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Cellulase Production by Pink Pigmented Facultative Methylotrophic Strains (PPFMs)

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Abstract Pink pigmented facultative methylotrophs (PPFM) isolated from water samples of Cooum and Adyar rivers in Chennai and soil samples of forests located in various districts of Tamil Nadu, India were screened for cellulase production using carboxymethylcellulose agar (CMC agar) medium. The strains showed wide variations in the production of clearing zones around the colonies on CMC agar medium flooded with Congo red. CMCase and filter paper assays were used to quantitatively measure the cellulase activity of 13 PPFM strains. Among the strains, *Methylobacterium gregans*, MNW 60, MHW 109, MSF 34, and MSF 40 showed cellulolytic activity ranging from 0.73 to 1.16 $U mL^{-1}$ with wide temperature (35–65°C) and pH (5 to 8) tolerance. SDS-PAGE analysis of the crude enzyme of PPFM strain MNW 60 exhibited several protein bands, and zymogram analysis revealed two dimeric cellulase bands with molecular mass of ~92 and 42 kDa. Scanning electron microscopic studies revealed significant morphological differences between the cells grown in normal and CMC amended medium. The strain MNW 60 was identified as *Methylobacterium* sp. based on biochemical, physiological, and morphological analyses, and the methylotrophic nature was authenticated by the presence of *mxaF* gene, encoding methanol dehydrogenase as a key indicator enzyme of methylotrophs, with 99% similarity to *Methylobacterium lusitanum.* With the 16S ribosomal RNA sequence showing 97% similarity to *M. lusitanum* strain MP2, this can be proposed as a novel taxon of the genus *Methylobacterium*. The study forms the first detailed report on the extracellular cellulase production by pink pigmented *Methylobacterium* sp., and it is expected that this might be the basis for further studies on cellulase production by PPFMs to explore the molecular mechanism, strain improvement, and large-scale cellulase production for its application.

Keywords CMC · CMCase · FPase · *mxaF* · *Methylobacterium*

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Introduction

Microbial degradation of lignocellulosic waste and the downstream products resulting from it are accomplished by a concerted action of several enzymes, among which the most prominent are the cellulases [1]. Cellulases assume greater importance in the current economic scenario due to their versatile industrial and commercial applications [2–4]. Though cellulase research has been concentrated mostly in fungi, actinomycetes, and protozoa, there is an increasing interest in cellulose production by bacteria [5–7].

Pink pigmented facultative methylotrophs (PPFMs) are a physiologically interesting group of bacteria that are capable of growing on single carbon such as methanol and methylamine, as well as on C2, C3, and C4 compounds [8]. The practical significance of these bacteria, such as bioconversion of some substrates unusable by other organisms into products with economic value, has brought them into prominence [9]. They are described with the ability to stimulate seed germination, plant development, hormone or vitamin production, ACC deaminase activity, siderophore production, and flavor development [10]. Though earlier studies indicate cellulase production by PPFMs [11], a detailed study is lacking. Similarly, while few attempts have been made to study the diversity of PPFMs in Tamil Nadu, India [12, 13], knowledge on their diversity in environments like in the rivers of Chennai and in the forests of Tamil Nadu and evaluation of their potential are lagging. Hence, the present study has been carried out to evaluate PPFM strains isolated from water samples of Cooum and Adyar rivers in Chennai and soil samples of various forests located in Tamil Nadu, India along with standard strains of PPFMs viz *Methylobacterium extorquens*, *Methylobacterium organophilum*, *Methylobacterium gregans*, and *Methylobacterium komagatae* for cellulase production. The study was directed towards screening of selected PPFM isolates for cellulolytic activity on carboxymethylcellulose (CMC) amended media, estimation of the cellulolytic activity through CMCase and filter paper (FPase) assays, optimization of pH and temperature for maximum cellulase activity by selected strains, characterization of crude enzyme present in the culture filtrate through SDS-PAGE and zymogram analysis, study of the cellulose-induced changes on the cells of PPFMs using a scanning electron micrograph (SEM), and identification of a promising PPFM strain, MNW 60, through biochemical and molecular methods.

Materials and Methods

Screening of PPFM Strains for Cellulase Production

Sixteen PPFM strains isolated from water samples of Cooum and Adyar rivers in Chennai and soil samples of forests in Tamil Nadu, India (Table 1) along with standard PPFM strains, *M. extorquens*, *M. organophilum* (source: Dr. Mary Lidstrom, University of Washington, Seattle, USA), *M. gregans*, and *M. komagatae* (source: Dr. Yuka Kato, Microbiological and Analytical Group, Food Research Laboratories, Japan), were subcultured on methanol mineral salts medium (MMS) (L^{-1} distilled water: KNO_3 1 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, Na_2HPO_4 0.23 g, NaH_2PO_4 0.07 g, $FeSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 5 μg, H_3BO_3 10 μg, $MnSO_4 \cdot 5H_2O$ 1 μg, $ZnSO_4 \cdot 7H_2O$ 70 μg, MoO_3 10 μg, and 0.5% methanol, pH 6.8) and were preserved at 4°C. Preliminary screening of PPFM strains for cellulase production was carried out on selective CMC agar plates consisting of (g L^{-1}) K_2HPO_4 0.165 g, $(NH_4)_2SO_4$ 0.16 g, yeast extract 0.1 g, NaCl 0.096 g, cysteine HCl 0.05 g, $CaCl_2$ 0.0096 g, $MgSO_4$ 0.0096 g, 1% (*w*/*v*) carboxymethylcellulose

