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RESEARCH ARTICLE

Hydrolytic enzymes production by thermotolerant *Bacillus altitudinis* IARI-MB-9 and *Gulbenkiania mobilis* IARI-MB-18 isolated from Manikaran hot springs

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Abstract

Thermostable bacterial enzymes play a major role in hydrolysis of lignocellulosic compounds to fermentable sugars at high temperatures. A total 154 bacteria were isolated from Manikaran hot springs using standard serial dilution method. Among 154, 27 bacterial strains could grow at temperatures >70 °C. The thermotolerant bacteria were screened for hydrolytic enzyme activities at high temperatures (50-70 °C). On the basis of stability and efficient activity of hydrolytic enzymes, two bacterial strains IARI-MB-9 and IARI-MB-18 were selected for present study. Production of hydrolytic enzymes under submerged and solid state fermentation condition was done using paddy straw as sole carbon source. 16S rRNA gene sequencing and phylogenetic analysis revealed that these bacterial strains were belonged to Firmicutes and Proteobacteria. The closest phylogenetic neighbours according to the 16S rRNA gene sequence data for the two isolates IARI-MB-9 and IARI-MB-18 were *Bacillus altitudinis* and *Gulbenkiania mobilis* respectively. *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) were found to be the efficient cellulase producers 32.8 and 37.0 IU $ml^{-1}g^{-1}$ under submerged and 32.5 and 35.5 IU $ml^{-1}g^{-1}$ under solid state fermentation condition at 60 ºC respectively. To our knowledge, this is the first report for the presence of *Bacillus altitudinis* and *Gulbenkiania mobilis* in thermal spring with multifarious hydrolytic enzymes production. Such thermotolerant bacterial strains have potential to be used to develop as consortia for bioconversion of lignocellulosic residue to fermentable sugars preferably at high temperature.

Introduction

Hydrolytic enzymes are widespread in nature, microbes serve as an ideal source of these enzymes because of their rapid growth, the limited space required for their cultivation. Thermophilic microorganisms unequivocally represent a valuable source of highly thermostable hydrolytic enzymes, with numerous advantages towards biotechnological applications due to their overall inherent stability and high reaction rates at elevated temperatures (Vieille and Zeikus 2001; Turner et al. 2007). One of the natural habitats of the thermophilic bacteria is the hot springs where the temperature of the water is between 70 to 100 °C. Extremophiles inhabiting hot springs are considered to be the closest living descendants of the earliest life forms on Earth. Therefore, these springs provide insights into the origin and evolution of life. In addition, thermophiles and hyperthermophiles produce a variety of hydrolytic enzymes such as cellulase, xylanase and other biomolecules, which are of industrial interest (Daniel 2013).

Microbial cellulose and xylanase are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Cellulose is the most abundant and renewable biological carbon resource on earth. In nature, cellulase from bacteria and fungi hydrolyze crystalline cellulose into oligosaccharides,

which are ultimately hydrolyzed into glucose by the synergistic action of at least three types of cellulolytic enzymes, namely, endo-1, 4-β-D-glucanase, cellobiohydrolases, and β-glucosidase (Zhang and Lynd 2004), while, hemicellulase mainly include xylan which is the second most abundant natural polysaccharide on earth. Xylanase refers to a class of enzymes that specifically degrade xylan into oligosaccharides and xylose (Collins et al. 2005). Hydrolysis of xylan is undoubtedly an important step toward proper utilization of abundantly available lignocellulosic material in nature. Xylan hydrolysis using enzymes such as xylanases provides a viable alternative to chemical hydrolysis as it is highly specific in nature apart from being an environment friendly process (Kuhad et al. 1997). Cellulases play important role in production of second generation biofuel from lignocellulosic biomass. Besides their use for biofuel production, cellulases are being used in textile industry for cotton softening and denim finishing and in detergent markets for colour care, cleaning and anti-redeposition in washing powders. Cellulases may be used in the pulp and paper industry together with hemicellulases to improve the drainage and runnability of the paper machines and to enhance the deinking of recycled fibres (Vyas and Lachke 2003; Pandey et al. 2013; Yadav et al. 2015c). Xylanase have also been widely used in energy, food, animal feed, medical, papermaking, and textile industries (Demain et al. 2005; Yadav et al. 2015c).

