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In vitro Study of Plant Growth Promoting Methylotrophic Bacterial Consortium as a Plant Probiotics for Paddy

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A B S T R A C T

Three efficient native phyllospheric methylotrophic isolates selected for in vitro compatibility test with existing rhizospheric methylotrophic reference cultures for liquid consortium development for testing efficacy on paddy cv. Gurjari. Chess medium found best for good growth and sporulation of isolates compared to other media. Beneficial native methylotrophic bacteria inherited capacity of methane degradation, have additional ability to promote plant growth through one or more mechanisms. Among all individual isolates consortium were found maximum potash solubilization efficiency, nifH gene presence and nitrogen fixation ability, inhibitory effect on soil borne pathogenic fungi by producing protease, cellulase and lipase enzymes, *in vitro* efficacy of individual and consortium application of methylotrophic bacteria on rice growth. Consortium application showed significant increase in seed germination, root length, shoot length and seedling vigor index of seedlings compared to individual culture inoculation, viz. *S. saprophyticus, B. subtilis, B. methylotrophicus, B. aerius, P. illinoisensis* and *B. megaterium* respectively.

Introduction

Extensive use of chemical fertilizers in farming assures high yield but simultaneously causes environmental problems. Because of this resurgence of interest for eco-friendly sustainable and organic agricultural practices is recently awaked (Esitken *et al.*, 2006). The positive relationship between microorganisms and plants are known since time immemorial, wherein, both partners benefit from each other directly or indirectly. Bacteria are among the most abundant microorganisms that colonize plant leaves (i.e., the phyllosphere) and so-called ‘‘phyllobacteria’’ or “epiphytes”.

These bacteria inhabit a harsh environment which is poor in nutrients and exposed to sun, wind and rain. In contrast to phyllosphere organisms, the rhizospheric microbes occur in the below-ground area and remaining in a dark and moist environment, which is relatively rich in organic nutrients. Most of these organic compounds (root exudates) are released by the growing cells of plants, the host organism for bacteria (Kutschera, 2007).

Food and Agriculture Organization (FAO) and World Health Organization (WHO) have

developed an operational definition for beneficial bacteria as Probiotics, "Live microorganisms which when administered in adequate amounts confer a health benefit on the host." It should come as no surprise that humans are not only the organisms that benefited from relationships with the right kind of bacteria, but soil beneficial bacteria can also be called as probiotics of plants.

Researcher has reported isolations of PPFMs from plant materials, in particular from root and leaf surfaces (Anitha, 2010). Their association is proved with more than 70 plant species and makes them interesting to study as potential agents improving plant growth and suppressing disease. However, there are few reports focusing on these aspects in India. Certain isolates are known to produce auxins, cytokinins and vitamin B12 providing them as best PGPB (Plant Growth Promoting Bacteria). Interactions with the plant nitrogen metabolism mediated by bacterial urease and the possible role of this in seed germination physiology have also been described. To evaluate dual role of native methylotrophic isolates like methane consumer cum growth promoter to develop plant probiotics liquid formulation for rice crop, the present research work was planned and conducted.

Materials and Methods

Consortium development

Sources of native methylotrophic bacterial isolates

Rhizospheric methylotrophic bacterial isolates like *Bacillus aerius* AAU M-8, *Paenibacillus illinoisensis* AAU M-17, *Bacillus megaterium* AAU M-29 were collected from the Department of Agril. Microbiology, B. A. Collage of Agriculture, Anand Agricultural University, Anand and three rice native phylospheric methylotrophic isolates (Prajapati, *et al.*, 2017 in printing) M-3, M-10 and M-15 were used for consortium development for paddy.

Compatible test

Each methylotrophic bacterial isolate was grown in AMS broth for 5-6 days. They were cross streaked on nutrient agar medium plates and their growth was checked after 48 hrs of incubation. Native isolates M-1 to M-15, AAU M-8, AAU M-17 and AAU M-29 were tested for compatibility by cross streak assay in nutrient agar medium. To test the compatibility of M-29 with other cultures, the M-29 was streaked as a strip at one end of the plate and inoculated for 24 hours to form a thick growth (Sateesh and Sivasakthivelan, 2013).

Consortium preparation

All the native Phyllospheric and Rhizospheric methylotrophic bacterial isolates were grown separately in respective broth media (AMS) to ensure maximum resting structures (cyst/spore) formation. Determination of population density of each isolates in broth was done by direct microscopic count. Individual culture in specific proportion was mixed to reach population density of 5 x 109 in final product (Dabhi, *et al.,* 2014). Longevity of the product monitored through determination of microbial population in the finished product at monthly interval up to 1 year (As per FCO gazette notification for introduction of NPK consortia biofertilizers, Dept. of Agriculture & co-operative, Ministry of Agriculture, GOI vide S. O. 1181(E) dated 30.04.2014).

In vitro evaluation of PGP traits of methylotrophic bacterial consortium

Detection of nifH gene

Genomic DNA of all native diazotrophic bacterial isolates and standard strains were

isolated using the protocol described Sambrook *et al.,* (1989). Fragments of nifH genes were amplified by two PCR reaction. PCR was performed in PCR reaction mixture (25 µl) containing 2.5 µl Taq Buffer (10 X), 0.5 µl dNTPs (2.5 mM each) mix, 2.0 µl Template DNA (25 ng/µl), 0.4 µl Taq polymerase (5U/µl), 17.8 µl Millipore Sterilized Water using degenerated following primers (Poly *et al.*, 2001) 1.0 µl Primer 1 (Pol F- 5' TGCGAYCCSAARGCBGACTC 3') and 1.0 µl Primer 2 (Pol R- 5'ATSGCCATCATYTCRCCGGA 3') and the primers synthesized at MWG Bio-tech Pvt. Ltd., Germany. PCR was successful to amplify a 360 base pair (bp) nifH fragment from the 3 different native diazotrophic bacterial isolates. PCR reaction mixture was prepared from the stock solutions of each individual component. The reagents were mixed thoroughly by a short spin using microfuge. The tubes were placed in Mastercycler personal (Eppendorf) and subjected to PCR, according to the following protocol. Initial denaturation at 94°C for 5 min, Denaturation 94°C for 5 min, annealing at 62°C for 1 min, extension at 72 °C for 1 min, final extension step at 72 °C for 5 min were performed. PCR reactions were run for 30 cycles. PCR products were analyzed by gel electrophoresis with molecular marker DNA (100 bp ladder) of known molecular weight on 1.8 % agarose gel at 80 V using 1 X TAE buffer and ethidium bromide (0.5 µg/ml). Gels were visualized under UV light and photographed using gel documentation system.