Table 1 Source and cellulolytic activity of PPFM isolates

PPFM strains[a]	Source	Sample type	Cellulolytic activity
Methylobacterium extorquens	University of Washington, Seattle	–	+
M. organophilum	University of Washington, Seattle	–	−
M. komagatae	Microbiological and Analytical Group, Food Research Laboratories, Japan	–	++
M. gregans	Microbiological and Analytical Group, Food Research Laboratories, Japan	−	++++
MLW 1	Cooum River	Water	++
MLW 6	Cooum River	Water	−
MNW 60	Cooum River	Water	++++
MDW 80	Adyar River	Water	++
MDW 83	Adyar River	Water	++
MHW 109	Adyar River	Water	++++
MSF 28	Anamalai Hills	Soil	++
MSF 29	Anamalai Hills	Soil	+++
MSF 31	Gugal Shola	Soil	++
MSF 32	Killai Forest	Soil	−
MSF 34	Kodaikanal Forest	Soil	++++
MSF 39	Mangroove Forest	Soil	+++
MSF 40	Mangroove Forest	Soil	++++
MSF 44	Tiger Shola	Soil	−
MSF 46	Erode Thorn Forest	Soil	+
MSF 48	Pambar Shola	Soil	+

[a] PPFMs were isolated by serial dilution method on MMS medium containing 0.5% (*v/v*) methanol as carbon source

(pH 7.0) [14]. After incubating at 30±2 °C for 3 days, the plates were flooded with aqueous solution of 1% (*w/v*) Congo red for 15 min and destained with 1 M NaCl for 15 min. Formation of a clear zone of hydrolysis around the colonies was construed as positive.

Enzyme Production

Based on CMC plate assay, 13 selected PPFM strains were inoculated in CMC broth amended with 1% (*w/v*) CMC as carbon source. After 72 h, the cultures were centrifuged at 10,000 rpm and the supernatants were assayed for enzyme activity.

Enzyme Assay

The cellulase activity was estimated by both CMCase and FPase assay methods [15] using glucose as standard. Reducing sugars released were determined using dinitrosalicylic acid (DNS) [16].

CMCase Activity A 0.5-mL culture filtrate was added to 0.05 g CMC in 0.1 M phosphate buffer (pH 7) in a test tube and incubated at 50 °C for 30 min. Later, 3 mL

of DNS reagent was added to the test tube and incubated in a boiling water bath for 5 min. After cooling, the glucose released in the assay liquid was measured by optical density method at 540 nm.

FPase Activity A 0.5-mL culture filtrate was added to a test tube containing 0.05 g filter paper strip (1×6 cm) and 1 mL of 0.1 M phosphate buffer (pH 7). The test tubes were incubated at 50 °C for 30 min. After that, 3 mL of the DNS reagent was added to a test tube and incubated in a boiling water bath for 5 min. The test tubes were cooled and the glucose released was measured using a UV spectrometer at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 μM of glucose per milliliter per minute and was expressed as U mL^{-1} min^{-1} under standard assay conditions.

Enzyme Characterization

Effect of pH and Temperature on the Activity and Stability of the Enzyme

Five PPFM strains viz *M. gregans*, MNW 60, MHW 109, MSF 34, and MSF 40 showing significant cellulase activity were further characterized to determine the optimum pH and temperature for cellulolytic activity. The optimum pH for the enzyme activity was determined over a pH range of 2–10 at 50 °C using the following buffers: 50 mM citrate (pH 2–5), 50 mM phosphate (pH 6–7), and 50 mM glycine–NaOH (pH 8–10). The effect of temperature on the cellulase activity was determined by assaying the enzyme activity between 25 and 75 °C.

To determine pH stability, the enzyme was kept at room temperature for 1 h in the above mentioned buffers, and the residual cellulolytic activity was determined under standard assay conditions. Thermostability was measured by incubating the enzyme samples in 50 mM phosphate buffer (pH 7) for 1 h at 20 to 60 °C. The enzyme solution was chilled in an ice bath for 5 min and then assayed using the standard assay procedures at 55 °C. For all the assays, filter paper was used as the substrate.

SDS-PAGE and Zymogram Analysis

The crude enzyme preparation of MNW 60 was subjected to SDS-PAGE analysis [17]. Zymogram analysis was done with the modified method reported earlier [18, 19]. The culture supernatant along with the sample buffer was subjected to electrophoresis on SDS-PAGE (10%) containing 0.2% (*w*/*v*) CMC. After electrophoresis, the gel was soaked in solution A (sodium phosphate buffer, pH 7.2, containing 40% (*v*/*v*) isopropanol) for 1 h followed by solution B (sodium phosphate buffer, pH 7.2 containing 0.1% (*v*/*v*) Triton X-100) for 1 h at room temperature with gentle shaking to remove the SDS and to allow the proteins in the gel to renature. The gel was then washed four times in sodium phosphate buffer (pH 7.2) for 30 min at 4 °C. After incubation for 60 min at 50 °C, the gel was soaked in 0.1% (*w*/*v*) Congo red solution for 30 min at room temperature and washed with 1 M NaCl until the activity bands were observed as clear colorless areas.