Application of enzymes in detergent, leather and paper industries demands identification of highly stable enzymes active at extreme temperature and pH. The search for thermophilic organisms is one of the means for obtaining enzymes with properties suitable for industrial applications. In the present study, thermotolerant have been isolated and screened for hydrolytic enzyme activities. Sequencing of 16S rRNA gene of novel and efficient enzymes producing bacteria was undertaken for identification. These hydrolytic enzymes producing bacteria could be utilized for different applications in agriculture, biotechnology and medicine.

Materials and methods

Sample collection and isolation of thermotolerant bacteria

Water and sediment samples were collected from Manikaran hot springs (32° 01' 60 N: 77° 20' 60 E), Himachal Pradesh, India. Forty five samples, nine from each sites were collected from Gurudwara kund (100 °C), Vishnu kund (90 °C), Shiv kund (85 °C), Ram kund (60 °C) and Sita kund (50 °C), in sterile polythene bags/bottles, labelled, transported on ice and stored at 4 °C until analyses. The temperature and pH of the hot water springs was recorded at sampling sites. Thermotolerant bacteria were isolated using standard serial dilution method using different growth medium as described earlier (Yadav et al. 2015e). All isolates were screened for tolerance to pH, temperatures and salt as described earlier by Yadav et al. (2015e). Thermotolerant bacterial isolates were preserved as slants at 4 °C and suspension in 25 % (v/v) glycerol stocks at -80 °C for further use.

Hydrolytic enzyme activities

All the thermotolerant bacteria were screened qualitatively for hydrolytic enzyme activities. All isolates were screened for the presence of cellulase and xylanase activity on Reese's minimal media (composition per liter: 2.0 g KH_2PO_4, 0.3 g $MgSO_4.7H_2O$, 0.3 g KNO_3, 1.4 g $(NH4)_2SO_4$, 0.3 g $CaCl_2$, 5.0 mg $FeSO_4.H_2O$, 1.4 mg $ZnSO_4.7H_2O$, 2.0 mg $CoCl_2$, 1.6 mg $MnSO_4.H_2O$, 20 agar) supplemented with 1 % caboxymethylcellulose (CMC) and 1 % xylanase respectively (Teather and Wood 1982). To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1 % Congo red for 15 min and washed with 1M NaCl. Thermotolerant isolates exhibited extracellular hydrolytic enzyme activities were further screened quantitatively using paddy straw as substrate under submerged (SmF) and solid state fermentation (SSF). All the experiments were carried out in triplicates with control. Submerged fermentation was carried out in 250 mL flask containing 100 mL Reese minimal medium (Reese 1956) with 1 % chopped paddy straw (area 6 cm^2). The suspension was autoclaved at 121 °C for 20 min. Two milliliter pure cultures were inoculated into suspension and incubated at 50, 60 and 70 °C for 15-30 days. Fermentation was carried out in an orbital shaker incubator (New Brunswick Scientific) under shaking conditions (120 rpm) for hydrolytic enzyme production. Solid state fermentations was carried out in 250 mL Erlenmeyer flask containing 15 g paddy straw per 5 mL of Reese minimal medium and incubated at 50, 60 and 70 °C. All the conditions were similar to submerged fermentation. Sampling was done using 5 g of fermented paddy straw suspended in 10 mL sodium citrate buffer (pH 4.8).

Enzyme extraction and activity assay

Crude enzyme was obtained by centrifugation of a 5 mL volume of fermentation broth at 10000 *g* for 15 min at 4 ^oC and supernatant as crude enzyme was used for enzyme assay. Hydrolytic enzymes (xylanase, carboxymethyl cellulase, cellobiose and FPase) activities were assayed using CMC and Whatman no 1 filter paper respectively as substrate described by Ghose (1987) and measuring the amount of reducing sugars (Miller 1959), whereas β-

glucosidase activity was assayed by measuring the amount of p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per minute under the assay conditions. All enzymatic activities were determined on different temperature and interval used for lignocellulosic hydrolysis. Protein content in supernatant was estimated by Lowry's method using bovine serum albumin as standard (Lowry et al. 1951).