Nitrogen fixation

The plant growth promoting effect showed by phyllospheric and rhizospheric methylotrophs is directly attributed to its capacity to fix atmospheric nitrogen into the forms utilized by plants. Isolates were inoculated into the nitrogen free broth containing sucrose as carbon source and cultures were grown at 30±2°C for 5-7 days and nitrogen fixation was measured by Micro-Kjehldahl method (Bremner, 1958). Sugar utilization was estimated by DNS method. The rate of nitrogen fixation was expressed as mg nitrogen fixed per gram of sucrose consumed.

Phosphate solubilization capacity

Phosphate solubilization efficiency in solid medium

All the isolates were spot inoculated on sperbor medium. Plates were incubated at 30+2˚C and examined for the colonies showing clear zones of calcium released at 6–7 days (Jackson, 1973).

Phosphate solubilization efficiency in liquid medium

Erlenmeyer flasks (250 ml) containing 100 ml of the liquid PKVK medium were inoculated with 100 µl of bacterial suspension (approx. 107 cfu/ml). For each isolate three flasks were inoculated. The flasks were incubated on rotary shaker (150 rpm) at 30+ 2˚C. After 3, 5 and 7 days, measurement of pH using pH meter and liberated P following Vanado-molybdate method was carried out (Jha *et al.*, 2009). The graph of OD versus concentration of phosphate in µg was plotted for the standard and samples were compared to calculate P concentration.

Indole acetic acid (IAA) production

In vitro IAA production by selected isolates was determined using the protocol described by Khalid *et al.,* (2004). For this purpose, 10 ml Glucose Phosphate Broth (GPB) medium was prepared in 100 ml Erlenmeyer flasks, autoclaved and cooled. L-Tryptophan was filter sterilized passing through 0.2 µm membrane filter and added at desired

concentration (1 µg/ml) to the liquid medium. The flasks were inoculated with 1.0 ml of 3-days old bacterial broth (107 CFU/ml) and incubated at 30+2˚C for 48 h. Un-inoculated control was kept for comparison. After incubation, the contents were filtered through Whatman filter paper No. 2. For measuring IAA, 3.0 ml of filtrate was taken in test tube and 2.0 ml of Salkowski reagent was added. The contents in the test tubes were allowed to stand for ½ h for color development. Similarly, color was also developed in standard solutions of IAA. The intensity of color was measured at 535 nm by spectrophotometer. Standard curve was prepared and used to calculate IAA produced by methylotrophic isolates.

ACC-deaminase activity

Qualitative screening of bacterial isolates for ACC deaminase enzyme production was carried out based on their ability to use ACC (1-Aminocyclopropane-1-Carboxylate) as a sole nitrogen source in the sugar free minimal salt medium. Cultures were spot inoculated on petri plates containing DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC substrate. Plates containing DF minimal medium without ACC served as negative control and with (NH4)2SO4 (2.0 gm/l) as a nitrogen source serve as positive control. The plates were incubated for 3-4 days at 30+2˚C. Growth of isolates on ACC supplemented plates was compared with positive and negative control plates. Isolates grown well on ACC plates were considered as ACC deaminase enzyme producers (Daun *et al.*,2009).

Potash solubilization efficiency

All the isolates were spot inoculated on Glucose Yeast Calcium agar medium (GYCaA). Plates were incubated at 30+2˚C and examined for the colonies showing clear zones of calcium released at 6–7 days. Colonies showing clear zone were further inoculated on Alendreskov's media containing mica and feldspar as a raw insoluble potash substrate to check their potash mobilization activity (Hu *et al.,* 2006).

Biocontrol potential of native potential methylotrophic consortium

Bioassay against plant pathogenic fungi

Methylotrophic isolates were tested in vitro for their biocontrol potential by dual inoculation technique (Foldes *et al.*, 2000) against three fungal plant pathogens viz. *Macrophomina* sp., *Fusarium* sp. and *Pythium* sp. Each fungal pathogen was grown on the Potato Dextrose Agar (PDA) plate till it covered the whole surface of the agar plate. With the help of sterile cork borer, a agar disc having fungal growth from plate was taken and placed at the centre of the fresh PDA plate. Test bacterial culture suspension (50 µl) was inoculated in the wells 3 cm away from fungal disc and kept for incubation at 30+2˚C for 7 days. Inhibition of fungal growth was recorded at 5th and 7th days after co-incubation and compared with normal fungal growth.

Siderophore production

The production of siderophore by isolates was assessed through plate assay. Chrome Azurol S blue agar medium (CAS) was used to detect siderophore production by the isolates as per method described by Alexander and Zuberer (1991). CAS medium (1 ltr) was prepared by dissolving 60.5 mg Chrome Azurol S (CAS) (HiMedia) in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl3.6H2O in 10 mM HCl). With continuous stirring, the solution was slowly added to 72.9 mg hexadecyl trimethyl ammonium bromide

(HDTMA) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved.

In 100 ml of 10XMM salt broth, 30.2 g of PIPES, 18 g agar and 750 ml double distilled water were added. pH of the medium was adjusted to 6.8 by the addition of NaOH solution (w/v) and autoclaved. After cooling of medium up to 50˚C, the previously prepared sterile CAS dye solution was added rolling down from glass wall of flask with gentle agitation to avoid formation of foam or bubble and uniform mixing of two liquids. The medium was poured into sterile petri plates. The plates were stored in refrigerator at 4˚C till used. The overnight grown test bacterial cultures were spot inoculated on individual CAS plates and incubated at 30+2˚C for 24 h. The cultures showing yellow to orange coloured ring around the colonies were considered as siderophore production positive.