Scanning Electron Microscopy

After 72 h of growth, the cells of MNW 60 grown in the medium amended with cellulose and without cellulose were harvested and subjected to SEM. The cells were washed in

cacodylate buffer (0.1 M, pH 7.2) containing 0.22 M sucrose and were fixed in 2% (*v*/*v*) glutaraldehyde in cacodylate buffer at 4 °C for 2 h. The suspension was again centrifuged and washed in cacodylate buffer. The samples were post-fixed in 1% (*v*/*v*) OsO_4 in cacodylate buffer at 4 °C for 2 h, dehydrated by increasing the concentrations of alcohol, dried in hexamethyldisilazane, and mounted on aluminum stubs. The samples were sputter-coated with gold/palladium and examined using a FEI quanta 200 SEM operating at an accelerating voltage of 10 keV.

Strain Characterization

The morphological, cultural, physiological, and biochemical characteristics of the cellulase producing pink pigmented facultative methylotropic strain, MNW 60, were determined by following Bergey's Manual of Systematic Bacteriology [20]. Gram stain reaction, catalase and oxidase activity, indole production, methyl red and Voges–Proskauer reactions, urease activity, nitrate reduction, and citrate production were carried out using standard procedures [21]. The ability of the PPFMs to grow in the presence of different substrates (ethanol, formaldehyde, acetic acid, methylamine, succinic acid, fumaric acid, formic acid, pyruvic acid, betaine, aspartic acid, glutamic acid, glycerol, glucose, fructose, lactose, maltose, mannose, ribose, rhamnose, xylose, and mannitol) other than methanol was studied using the MMS liquid medium with 0.5% for each amendment. Thermolabile substrates were filter-sterilized using a 0.22-μm membrane filter (Millipore) and added to the medium. For DNA extraction, the strain was grown in 100 mL of MMS medium supplemented with 0.5% (*v*/*v*) methanol for 72 h at 35 °C. The biomass was harvested by centrifugation at 10,000 rpm for 10 min and washed twice in sterile Tris–EDTA buffer (10:1 M ratio, pH 8.0). The extraction of DNA was carried out by the phenol chloroform method [22]. The presence of *mxaF* gene was detected using specific primers 1003f (5′-GCG GCA CCA ACT GGG GCT GGT-3′) and 1561r (5′-GGG CAG CAT GAA GGG CTC CC-3′) [23, 24]. For amplification of 16S ribosomal RNA (rRNA) gene, universal 27F and 1492R bacteria-specific primers were used [25]. Both the amplified DNA fragments were gel-purified using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and sequenced using an ABI377 sequencer (Applied Biosystems). The nucleotide sequence obtained was compared with GenBank data (http://www.ncbi.nlm.nih.gov/) using BlastN search [26]. Sequences were then aligned with the ClustalW software [27]. A phylogenetic tree was constructed by using the neighbor-joining DNA distance algorithm [28] with 1,000 bootstrap resamplings.

Statistical Analysis

All the experiments were carried out in triplicates, and the data were evaluated by analysis of variance and means were compared by applying Tukey's test using SPSS 14.0 statistical package. The level of significance was accepted as $P<0.05$.

Results and Discussion

The importance of cellulases has attracted much interest because of their diversity in biological, chemical, and industrial applications [29, 30]. Therefore, there is a continuous thrust to isolate efficient cellulose-producing microbes and optimize their ability to produce more. In this study, we report cellulase production by PPFMs isolated from the water

samples of Cooum and Adyar rivers in Chennai and soil samples of forests in Tamil Nadu along with four reference *Methylobacterium* strains (Table 1).

Screening for Cellulase-Producing PPFM

Among the 20 PPFM isolates, 13 strains showed clearing zones around the colonies in CMC agar plates flooded with Congo red solution indicating cellulase production (Table 1). In this study, detection of cellulolytic ability among the PPFMs isolated from different environments with good organic components supports the argument that the presence of decaying organic matter acts as a natural enrichment for cellulolytic microbes [31–33].

Enzyme Activity and Characterization

All the 13 cellulase-positive PPFM isolates were able to grow in the liquid medium with cellulose as a sole carbon source which induced cellulase production. The enzyme production reached maximum after 72 h, indicating a slow cellulose degradation process which could be related to catabolite repression by glucose [34]. Although many cellulolytic bacteria have been reported to produce cellulases that show both endo- and exoglucanase activities [35, 36], in this study, significantly higher FPase activity was observed than CMCase activity. *M. gregans*, MNW 60, MHW 109, MSF 34, and MSF 40 showed CMCase activity in the range of 0.23–0.54 UmL^{-1} and higher FPase activity between 0.49 and 0.84 UmL^{-1} than the other strains studied (Fig. 1). This could be related to the influence of substrate on the growth of cellulolytic organisms [37, 38], which seem to be regulated by induction repression mechanisms [39]. Observations on maximum FPase activities compared to CMase activity are similar to the studies expressed by Belghith et al. [40] and Emtiazi et al. [41].