Thermal stability of enzymes

The optimum temperature was determined ranging from 50 to 70 °C with 10 °C increments by conducting enzyme assays. The reducing sugars released from lignocelluloses during the reactions were measured by the DNS method (Miller 1959). The heat stability of the enzyme was studied by both submerged and solid state fermentation with paddy straw as supplemented substrate for different time interval at temperatures ranging from 50 to 70 °C. The residual activities were then measured by the DNS method (Miller 1959).

Molecular identification of bacterial isolates

Genomic DNA was extracted as described earlier by Verma et al. (2015a). Amplification of 16S rRNA gene was done by using the universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Yadav et al. 2015d). The amplification conditions were used as described earlier (Pandey et al. 2013). The PCR amplified 16S rRNA gene was purified by Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA). 16S rRNA gene sequencing and phylogenetic analysis was carried out as described earlier by Verma et al. (2015b). The partial 16S rRNA gene sequences of bacterial isolates were submitted to NCBI GenBank and assigned accession numbers JN411334 and JN411342.

Results and Discussion

Isolation, screening and characterization of the thermotolerant bacterial isolates

In present investigation 154 bacteria were isolated from thermal springs of Manikaran and 27 isolates found to exhibited multifarious hydrolytic enzymes production at high temperatures (>70 °C). Hot water springs represent extreme niches whose pristine quality is maintained over a period of time. The terrestrial hot springs that exist on Earth represent hot spots for unusual forms of life, genes and metabolites (Tobler and Benning 2011). In the last two decades, a number of researchers have investigated various facets of the bacterial abundances in hot springs of different parts of world (Meyer-Dombard et al. 2005; Pagaling et al. 2012; Kumar et al. 2014a; Kumar et al. 2014b; Yadav et al. 2015e). There are some reports of isolation of hemicelluloses degrading thermophiles from the hot springs in Bulgaria (Dimitrov et al. 1997) and Portugal (Bataillon et al. 1998). Manikaran thermal springs located in Himachal Pradesh, India is famous for its hot water springs where the temperature is near boiling. It was pertinent to isolate thermotolerant hemicellulolytic bacteria from these springs as they have earlier been reported to be a good source of thermotolerant amylolytic bacteria (Sodhi et al. 2005). Therefore, we explored the novel cellulase and hemicellulase degrading bacteria from thermal hot springs which led to a first time report of bacteria like *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) as hydrolytic enzyme producers from hot spring.

Microbes have been heavily exploited for their abilities to produce a wide variety of cellulase and hemicellulase for the hydrolysis of lignocellulosic materials (Sun and Cheng 2002). Cellulase and hemicellulase research has been concentrated mostly in fungi but there is increasing interest in hydrolytic enzyme production by bacteria due to their higher growth rate and thermostable properties. From the examination of the growth curve (Fig. 1), it is clear that the bacteria attained exponential growth before 30 h when incubated at 50-70 ºC. Based on the maximum identity score, sequences were selected and aligned using multiple alignment software Clustal W. Phylogenetic tree was constructed using MEGA 5.05 software (Fig. 2). Analysis of 16S rRNA gene sequence indicated that isolate IARI-MB-9 and IARI-MB-18 showed similarity with *Bacillus altitudinis* and *Gulbenkiania mobilis* respectively.

Hydrolytic enzymes production

Bacteria were screened for hydrolytic enzymes production in submerged and solid state fermentation at different temperatures 50, 60 and 70 °C for times interval of 15 and 30 days. In submerged fermentation, *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) were observed for maximum CMCase, xylanase and FPase activity after 15 days of incubation period while cellobiose showed best activity after 30 days of incubation. In solid state fermentation, maximum CMCase activity was showed after 15 days while above three enzymes showed

maximum production after 30 days of incubation (Fig. 3; Fig. 4). Time interval play very essential role for fermentation, microbial culture population range dependent on time duration.

Effect of fermentation

Bacillus altitudinis (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) applied for solid state (SS) and submerged (Sm) fermentation for *in vitro* production of hydrolytic enzymes. Both the isolates were able to degrade paddy straw in higher quantity which is responsible for maximum production of hydrolytic enzymes. *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) were found to be the maximum enzyme production of CMCase 32.8 and 37.0 IU ml^{-1} g^{-1} under submerged state fermentation condition (Fig. 3) and 32.5 and 35.7 IU ml^{-1} g^{-1} after 30 days under solid state fermentation condition respectively (Fig. 4).