Production of cell wall degrading enzymes

The lipolytic activity was determined by streaking isolates on Tributyrin agar plates (Lawrence *et al.,* 1967) in laboratory and recorded growth. The protease production was determined using skimmed milk agar. Bacterial cells were spot inoculated and incubated for 2 days at 30+2˚C. Proteolytic activity was identified by clear zone around the colony (Smibert and Krieg, 1994). The cellulase activity was determined by streaking isolates on cellulose agar plate and after incubation assayed as per method suggested by Ibrahim and El- diwani (2007).

PGPR effects of proven isolates consortium on rice cv. Gurjari

Rice seeds cv. Gurjari were surface sterilized by washing in 95 % ethanol solution for 5 min, 0.1 % HgCl2 solution for 2 min and rinsed thoroughly with distilled water 3-5 times. Thoroughly washed seeds were kept on previously sterilized filter paper sheet placed in Petri plates and incubated at room temperature for 5 days, seed germination was examined at 96 hrs interval and germination percentage were calculated. In vitro efficacy of isolates was tested on solid water agar in tubes on Rice cv. Gurjari. Surface sterilized seed were treated with 0.01 ml of previously grown starter cultures of methylotrophic isolates for 30 min. Individual treated seeds were inoculated on butt agar (1 %) and allowed to grow in a growth chamber at 28±2˚C. Control seeds without treatment were also used as check and each treatment was repeated three times. After 10 to 12 days of incubation the plantlets were removed carefully from water agar and root length, shoot length and fresh weight were measured. Vigor Index (VI) has been calculated using following formula (Haque *et al.,* 2007).

Vigor Index (VI) = Germination % X (Root length + Shoot Length)

Results and Discussion

Consortium development

In vitro compatibility of chosen phyllospheric and rhizospheric methylotrophic bacterial isolates for consortium

An important prerequisite for successful development of microbial culture mixture (consortium) depend on the compatibility (tolerance) of co-inoculated microorganisms. Three native rhizospheric methylotrophic bacterial cultures (*B. aerius* AAU M-8, *P. illinoisensis* AAU M-17 and *B. megaterium* AAU M-29) already proven as methane degrader (Jhala *et al.,* 2015) as well as proved as good plant growth promoter were tested for compatibility with three phyllospheric methylotrophic bacterial isolates (*S.*

saprophyticus, B. subtilis and *B. methylotrophicus*) on Nutrient agar media in vitro. All the bacterial cultures were found compatible with each other (Plate 4.8) and were selected for preparing a rhizospheric and phyllospheric methylotrophic bacterial liquid consortium for trapping or capturing emitted methane as a sole carbon source from paddy field and feedback provides plant growth promoting substances for growth and development paddy.

Consortium preparation

All phyllospheric and rhizospheric methylotrophic cultures were grown separately in five different medium viz., Ammonium mineral salt (NMS), Nutrient broth (NB), Chess medium, Lurial broth (LB) and Rocket medium respectively, to ensure maximum sporulation. Result showed that among above five medium Luria broth (LB) showed maximum growth (1.78 × 106) but in case of spore formation chess medium showed fast sporulation compared to other medium (1.96 × 107) (Table-4.16, Plate 4.9). Chess medium was employed for development of phyllospheric and rhizospheric methylotrophic bacterial consortium. For determination of population density of each isolates in broth direct microscopic count was carried out in neuberger's chamber. Individual culture when obtained population density of 5 x 109 (cfu/ml) where stored at room temperature in laboratory. It was observed that bacteria has no inhibitory effect on each other in consortium indicating all chosen cultures used in consortium preparation were compatible with each other.

In vitro evaluation of liquid plant probiotic properties of methylotrophic consortium

Detection of nifH gene: All three native chosen phyllospheric methylotrophic isolates *S. saprophyticus, B. subtilis and B. methylotrophicus* gave single band of ~ 360 bp indicating these isolates have presence of nif gene providing capability to fix atmospheric nitrogen (Plate 4.10). The nifH gene is widely used as marker gene for screening nitrogen fixing prokaryotes in soil. Jhala (2015) has already reported nifH in native methylotrophic bacteria of Gujarat like *Bacillus aerius* AAU M 8 (Accession no. KC787582) the same bacterium is also incorporated as rhizospheric methylotrophic culture in formulated consortium for rice field testing on cv. Gurjari.

Nitrogen fixing capacity

The results of this experiment are mentioned in Table 4.17. All the isolates and consortium were confirmed to have ability of fixing atmospheric nitrogen. It was revealed from the results that nitrogen fixing potentiality of these isolates ranged from 5.56 to 17.06 mg Ng-1 of sucrose consumed and consortium showed the highest nitrogen fixation capacity (17.06 mg Ng-1 of sucrose consumed) followed by *B. methylotrophicus, S. saprophyticus* and *B. subtilis* (9.70, 7.79 and 5.56 mg N/g of sucrose consumed respectively). Satapute *et al.,* 2012 studied Bacillus subtilis strain AS-4 free living nitrogen fixing bacteria that could be exploited as soil inoculants and can be used for nitrogen fixation in soil for long run, eco-friendly and cost ineffective.

Phosphate solubilization capacity

Phosphate solubilization efficiency in Solid medium

All the tested isolates and their consortium were studied for phosphate solubilization capacity on Sperber's agar media. Methylotrophic consortium (combine inoculation) showed the maximum

solubilization zone (5 mm) followed by individual inoculation of *S. saprophyticus*, *B. subtilis*, *B. aerius*, *P. illinoisensis* and *B. megaterium* (Table 4.18).