The crude enzyme-hydrolyzed CMC in a broad range of pH (6.0–8.0) with optimum pH at 8.0 (Fig. 2a) corroborated well within the reported pH range of 3–9 for different microbial cellulases [42–47]. The strain MNW 60 showed higher enzyme activity of 0.93 U mL^{-1} at optimum pH. Cellulase has been reported to have high temperature optima of about 35–60 °C like other enzyme systems [16, 44, 48], with exceptions of thermostable cellulases showing optimum temperatures at 70 [43] and 100 °C [49]. In the present study,

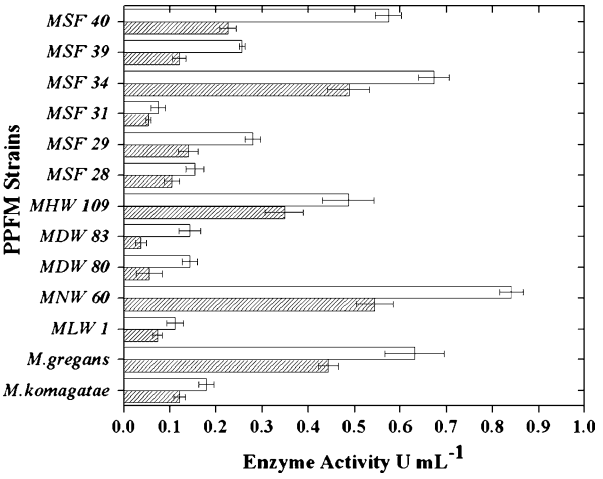


Fig. 1 CMCase (carboxymethyl-cellulase) and FPase (total cellulase) activity of PPFM strains after 7 days of culturing in the medium with 1% carboxymethyl cellulose (*w*/*v*) as a sole carbon source. The assays were performed at 50 °C in 0.1 M phosphate buffer pH 7 for 30 min (CMCase—*crossed bars*) and 1 h (FPase—*open bars*). *Each bar* represents the mean of three replicates of enzymatic activity (unit per milliliter). *Error bars* (±S.D.) are shown when larger than the symbol