Effect of temperature

Under submerged fermentation condition, *Bacillus altitudinis* (IARI-MB-9) was found to be the maximum enzyme produced at 70 °C for xylanase (25.6 IU ml^{-1} g^{-1}) and Fpase (13.3 IU ml^{-1} g^{-1}); at 60 °C for CMCase (32.8 IU $ml^{-1}g^{-1}$) and cellobiose (3.0 IU ml^{-1} g^{-1}) after 15 and 30 days respectively. Same type of pattern was recorded by *Gulbenkiania mobilis* (IARI-MB-18) (Fig. 3). Under solid state fermentation condition, *Bacillus altitudinis* (IARI-MB-9) produced maximum amount of CMCase (32.5 IU ml^{-1} g^{-1}) and cellobiose (4.4 IU ml^{-1} g^{-1}) at 60 °C; xylanase (21.6 IU ml^{-1} g^{-1}) and FPase (6.9 IU ml^{-1} g^{-1}) at 70 °C (Fig. 4). *Gulbenkiania mobilis* (IARI-MB-18) produced maximum amount of CMCase (32.5 IU ml^{-1} g^{-1}) and cellobiose (6.2 IU ml^{-1} g^{-1}) at 60 °C; xylanase (25.8 IU ml^{-1} g^{-1}) and FPase (8.8 IU ml^{-1} g^{-1}); at 50 °C (Fig. 4).

To our knowledge, this is the first report for the presence of *Bacillus altitudinis* and *Gulbenkiania mobilis* in thermal spring with multifarious thermostable hydrolytic enzymes production. These species have been earlier isolated from different habitat. For example *Bacillus altitudinis* is a species of bacteria first isolated from cryogenic tubes used for collecting air samples from high altitudes (Shivaji et al. 2006), and later from the gut of marine fish *Sardinella longiceps* (Esakkiraj et al. 2012), spring silt (Mao et al. 2013) and Rohtang Pass cold desert of NW Indian Himalayas (Yadav et al. 2015b). It is white coloured on nutrient agar. Growth occurs between 37 to >70 °C and at pH 65–9 and could tolerate up to 5 % NaCl. *Gulbenkiania mobilis* isolated from treated municipal wastewater (Vaz-Moreira et al. 2007), and later from the Vashist hot springs (Kumar et al. 2014a). It is white coloured and alkalitolerant bacterium that could tolerate 5 % NaCl and upto 70 °C.

The carbon source plays major role in the economics of xylanase and cellulase production. In order to replace the cost of the xylan, cost effective natural lignocellulosic substrates like paddy straw, wheat bran, sugarcane bagasse, corn cobs etc., are used for the production of cellulase and hemicellulase. A number of studies have been done on lignocellulosic wastes. It was investigated that the *Thermoascus aurantiacus* ATCC 204492 is able to produce a high level of thermostable xylanase when sugar cane bagasse is used as a substrate (Milagres et al. 2004). Enhanced production of xylanase is obtained from a local soil isolate *Trichoderma viride*, using various lignocellulosic substrates like maize straw, bajra straw, jowar straw, wheat straw, oat hay and barseem hay in submerged culture fermentation (Goyal et al. 2008). The production of extracellular xylanase, β-xylosidase and α-L-arabinofuranosidase by the mesophilic fungus *Penicillium janczewskii* under submerged cultivation was investigated with different carbon sources like sugarcane bagasse, oat bran, wheat bran, corncobs, rice straw, orange waste and cassava peel (Terrasan et al. 2010). Paddy straw is one of the most abundant lignocellulosic crop residues in India, with an annual availability of 112 million metric tons so finding of current study has great potential in generation of biofuel from lignocellulosic waste like paddy straw.