Phosphate solubilization efficiency in liquid medium

Data regarding phosphate solubilization, all the strains solubilized and released P from tri calcium phosphate (TCP), *S. saprophyticus* recorded maximum phosphate solubilization with increasing the time interval (30, 64 and 122 µg /ml at 2, 4 and 6 DAI respectively) followed by other strains, *B. subtilis* (15 and 18 µg/ml at 4 and 6 DAI respectively), *B. methylotrophicus* (7, 13 and 95 µg/ml at 2, 4 and 6 DAI respectively), *B. aerius* (20, 27 and 47 µg/ml at 2, 4 and 6 DAI respectively), *P. illinoisensis* (17, 25 and 48 µg/ml at 2, 4 and 6 DAI respectively) and *B. megaterium* (15, 25 and 54 µg/ml at 2, 4 and 6 DAI respectively) while methylotrophic consortium showed maximum phosphate solubilization with increasing duration (49, 49 and 80 µg/ml at 2, 4 and 6 DAI respectively) as compared to individuals Table 4.19. The results indicated that methylotrophic consortium having capacity to utilize atmospheric methane as carbon and energy source, additionally have capacity to convert the unavailable phosphorus to available form for crop.

Indole 3-Acetic Acid (IAA) production

All methylotrophic isolates and their consortium were grown in Glucose Phosphate Broth supplemented with 0.5 µg/ml of tryptophan for IAA production. With increasing the incubation time viz., 2, 4 and 6 DAI increase in the IAA concentration (µg/ml) ranging from 2.3-5.2, 3.4-7.6 and 4.7-12.2 µg/ml respectively, was observed. Among all treatments, consortium having (*S. saprophyticus* + *B. subtilis* + *B. methylotrophicus*, *B. aerius* + *P. illinoisensis* + *B. megaterium*) produced maximum IAA (5.2, 7.6 and 12.2 µg/ml at 2, 4 and 6 DAI respectively) followed by individual inoculum of *S. saprophyticus* (3.3, 6.6 and 10.5 µg/ml at 2, 4 and 6 DAI respectively), *B. subtilis* (4.3, 6.4 and 8.0 µg/ml at 2, 4 and 6 DAI respectively) and *B. methylotrophicus* (2.7, 5.9 and 9.5 µg/ml at 2, 4 and 6 DAI respectively) as well as standard cultures *B. aerius* AAU M 8 (2.3, 3.4 and 4.7 µg/ml at 2, 4 and 6 DAI respectively), *P. illinoisensis* AAU M 17 (4.1, 6.4 and 4.8 µg/ml at 2, 4 and 6 DAI respectively) and *B. megaterium* AAU M 29 (3.1, 5.4 and 6.7µg/ml at 2, 4 and 6 DAI respectively) Table 4.20.

These results showed that combined inoculation (consortium) of native methylotrophic six isolates may have capacity to improve plant growth. Many phyllospheric and rhizospheric microorganisms are able to synthesize and secrete auxin, primarily IAA due to which they influence the growth of the plants. Yim *et al.*, (2010) carried out quantitative analysis of IAA using Salkowski reagent from culture liquids of the *Methylobacterium* strains CBMB20 and CBMB110 in the presence of L-tryptophan and obtained 2.33 and 4.03 µg/ml respectively after 5 days of inoculation.

Measurement of ACC-deaminase activity

All the methylotrophic bacterial isolates and their consortium were found to grow luxuriously on plates containing (NH4)2SO4 as nitrogen source, whereas, grew poorly on plates containing nitrogen free MS media, moreover, combined inoculum (consortium), *B. subtilis*, *B. aerius* AAU M 8 and *B. megaterium* AAU M 29 showed luxurious growth on plates having ACC as sole source of nitrogen showing their ability to produce enzyme ACC deaminase (Table 4.21).

Table.1 Effect of different synthetic medium on sporulation after 72 hrs inoculation of methylotrophic bacteria

Sr. No.	Synthetic medium	Microscopic count	Plate count
		Cells/ml	CFU/ml
1	Ammonium mineral salt (AMS)	1.78×10^6	2.12×10^6
2	Nutrient broth (NB)	1.52×10^6	1.69×10^6
3	Chess medium	1.11×10^6	1.56×10^7
4	Luria broth (LB)	1.91×10^6	2.48×10^5
5	Rocket medium	1.46×10^6	1.89×10^6

Table.2 *In vitro* nitrogen fixation capacity of methylotrophic isolates

Isolate	Nitrogen fixation capacity mg N/g of sucrose consumed
S. saprophyticus	7.79
B. subtilis	5.56
B. methylotrophicus	9.70
Consortium	17.06

Table.3 Solubilization of tri-calcium phosphate by methylotrophic isolates

Isolate	TCP solubilization Zone (mm)
S. saprophyticus	3
B. subtilis	2
B. methylotrophicus	3
***B. aerius* AAU M 8**	3
***P. illinoisensis* AAU M 17**	2
***B. megaterium* AAU M 29**	3
Consortium	5

Table.4 Solubilization of tri-calcium phosphate by methylotrophic isolates

Isolate	TCP solubilization Zone (mm)
S. saprophyticus	3
B. subtilis	2
B. methylotrophicus	3
B. aerius AAU M 8	3
P. illinoisensis AAU M 17	2
B. megaterium AAU M 29	3
Consortium	5

Table.5 *In vitro* phosphate solubilization efficiency of isolates

Isolates	At 2 DAI	At 4 DAI	At 6 DAI
	P µg /ml	P µg /ml	P µg /ml
Initial	-	-	-
S. saprophyticus	30	64	122
B. subtilis	ND	15	18
B. methylotrophicus	7	13	95
B. aerius AAU M 8	20	27	47
P. illinoisensis AAU M 17	17	25	48
B. megaterium AAU M 29	15	25	54
Consortium	49	49	80

Note: ND-not detected

Table.6 *In vitro* IAA production efficiency of methylotrophic isolates

Isolate	IAA concentration (µg/ml)		
	2 DAI	4 DAI	6 DAI
S. saprophyticus	3.3	6.6	10.5
B. subtilis	4.3	6.4	8.0
B. methylotrophicus	2.7	5.9	9.5
B. aerius AAU M 8	2.3	3.4	4.7
P. illinoisensis AAU M 17	4.1	6.4	4.8
B. megaterium AAU M 29	3.1	5.4	6.7
Consortium	5.2	7.6	12.2

Table.7 *In vitro* ACC deaminase activity of isolates

Isolates	ACC deaminase activity
S. saprophyticus	+
B. subtilis	++
B. methylotrophicus	++
B. aerius AAU M 8	++
P. illinoisensis AAU M 17	+
B. megaterium AAU M 29	+++
Consortium	+++