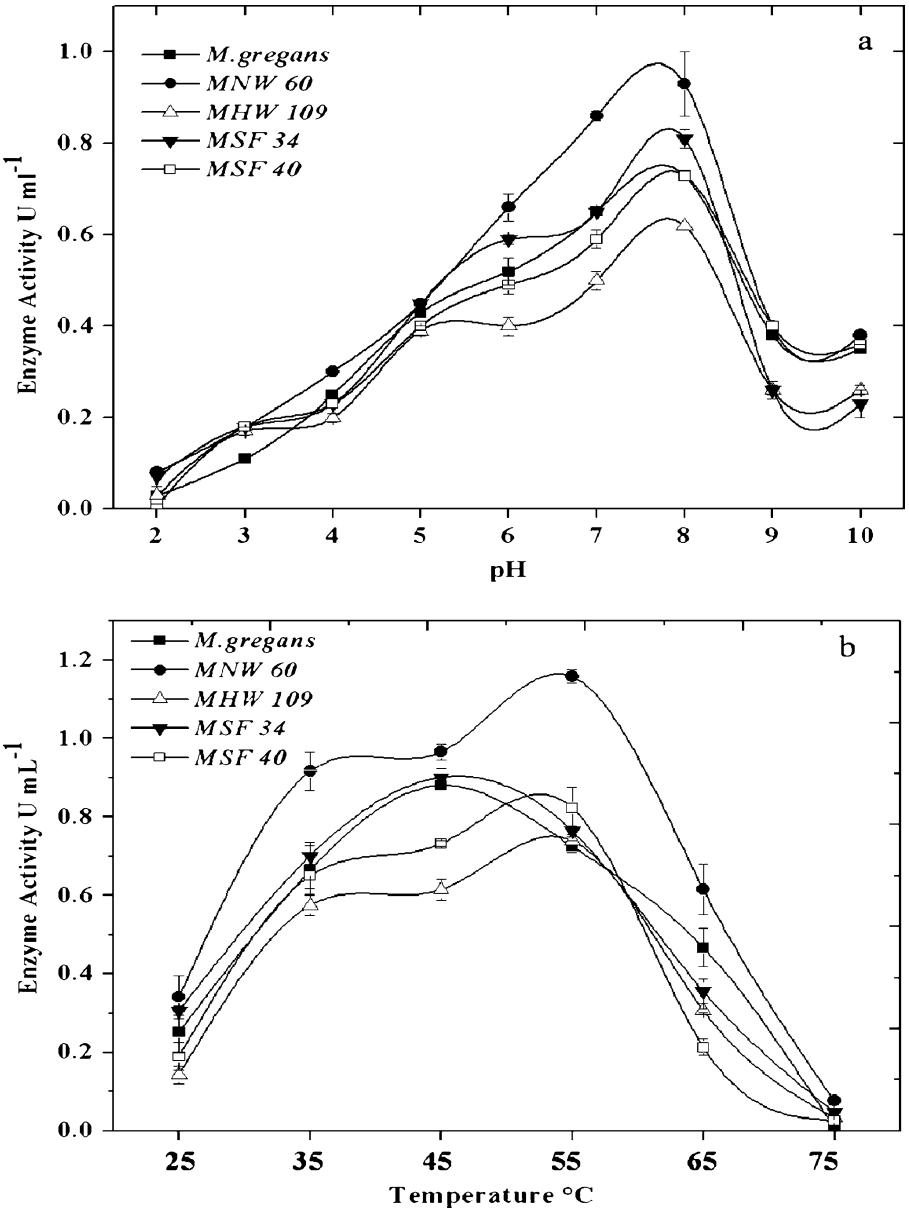


Fig. 2 Effect of pH (**a**) and temperature (**b**) on enzyme activity. **a** Enzyme activity: Assays were done under standard FPase assay conditions with the following buffer systems: 50 mM citrate buffer (pH 2–5), 50 mM phosphate buffer (pH 6–7), and 50 mM glycine–NaOH buffer (pH 8–10) at 50 °C for 1 h. **b** Enzyme activity: Cellulase activity was measured under standard FPase assay conditions using 50 mM glycine–NaOH buffer pH 8 at different temperatures (25–75 °C). Values are expressed as mean ± standard deviation of three independent readings

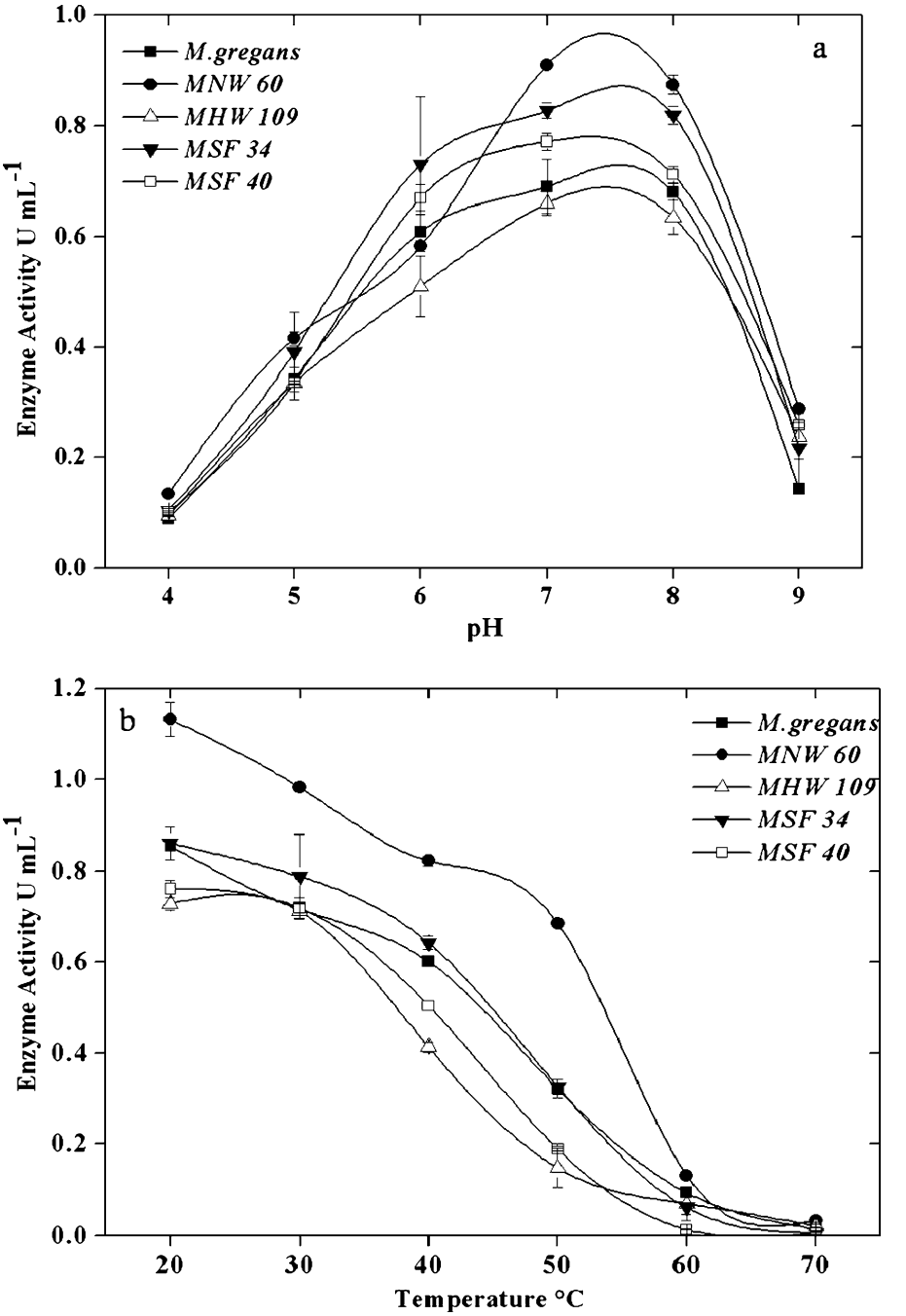


Fig. 3 Effect of pH (**a**) and temperature (**b**) on the enzyme stability. **a** Enzyme stability was determined by incubating the crude enzymes without substrate in the above mentioned buffers (pH 4–9) at room temperature for 1 h. The activity of the enzyme was determined under standard FPase assay using 50 mM glycine–NaOH buffer (pH 8). **b** Enzyme stability was determined after incubating the crude enzyme samples in 50 mM phosphate buffer (pH 7) up to 1 h at temperatures from 20 to 60 °C. Activity was measured using standard FPase assay conditions under optimal conditions (50 mM glycine–NaOH buffer, pH 8 at 55 °C for 1 h)