Cellulase producing bacterial strains of *Acidothermus cellulolyticus*, *Bacillus polymyxa*, *Cellulomonas fimi*, *Clostridium stercorarium*, *Pyrococcus furiosus*, *Rhodospirillum rubrum* and *Saccharophagus degradans* have been extensively reviewed (Singh and Kaur 2012; Pandey et al. 2013; Suman et al. 2015; Yadav et al. 2015c). Many bacterial strains like *Bacillus circulans*, *Bacillus amyloliquefaciens*, *Clostridium thermocellum*, *Thermobacillus xylanolyticus*, *Bacillus subtilis*, *Dictyoglomus thermophillus*, and *Streptomyces halstedi* are found to be sources of hemicellulase (Maki et al. 2009; Yadav et al. 2015c). Finding of this study suggested that the novel strains *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) from Manikaran hot spring prove more efficient in producing hydrolytic enzymes.

It has been reported that solid state fermentation is an attractive process to produce cellulase which is economical due to its lower capital investment and lower operation expenses. Production of cellulase by fungi in SSF using agricultural wastes has been reported (Jatinder et al. 2006; Dogaris et al. 2009). A new species of *Bacillus licheniformis* produced extracellular xylanase under submerged fermentation when wheat bran is used as carbon source (Chaturvedi et al. 2013). Extracellular cellulase and xylanase synthesis during biodegradation of microwave

treated rice straw by *Myceliopthora thermophila* SH1 fungal strain at 50 °C was reported (Matsakas et al. 2015). Xylanases are the endoactive enzymes which are generally produced in the medium containing xylan and also containing xylanase hydrolysate as the carbon source and attack the xylan chain in a random manner, causing a decrease in degree of polymerization of the substrates and liberating shorter oligomers, xylobiose and xylose. Cellulase free xylanase are of paramount significance in some of industries *viz.* paper and pulp industries to avoid hydrolysis of the cellulose fibres (Haltrich et al. 1996)

In conclusion, thermotolerant bacteria have attracted the attention of the scientific community due to their ability to produce thermostable hydrolytic enzymes. Microbial extracellular enzymes with optimal activity at high temperature provide opportunities to study the adaptation of life in thermal springs and the potential for biotechnological exploitation, besides those efficient in multiple plant growth promotion traits (Suman et al. 2015b; Verma et al. 2013; 2014; Yadav et al. 2015a). Diverse thermostable enzymes detected in this study may be used to serve various industrial, agricultural and medicinal purposes.