Note: +++ strong, ++ moderate, - absent

Table.8 *In vitro* potash mobilization activity of isolates

Isolates	Potash solubilization efficiency Zone diameter (mm)
	Alendreskov's (mica) media
S. saprophyticus	-
B. subtilis	3
B. methylotrophicus	3
B. aerius AAU M 8	4
P. illinoisensis AAU M 17	-
B. megaterium AAU M 29	4
Consortium	4

Table.9 Biocontrol activity of potential methylotrophic isolates against plant pathogenic fungi

Isolate	Growth inhibition of test pathogenic fungi			
	Macrophomina spp.	***Pythium spp.***	***Rhizoctonia spp.***	***Fusarium spp.***
S. saprophyticus	ND	ND	ND	ND
B. subtilis	-	+	-	+
B. methylotrophicus	+	+	+	+
***B. aerius* AAU M 8**	+	+	+	+
***P. illinoisensis* AAU M 17**	-	-	-	+
***B. megaterium* AAU M 29**	+	+	+	+
Consortium	+	+	+	+

Note: ND- not detected, + Detected

Table.10 *In vitro* siderophore production activity of isolates

Isolates	Siderophore production on CAS agar medium
S. saprophyticus	ND
B. subtilis	+
B. methylotrophicus	+
***B. aerius* AAU M 8**	+
***P. illinoisensis* AAU M 17**	+
***B. megaterium* AAU M 29**	+
Consortium	++

Note: ND- not detected

Table.11 Cell wall degrading enzyme activity

Isolates	Lipase	Protease
S. saprophyticus	+	ND
B. subtilis	+	ND
B. methylotrophicus	ND	+
***B. aerius* AAU M 8**	ND	ND
***P. illinoisensis* AAU M 17**	ND	ND
***B. megaterium* AAU M 29**	+	ND
Consortium	+	ND

Note: ND- not detected

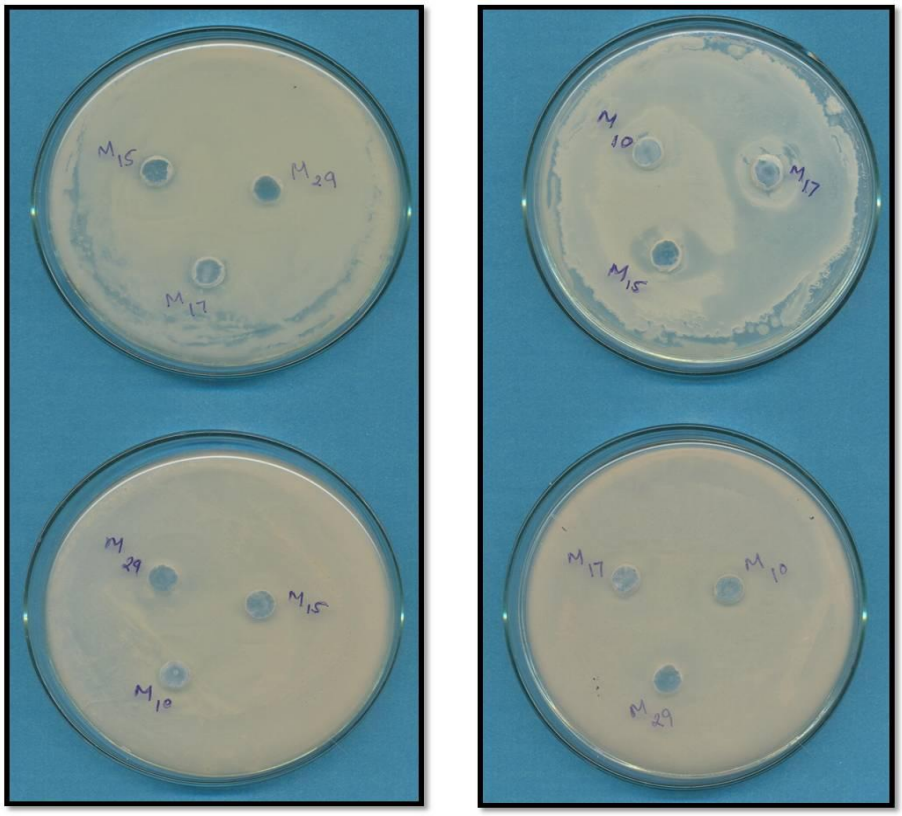

Plate.1 *In vitro* compatibility of phyllospheric and rhizospheric methylotrophic isolates on N-agar

Table.12 *In vitro* effect of methylotrophs on rice *cv.* Gurjari at 12 DAI

Treatment	Germination percentage (%)	Root length (cm)	Shoot length (cm)	Seedling vigor Index
Control	80	3.50	7.90	684.0
***S. saprophyticus* AAU M 3**	90	6.00	12.00	1620.0
***B. subtilis* AAU M 10**	100	4.00	11.00	1500.0
***B. methylotrophicus* AAU M 15**	100	6.50	11.70	1820.0
***B. aerius* AAU M 8**	100	6.00	12.20	1820.0
***P. illinoisensis* AAU M 17**	100	6.50	11.00	1750.0
***B. megaterium* AAU M 29**	100	5.50	11.70	1720.0
Consortium	100	7.00	12.7	1970.0
S.Em.±	-	0.135	0.157	-
CD at 5 %	-	0.395	0.462	-
CV %	-	4.78	2.80	-