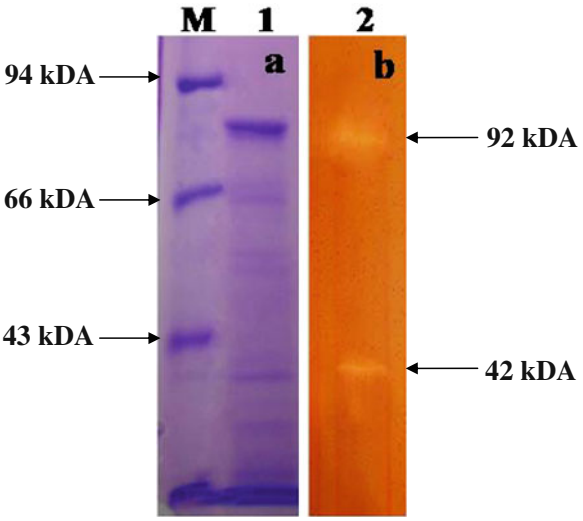


Fig. 4 SDS-PAGE (10%) (**a**) and zymogram analysis (**b**) of the crude cellulase enzyme of PPFM strain MNW 60. The proteins present in the crude supernatants of PPFM strain MNW 60 grown in media containing CMC as carbon source were used. *Lane M* molecular weight marker; *lane 1a* CBB-stained proteins present in the supernatant; *lane 2b* activity of crude cellulase enzyme. After electrophoresis in SDSpolyacrylamide gel containing 0.2% (*w*/*v*) CMC, the gel was stained with Congo red and destained with NaCl

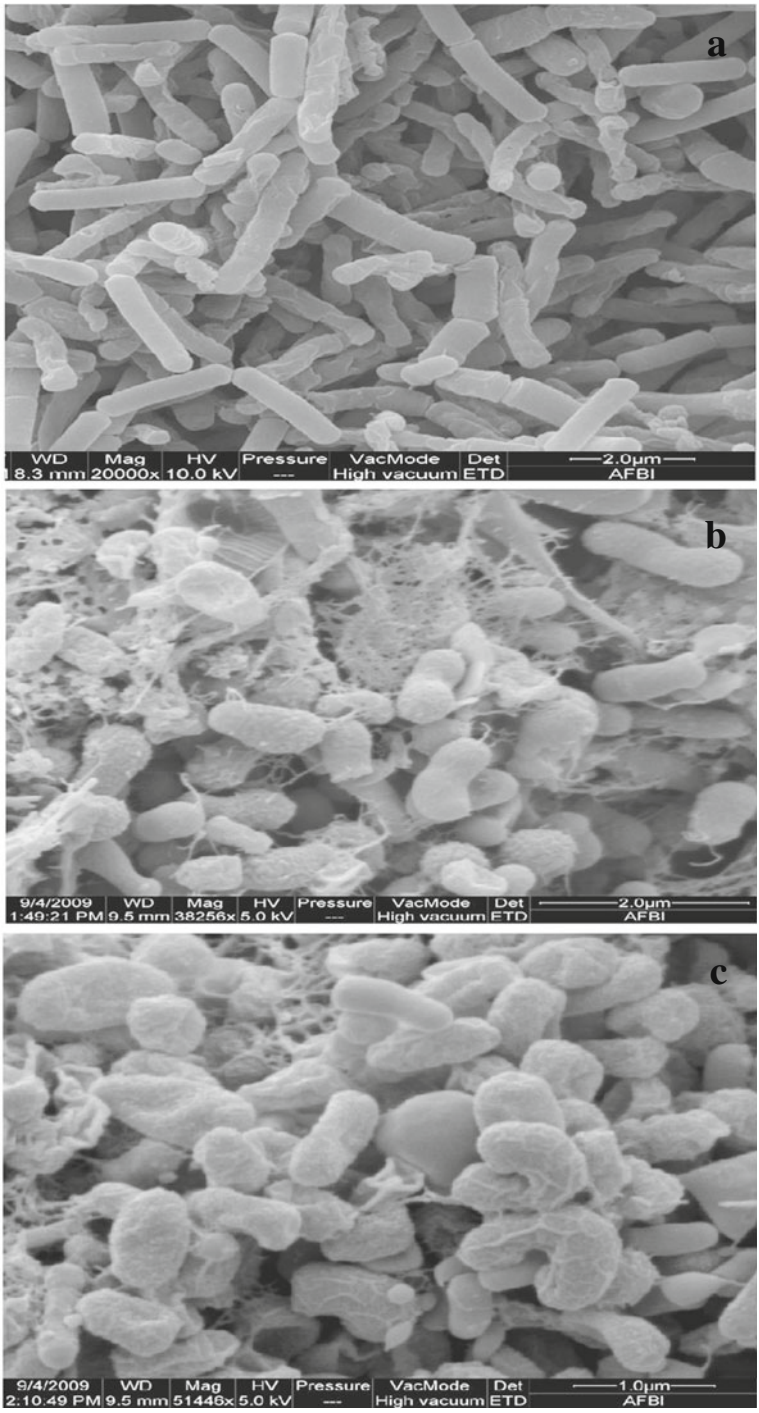
significant enzyme activity was observed at temperatures between 35 and 65 °C (Fig. 2b). Though maximum activity was observed at 45 and 55 °C in the strains *M. gregans*, MNW 60, MHW 109, MSF 34, and MSF 40 (0.74–1.12 $U mL^{-1}$), the abrupt reduction in cellulase activity above 55 °C could be related to the denaturation of thermal sensitive components of the cellulase system [50].