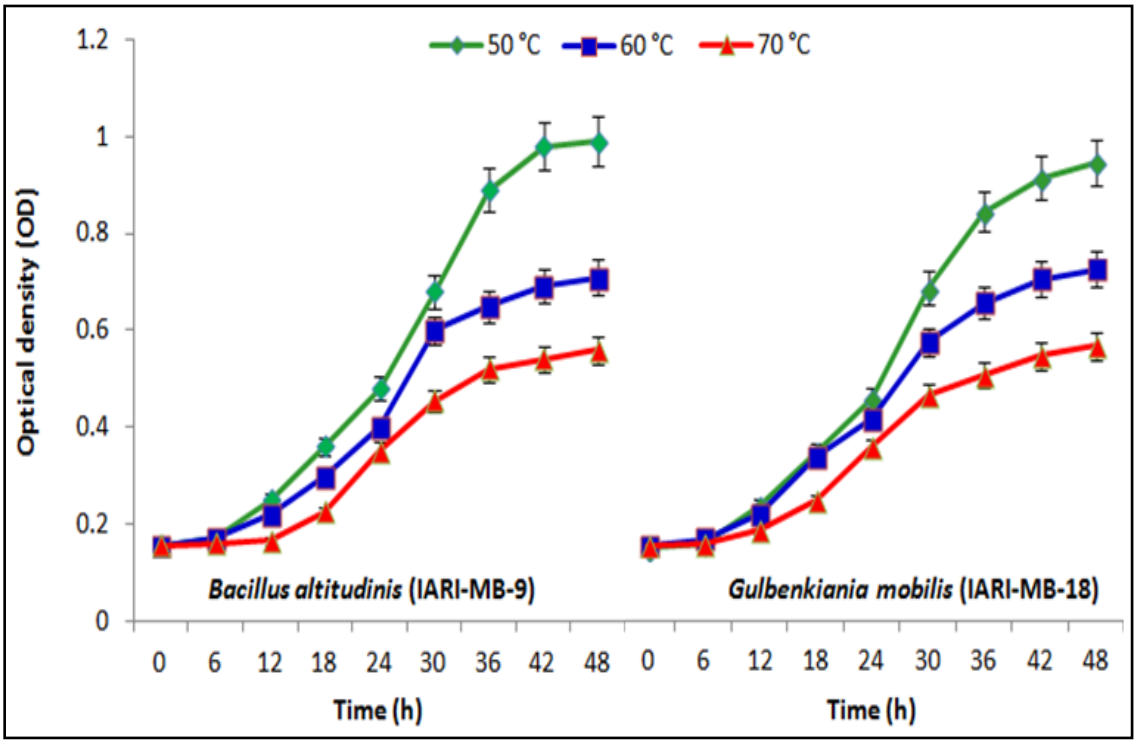

Fig. 1 Growth curve of *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) at three different incubation temperatures. Bar represents the standard deviation (SD).

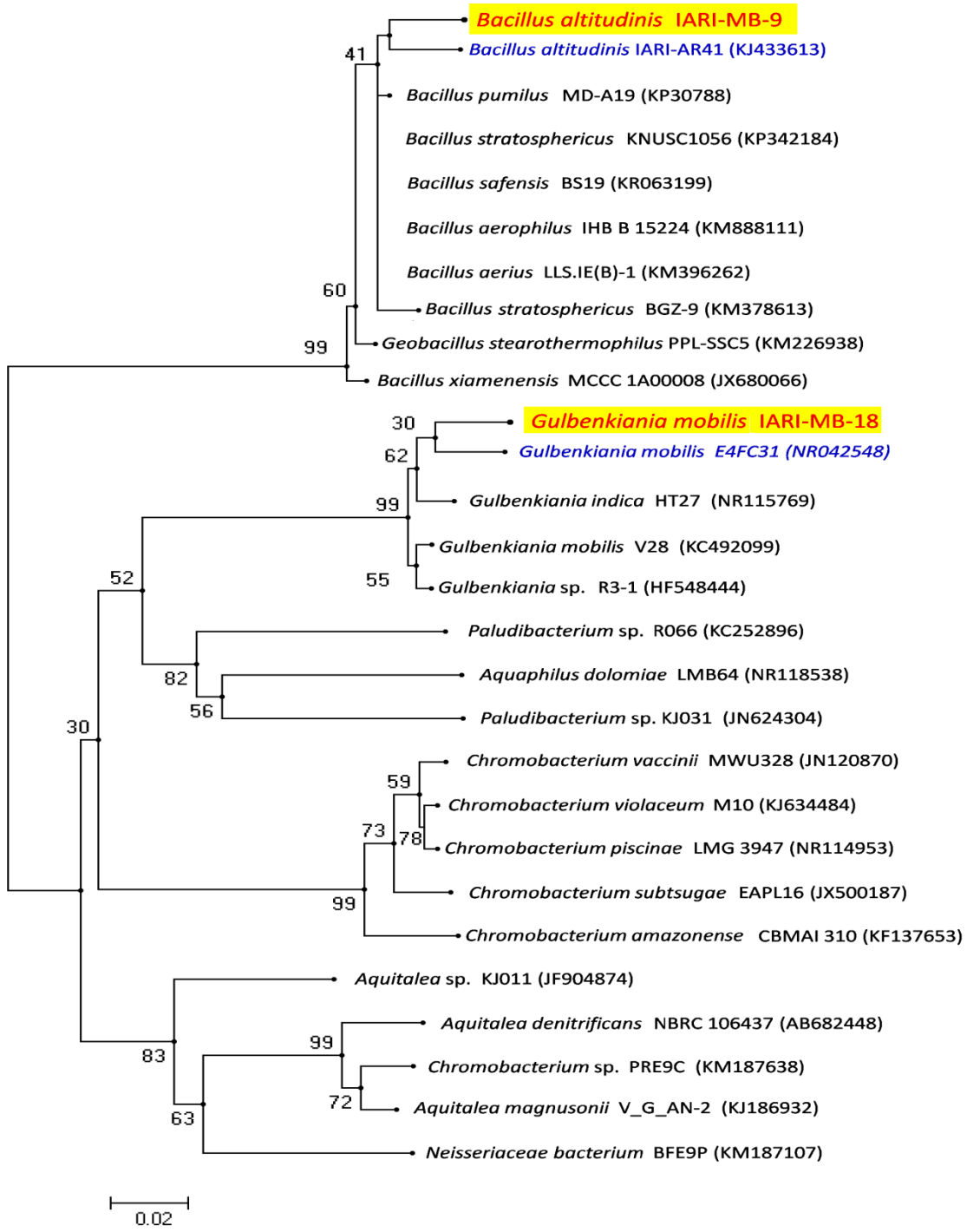

Fig. 2 Phylogenetic tree showing the relationship *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18) 16S rRNA gene sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTAL W program and trees were constructed using Neighbor Joining with algorithm using MEGA 4.02 software.