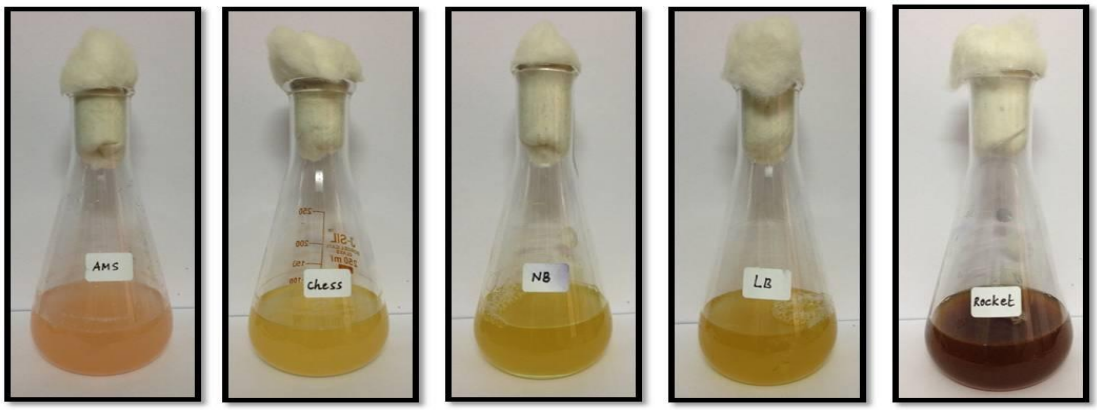

Plate.2 Different synthetic liquid media used for sporulation of methylotrophic bacterial liquid consortium.

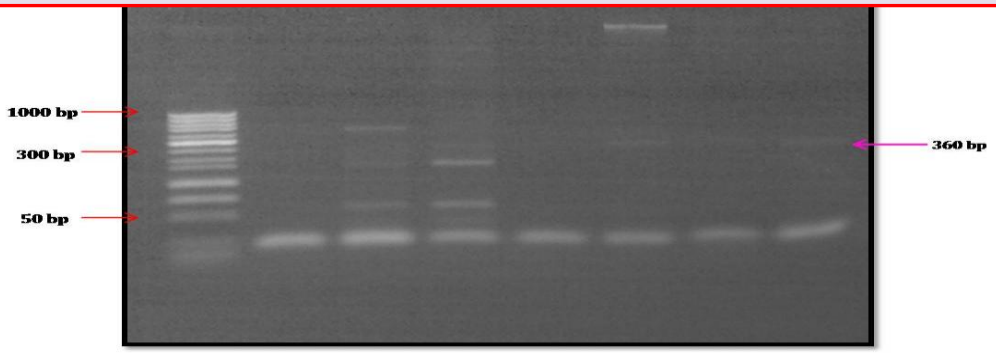

Plate.3 Amplification of the nitrogen fixation (*nif*H) gene from native phylospheric methylotrophic bacterial isolates with reference strains using degenerated universal *nif*H gene primer. Line: 1. M-10, 2. M-3, 3. M-15 and diazotrophic reference strains (R) MTCC-446 (*A. chroococcum*), MTCC-2306 (*A. lipoferum*)

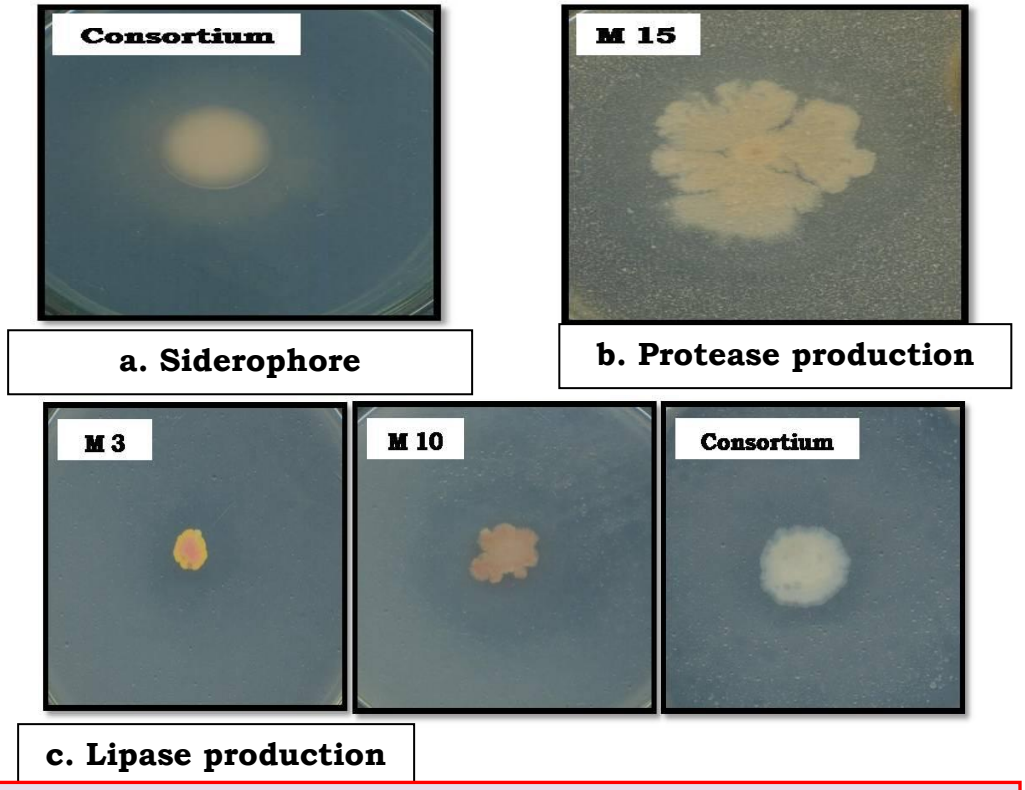

Plate.4 Siderophore and cell wall degrading enzyme production by methylotrophic isolate and consortium