Observations on the stability of the enzyme over the pH range of 5.0 to 8.0 with maximum activity at pH 7.0 were in agreement with earlier reports [48, 51]. The activity was 50% at pH 5, and at the alkaline pH 9, it was reduced to 30% (Fig. 3a). Thermostability studies showed that the increase in temperature had a negative effect on the enzyme activity. The enzyme was found stable at 20 to 50 °C, and although higher enzyme activity was observed at 20 °C, it retained 60% of the activity at 40°C (Fig. 3b).

SDS-PAGE and Zymogram Analysis

The cellulase enzyme systems have been studied extensively in many organisms, and the molecular mass of native cellulase has been reported to be highly variable among different organisms and organized in different structural subunit arrangements. In this study, the SDS-PAGE analysis of crude enzyme preparation of MNW 60 showed the presence of at least nine protein bands with the molecular masses ranging from 92 to 14 kDa comprising three major and several minor protein bands (Fig. 4a, lane 1). The major protein bands corresponded to molecular masses viz 92, 90, and 42 kDa. The zymogram analysis of crude enzyme using CMC as substrates coupled with Congo red staining determined the enzyme activities of the catalytic components. Two major proteins bands (92 and 42 kDa) were observed with cellulase activities as colorless areas, indicating them as a major extracellular enzyme (Fig. 4b, lane 1). The molecular weights of most of those reported from bacteria were in the range between 5.6 and 114 kDa [52–57]. Multiple forms of endoglucanases in cellulolytic bacteria [58] have been reported to be a

Fig. 5 Scanning electron microscopy of PPFM strain MNW 60. **a** MNW 60 grown in medium without CMC. **b**, **c** MNW 60 grown in medium with CMC showing changes in the morphology of cells, adherence of cellulose-like particles, and presence of some ultrastructural protuberances on the cell surface. The cultures were grown at room temperature for 3 days


a
WD Mag HV Pressure VacMode Det 2.0µm
8.3 mm 20000x 10.0 kV --- High vacuum ETD AFBI
b
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c
9/4/2009 WD Mag HV Pressure VacMode Det 1.0µm
2:10:49 PM 9.5 mm 51446x 5.0 kV --- High vacuum ETD AFBI

consequence of multiple genes [59]. However, the dimeric form of the enzyme observed in this study could be related to the dissociation of the two subunits induced by SDS-PAGE [55].

SEM Analysis

Changes in the cell morphology of *Methylobacerium* sp. grown in the presence of tricalcium phosphate-containing medium was observed earlier (Jayashree et al. [60] communicated to Archives of Microbiology). MNW 60 cells grown in methanol mineral salts medium were seen as long rods with smooth surfaces, and the cells grown in the medium containing CMC showed extensive morphological changes with adherence of cellulose-like particles and presence of some ultrastructural protuberances on the surface of the cells (Fig. 5a–c). This structural change in the presence of cellulose coupled with enzyme production shows the hydrolyzing nature of the strain and leads to the conclusion that these changes could be induced by the substrate, CMC, due to enzyme production. The presence of protuberance-like structures, though not prominent, seen in the bacterial cell mass has been reported earlier for efficient cellulose degradation [61–64]. Several cellulolytic *Clostridia* sp. have also shown to produce an ultrastructural cellular protuberance which is formed during the efficient degradation of cellulose and is confirmed to be an exocellular protein complex of cellulolytic enzymes termed cellulosome [65]. Adhesion of cellulose-like particles observed in the surface of the bacterial cells grown in the presence of CMC has also been reported earlier in many bacteria [62, 66, 67]. According to Wilson et al. [68], adherence of bacteria to the cellulosic material is important for cellulase degradation. For these reasons it is believed that the rate and extent to which microorganisms adhere to cellulose is important for the digestibility of the material, and therefore the enzyme activity [67].

