMS) agar medium

All cultures were given growth, pigment production in NMS agar after incubation at the methane saturated environment. All grown colonies appeared white except R3 and R4 that appeared in yellow. *Methylobacterium* strains are slow growers and require an extended time of incubation to get a measurable growth pattern. The cultures grown in methane were tabulated (Table 1) based on their utilization of methane as excellent (+++), medium (++), poor (+). The appearance of the growing colony as white in color implies the probability of assimilation pathway occurring may be ribulose mono/bis phosphate or serine pathway.

B. Naphthalene Oxidation Assay

Cultures which were grown in methane saturated environment where introduced with naphthalene, this was given sufficient time to ensure the oxidation. Produced napthol is directly proportional to the methane utilized further affirmed by the pigment produced with the addition of the dye. The results clearly indicated the presence of sMMO on the culture plate which was produced to facilitate methane assimilation. In our study, it was observed even though the ability to utilize methane was very minimum (faint brownish pigment formation), but could assimilate methane for its survival and multiplication (Fig. 1). The standard culture of methanotrophs (R3) shown very good efficacy on methane as a growing substrate.

S. No	Strains	Growth & Pigment	sMMO Activity
1	MEW 141	+, W	+++
2	MYW155	+, W	+++
3	MYW165	+, W	+++
4	MSY194	+, W	+++
5	MSY195	+, W	+++
6	MSY196	+, W	+++
7	MSY200	+, W	+++
8	MSY202	+, W	+++
9	MBW168	+, W	+
10	MBW169	+, W	+
11	MBW174	+, W	+++
12	MSB209	+, W	+
13	MSB217	+, W	+++
14	MFW124	+, W	+++
15	MFW125	+, W	+
16	MFW139	+, W	++
17	MSI69	+, W	+
18	MSI70	+, W	+++
19	MSI71	+, W	+
20	MSI73	+, W	++
21	MSI74	+, W	+++
22	MSI77	+, W	++
23	MSI83	+, W	++
24	MSI84	+, W	++
25	R1	+, W	+
26	R2	+, W	+++
27	R3	+, W	++
28	R4	+, W	++
29	R5	+, W	++

Growth (+). White (W), Yellow (Y)

Table 1: Naphthalene Oxidation Assay

The isolated strain (MFW139) expressed a competitive potential to utilize methane as its carbon source. Methane utilization efficiency was distinguishable between methanotrophs and *Methylobacterium*. *M. organophilum* reported ambiguously [7] for the utilization of methane as a carbon source.

Cultures were proven their purity in the successive steps of isolation and further in the applications. As previously mentioned we have taken preventive measures to the availability of access carbon sources other than methane. The result holds value hence they maintain purity and prevented any sort of contaminated substratum as a carbon source for their growth. The relevance of the study was further validated by MMO activity measurements. The obtained results may be clarified satisfactorily the objections to affirm methane utilization by genus *Methylobacterium* [7]. Preparation of cultures was done for methane accumulated growth media by growing on a multicarbon substrate for its growth and energy with a number of transfers of cultures grown on methane.

Fig. 1: Methane utilized by PPFMs

Initial inability of *M. organophilum* to utilize methane as this required an intracytoplasmic membrane, to metabolize this as primary growth substratum. The above mentioned procedure triggers to facilitate the requirements for methane assimilation. While growing on methane *M. organophilum* observed with an intracytoplasmic membrane, which was not exists at the time of growth on higher substrates such as methanol and glucose [8].

C. Fungal Contamination

During the long duration of incubation studies, plates with grid structure were to accommodate the maximum number of strains for methane utilization resulted in with, and without fungal contamination. On the plate culture it is clearly visible that absence of spreading brownish colour is an indication of purity without any fungal contamination. Here one decisive point to be noted that in our study we avoided addition of cyclohexamide, which is an antifungal agent used in the various phases of the study. As cyclohexamide ($C_{15}H_{23}NO_4$) contains carbon, inclusion of this may lead to an erratic assessment of carbon utilization by the cultures, as there is no mechanism to differentiate whether cultures are utilizing methane or cyclohexamide as their carbon source. One more interesting observation was made that, as the medium formulated with only methane as a nutritional source. The grown fungi must be posses with the caliber to utilize methane for their growth and development.