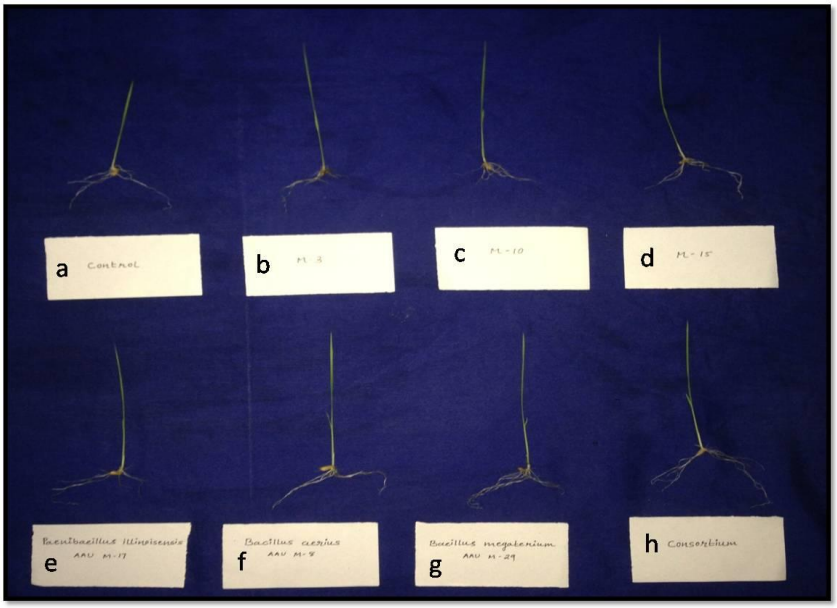

Plate.5 *In vitro* effect of methylotrophic isolates and their consortium on emerging seedlings of rice *cv*. Gurjari (a) Control, (b) M 3, (c) M 10, (d) M 15, (e) *Panibacillus illinoisensis* AAU M 17, (f) *Bacillus aerius* AAU M 8, (g) *Bacillus megaterium* AAU M 29 and (h) consortium

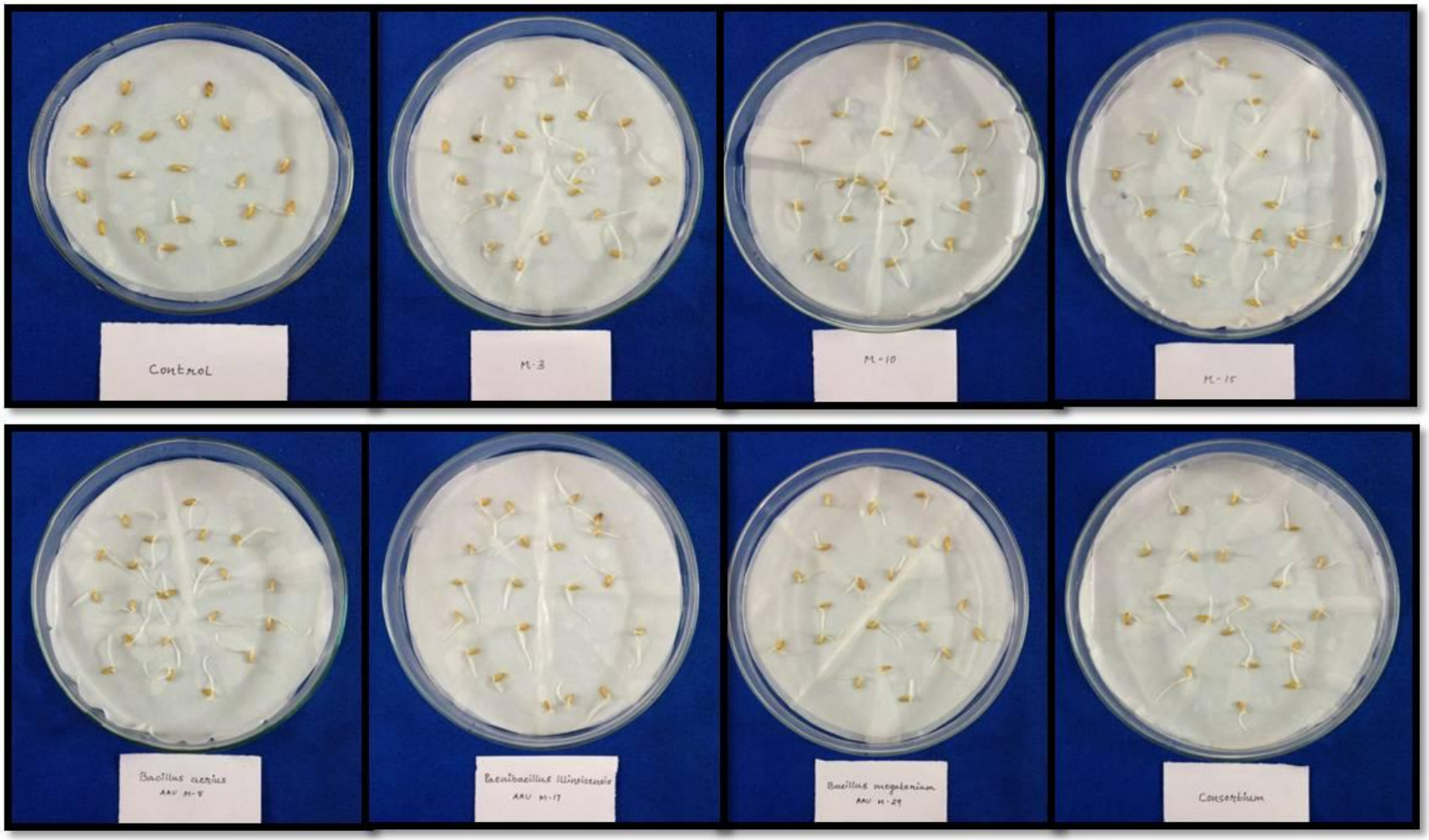

Plate.6 *In vitro* effect of methylotrophic isolates and consortium on germination of rice seeds *cv.* Gurjari

The ACC deaminase is useful to plants to fight against several biotic and abiotic stresses such as attack by phytopathogens, salinity, drought and higher concentration of heavy metals, so native methylotrophic bacterial consortium may be useful in rice (Glick *et al.,* 2007).

Enzyme ACC deaminase facilitates plant growth and development under stress condition by decreasing plant ethylene level and thereby protecting plants from stress. It converts ethylene precursor ACC in to 2-oxobutanote and NH3. Yim *et al.,* (2010) reported that M. oryzae strains CBMB20 and CBMB110 were capable of producing ACC deaminase 94.48 and 24.74 nmol α-ketobutyrate mg-1 protein h-1), respectively.