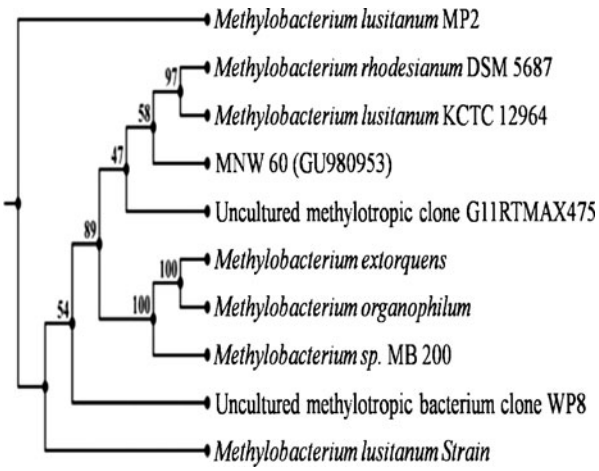

Fig. 6 Phylogenetic tree showing the relationships based on *mxaF* gene sequences between methylotrophic strain (MNW 60) and other related taxa. MNW 60 which had maximum cellulase activity showed the closest methanol dehydrogenase gene *mxaF* sequence relationships with the pink pigmented facultative methylotropic species of the genus *Methylobacterium* belonging to alpha proteobacteria with valid published names. Accession number is given within brackets and the tree was drawn using the neighbor-joining method

Strain Characterization

The strain MNW 60 was characterized as pink pigmented, Gram-negative, aerobic, and motile rods possessing sudanophilic granules. The strain was catalase and oxidase positive. It utilized nitrate, citrate, and urease and responded negatively to indole, methyl red, and Voges–Proskauer tests, indicating that the strain does not produce indole and strong acids which corroborates with earlier reports [69]. The isolate showed a wide range of substrate utilization patterns by exhibiting growth in the presence of ethanol, formaldehyde, glycerol, glutamate, betaine, citrate, acetate, aspartate, succinate, formate, propionic acid, malic acid, fumaric acid, fructose, lactose, maltose, mannose, ribose, rhamnose, xylose, and mannitol. The above characteristics of strain MNW 60 are consistent with its assignment to the genus *Methylobacterium* [69–71]. Further, the methylotrophy of the PPFM strain MNW 60 was confirmed by the presence of the *mxaf* gene which had the highest homology (99%) with the previously published *mxaF* sequence encoding the methonal dehydrogenase enzyme alpha subunit of *Methylobacterium lusitanum* strain MP2 [72]. The taxonomic position of the *mxaF* gene, a highly conserved locus and indicator of the presence of methanol-utilizing organisms in a natural habitat [23], is shown in the phylogenetic tree (Fig. 6). The *mxaF* gene sequence was deposited in the NCBI GenBank with accession number GU980953. The 1,449 bp of 16S rRNA gene sequence of strain MNW 60 submitted to NCBI GenBank (accession no. HQ 665015) had the highest homology of 97% with a previously published sequence of *M. lusitanum* strain MP2 (Fig. 7). Though the results of single carbon source assimilation and 99% similarity in *mxaF* gene sequence assign the strain MNW 60 under *Methylobacterium* sp., considering the 97% sequence similarity of 16S rRNA, it could be claimed as a new taxon belonging to the genus *Methylobacterium* under family Methylobacteriaceae [71].

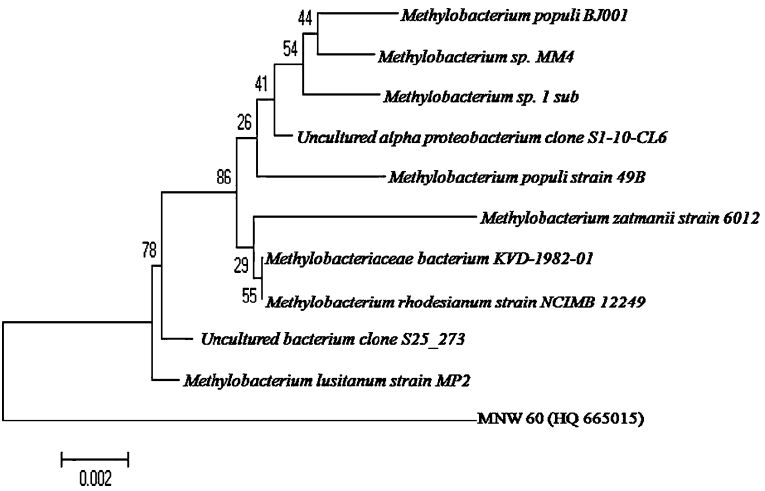

Fig. 7 Phylogenetic tree showing the relationships based on 16S rRNA gene sequences between PPFM strain (MNW 60) and other related taxa. 16S rDNA sequence alignment, corresponding NCBI gene accession number, and the phylogenetic tree depicting the relatedness of PPFM strain MNW 60 with the reported *Methylobacterium* sp. belonging to alpha proteobacteria with valid published names. Accession number is given within brackets and the tree was drawn using the neighbor-joining method with 1,000 bootstrap resamplings

Conclusion

The current study establishes a detailed report on the cellulase production by PPFM for the first time. A PPFM strain MNW 60 exhibiting higher cellulase activity (1.15 $U mL^{-1}$) at pH 8 and at 55 °C was found to be related to *Methylobacterium* sp. Further investigation on growth, culture conditions, strain improvement for cellulase production, purification, and characterization of the cellulose enzyme would add value for its use in large-scale application. Detection of genes responsible for cellulase production [73] in different *Methylobacterium* sp. would help explore their functional aspects. The results of this study also indicate that the Coum and Adyar rivers in Chennai and various forests in Tamil Nadu, India are rich sources of many pink pigmented facultative methylotropic bacteria which in turn could be a source of various enzymes, and further studies are recommended on these sites including study of microbial biodiversity and the associated biotechnological potential of the isolated strains.

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