D. Growth of PPFMs in NMS medium

The results clearly demonstrated that methanotrophs (R3) shown better growth than other experimental strains in the liquid NMS media methane as a carbon source (Fig. 2). Maximum OD was given by R3 (0.132) and none of the other cultures were given OD beyond 0.025. This strain is the one among the three methanotrophic reference strains (R3, R4 & R5) were studied for their methane utilization and the other two strains were given very minimal OD reflecting retarded growth rate.

Two reference strains of PPFM (R1, R2) were able to utilize methane in the liquid medium, whereas isolated strains were observed with comparatively better utilization of methane than the reference strains. Among the isolated PPFM cultures maximum growth was given by strain MFW139 followed by MSI70, MSY202, MSY200, MYW155, MYW165 and MMSI 84. The cultures inefficient to give measurable OD while grown in methane containing liquid media found were MBW169, MBW168, MSB217, MFW215 and MSI71. However, low growth rate is one of the important limiting factors for the application of methanotrophs. Methane is the most widely used substrate employed in cultivating this organism.

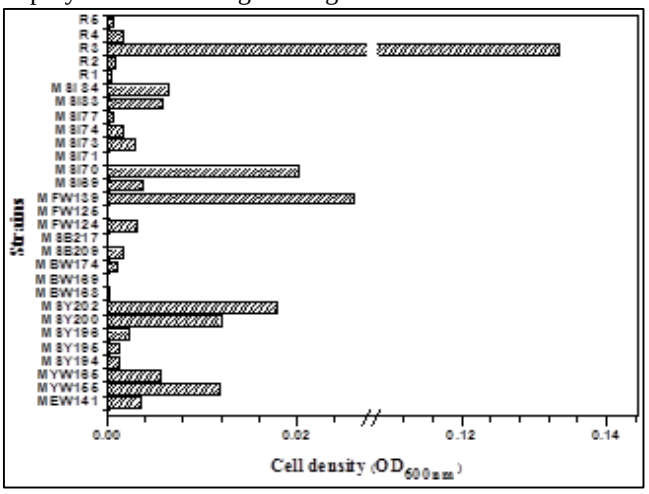

Fig. 2: Growth of experimental cultures in NMS media

A major problem encountered in high cell density cultivation of methanotrophs is the low aqueous solubility of methane. In the favorable growing environment, growth of the potent PPFM strains were primarily limited by the availability of the carbon source which exclusively depends on the mass transfer of gaseous methane to the aqueous phase [9]. Almost all methanotrophs are mesophiles (20–35°C) [10]. Better methane assimilation by methanotrophs in the lower temperatures of 0–4°C [11, 12, 13]. This indicated that shifting of temperature parameter from the above given experimental setup to lower temperatures may improve the efficiency of the PPFM strains to consume methane present in the liquid growth media.

S. No.	Strains	Methane utilization by PPFMs				Efficiency (%)
		Day 2	Day 4	Day 6	Day 8	
1	MEW 141	0.00	0.00	1.12 ± 0.17^{k}	0.04 ± 0.03^{l}	1.15 ± 0.19^{o}
2	MYW155	2.10 ± 0.12^{a}	1.91 ± 0.08^{d}	1.73 ± 0.21^{i}	1.49 ± 0.02^{g}	7.23 ± 0.18^{e}
3	MYW165	0.00	1.76 ± 0.02^{e}	4.82 ± 0.01^{d}	0.11 ± 0.02^{k}	6.69 ± 0.02^{f}
4	MSY194	0.00	0.94 ± 0.01^{j}	0.00	0.00	0.94 ± 0.01^{qr}
5	MSY195	0.34 ± 0.01^{b}	0.35 ± 0.01^{o}	2.51 ± 0.23^{f}	0.03^{l}	3.23 ± 0.23^{j}
6	MSY196	0.00	0.40 ± 0.01^{n}	0.00	0.00	0.40 ± 0.01^{s}
7	MSY200	0.00	4.35 ± 0.02^{b}	11.13 ± 0.02^{c}	19.40 ± 0.36^{a}	34.88 ± 0.37^{bq}
8	MSY202	0.00	0.00	17.10 ± 0.08^{b}	2.54 ± 0.09^{d}	19.63 ± 0.16^{c}
9	MBW168	0.00	0.22 ± 0.01^{q}	0.48^{m}	0.07 ± 0.01^{l}	0.77 ± 0.01^{r}
10	MBW169	0.00	0.00±0.00	4.27 ± 0.02^{e}	0.00	4.27 ± 0.02^{h}
11	MBW174	0.00	0.22 ± 0.01^{q}	0.87 ± 0.01^{l}	0.50 ± 0.01^{k}	1.59 ± 0.02^{n}
12	MSB209	0.00	0.05 ± 0.00^{r}	0.89 ± 0.02^{l}	0.04^{l}	0.98 ± 0.02^{pq}
13	MSB217	0.00	0.27 ± 0.01^{p}	1.09 ± 0.01^{k}	1.18 ± 0.02^{h}	2.54 ± 0.02^{l}
14	MFW124	0.00	0.76 ± 0.01^{l}	0.00	1.75 ± 0.03^{f}	2.50 ± 0.03^{l}
15	MFW125	0.00	1.47 ± 0.01^{g}	0.00	0.00	1.47 ± 0.01^{o}
16	MFW139	0.07 ± 0.12^{c}	2.24 ± 0.01^{c}	4.78 ± 0.01^{d}	5.81 ± 0.02^{c}	12.90 ± 0.13^{d}
17	MSI69	0.00	0.06^{r}	0.00	0.72 ± 0.02^{j}	0.78 ± 0.02^{r}
18	MSI70	0.00	1.34 ± 0.01^{h}	2.32 ± 0.02^{g}	1.90 ± 0.03^{e}	5.57 ± 0.05^{g}
19	MSI71	0.00	1.92 ± 0.01^{d}	0.00	0.00	1.92 ± 0.01^{m}