Potash solubilizing efficiency

Chosen methylotrophic bacterial isolates and their consortium were tested for their potash solubilizing efficiency on Alendreskov's media containing mica as natural 'K' substrate. Among all treatments, consortium (4 mm), M 10 (3 mm), M-15 (3 mm), *B. aerius* AAU M 8 (4 mm) and *B. megaterium* AAU M 29 (4 mm) showed potash solubilization efficiency zone diameter (mm) on Alendreskov's mica media plates (Table 4.22).

Biocontrol activity of potential methylotrophic consortium

Bioassay against plant pathogenic fungi

Antifungal activity of selected methylotrophic isolates and their consortium on plant pathogenic fungi are presented in Table 4.21. Among all the methylotrophic isolates, *B. methylotrophicus*, *B. aerius* AAU M 8, *B. megaterium* AAU M 29 and consortium inhibited growth of *Macrophomina* spp., *Pythium* spp., *Rhizoctonia* spp. and *Fusarium* spp. on Nutrient-Potato dextrose agar medium whereas isolate *B. subtilis* inhibited growth of *Pythium* spp., *Fusarium* spp. and isolate *P. illinoisensis* AAU M 17 inhibited growth of *Fusarium* spp.

A positive role is played by phyllosphere antagonistic microorganisms, which protect the plants from pathogenic microorganisms and thus improve their healthiness. The inhibition of phytopathogens by PPFM isolates has already been reported by Poorniammal *et al.,* (2010), they have documented that *Methylobacterium* sp. isolate CO 47 significantly reduced the linear mycelial growth of *R. solani* (Table 4.23).

Siderophore production

Selected six native isolates and their consortium were found capable of producing siderophores (Table 4.24). All isolates produced yellow-orange colour zone on CAS agar plate hence considered as siderophore producers (Plate 4.11) except *S. saprophyticus*.

Siderophore bind most of the available iron (Fe+3) in rhizosphere and thereby preventing proliferation of fungal pathogen in immediate vicinity due to lack of iron (Jhala *et al.,* 2015). Lacava *et al.,* (2008) reported 37 strains of *Methylobacterium* spp. positive on Chrom Azurol Sulphate (CAS) agar for siderophore production. *Methylobacterium* spp. are producing hydroxamate-type siderophores.

Production of cell wall degrading enzyme

Selected six native isolates and their consortium were grown on their specific medium. Among them consortium was capable of producing lipase and protease enzyme responsible for cell wall degradation of plant pathogen in vitro (Plate 4.11). *S.*

saprophyticus, *B. subtilis* and *B. megaterium* produced lipase where as *B. methylotrophicus* was capable to produce protease (Table 4.25).

Madhaiyan *et al.,* (2004b), reported induction of systemic resistance in rice cultivar Co-47 with 17.8% disease reduction through combined applications of seed imbibition and phyllosphere spray of *Methylobacterium* sp. and thereby reported increase in content of Phenylalanine Ammonia Lyase (PAL), peroxidase, β-1,3-glucanase and chitinase activity. *Methylobacterium* inoculation increased shoot length, number of effective tillers, plant biomass and grain yield as well as protected rice from *R. solani*.

Altogether this result confirms the antagonistic activity of isolates against common soil borne pathogens which benefits the succeeding crops of rice. The antagonistic activity of isolates may be due to their ability to produce Siderophore and cell wall degrading enzymes viz. lipase and protease that degrades lipids which are components of fungal cell wall and thereby have capacity to inhibit their growth.

Plant growth promoting effects of proven isolates on rice cv. Gurjari

Biopriming of seed by native methylotrophic bacterial isolates with single inoculation as well as combined inoculation (consortium) have significant effect on germination and development of rice cv. Gurjari.

Methylotrophic bacterial isolates, *B. subtilis*, *B. methylotrophicus*, *B. aerius*, *P. illinoisensis* and *B. megaterium* as well as its consortium (combine inoculation) showed 100% germination while *S. saprophyticus* isolate and control showed 90% and 80% germination of Gurjari seeds respectively (Plate 4.12). With regard to seedling vigor index (SVI), consortium of methylotrophic bacterial isolates showed higher SVI (1970.0) followed by *B. methylotrophicus*, *B. aerius*, *P. illinoisensis*, *B. megaterium*, *S. saprophyticus* and *B. subtilis* (1620.0, 1500.0, 1820.0, 1820.0, 1750.0 and 1720.0) respectively compared to control (684.0). All the isolates and its consortium showed increase in seed germination rate as compared to non-inoculated seeds which is represented as germination percentage in Table 4.26 and Plate 4.13.

Among all treatments consortium gave the significantly highest root and shoot length (7.00 cm and 12.7 cm) compared to single inoculation, *S. saprophyticus* (6.00 cm and 12.00 cm), *B. subtilis* (4.00 cm and 11.00 cm), *B. methylotrophicus* (6.50 cm and 11.70 cm), *B. aerius* (6.00 cm and 12.20 cm), *P. illinoisensis* (6.50 cm and 11.00 cm) and *B. megaterium* (5.50 cm and 11.70 cm) respectively (Table 4.26, Plate 4.13). Results indicated potential use of bacterial inoculation to reduce the time period required for raising seedlings for transplanted rice cultivation as the seed treated with bacterial isolates showed improved seedling growth parameters.

These result confirms that one or more PGPR traits of methylotrophic bacterial isolates are reflected in overall better growth in laboratory. Moreover, all the isolates were capable of producing IAA which may have played a central role in germination and seedling development as IAA regulator of numerous biological processes like cell division, elongation and differentiation to tropic responses, improves root growth providing a large surface area for nutrient and water uptake which can directly affects seedling development.

In addition, all the isolates showed production of biocontrol molecules such as siderophores, lipase and chitinase enzymes and antagonist to fungi as well as some of them were also

capable of producing stress release enzyme ACC deaminase, all such parameters may impacted for better seed germination and growth.

Madhaiyan *et al.,* (2005) studied the PPFM inoculation on quality of sugarcane true seed under in vitro conditions wherein inoculated seeds showed increased germination (RG) ranging between 5.14 to 7.18 suggesting that M. extorquens PPFMSo78 strain induced crop growth earlier and better.

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