20	MSI73	0.00	0.88 ± 0.01^{k}	0.00	0.00	0.88 ± 0.01^{qr}
21	MSI74	0.00	0.99 ± 0.01^{i}	0.00	0.00	0.99 ± 0.01^{pq}
22	MSI77	0.00	0.41 ± 0.01^{m}	1.96 ± 0.02^{h}	0.61 ± 0.02^{j}	2.99 ± 0.04^{k}
23	MSI83	0.00	0.99 ± 0.01^{i}	0.00	0.00	0.99 ± 0.01^{p}
24	MSI84	0.00	1.70 ± 0.01^{f}	0.00	0.00	$1.70+0.01^{n}$
25	R1	0.00	0.00	0.24 ± 0.01^{n}	0.06^{l}	0.30 ± 0.01^{s}
26	R2	0.00	0.87 ± 0.01^{k}	2.26 ± 0.03^{g}	0.71^{j}	3.84 ± 0.02^{i}
27	R3	0.00	20.49 ± 0.09^{a}	27.50 ± 0.16^{a}	7.29 ± 0.04^{b}	55.28 ± 0.11^{a}
28	R4	0.00	0.92 ± 0.01^{j}	1.32 ± 0.01^{j}	1.03 ± 0.02^{i}	3.27 ± 0.02^{j}
29	R5	0.00	0.00	2.34 ± 0.04^{g}	1.05 ± 0.02^{i}	3.40 ± 0.05^{j}

Table 2: GC Analysis for Methane Utilization

E. GC Analysis

The results are clearly indicates that, PPFM isolates could utilize methane as a carbon source (Table 2). All the 29 isolates were utilized methane, but in different criteria such as MYW155, MSY195 and MFW139 were started to utilize the methane from the second day of incubation and respectively the value was represented in %, 2.1, 0.34 and 0.07. Except for MEW141, MSY202, R1 and R5 isolates, all were started to utilize methane fourth day of incubation onwards. Out of 29, 10 isolates were shown utilization on 4th day only. The reference cultures (R1, R2, R3, R4 & R5) were shown the utilization in the range from 0.3 to 55.28%. In our isolates, the best one in methane utilization was MSY200 and that was on the 6th day of incubation. Methane is reported to be a non-preferred substratum among the common substrates used as carbon source, in the culturing of *M. organophilum.* Apart from this, other substrates used for carbon containing nutrients having better solubility than methane made availability of intended substrate in the growing media a limiting factor [14].

IV. Conclusion

This research work underlines the necessity for the screening of PPFMs for the utilization of methane. Interestingly, PPFMs showed excellent growth rate in the NMS medium with methane as a sole source of carbon. The naphthalene oxidation assays was performed to corroborate the expression of sMMO by the PPFMs with the utilization of methane. The findings of this research work could be scaled up for potential exploitation for environmental applications and benefits of human in the future.

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