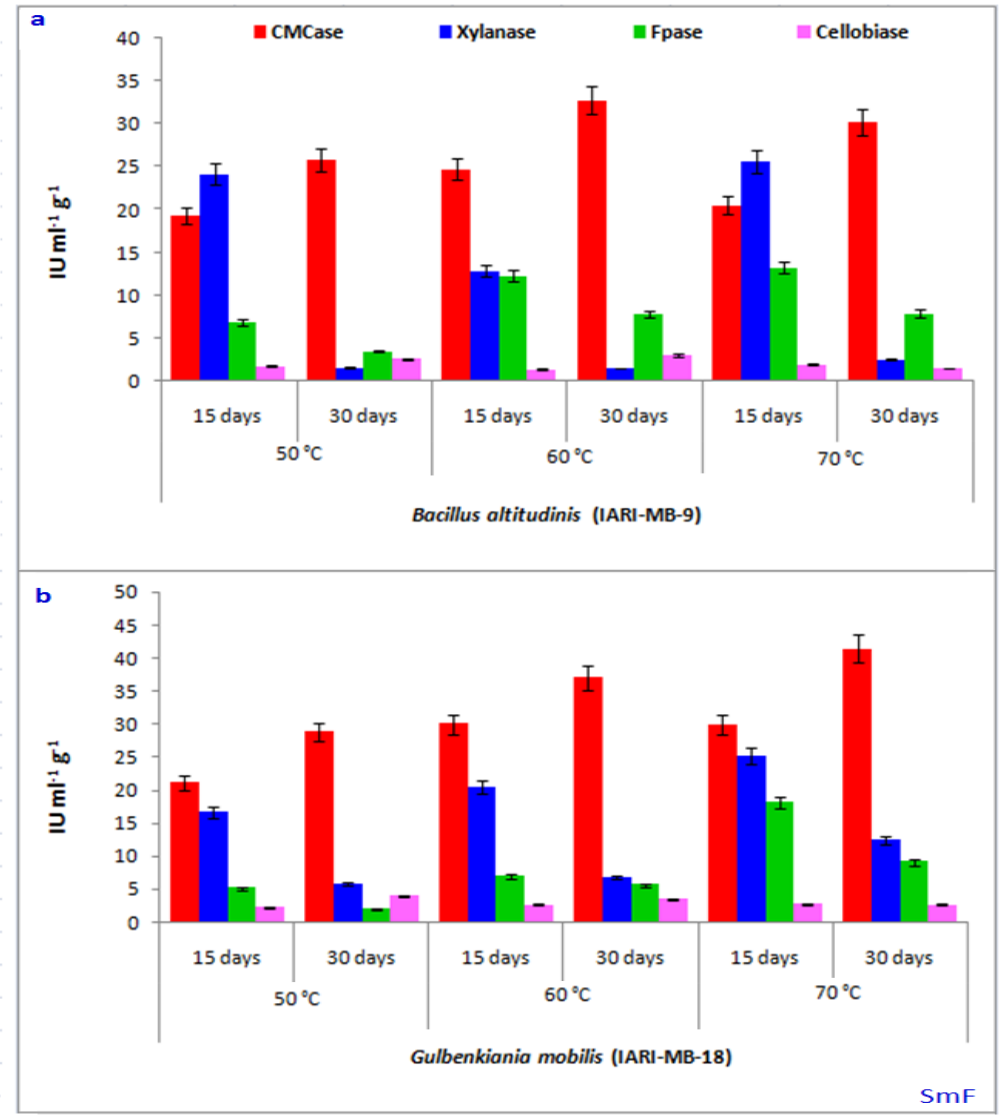

Fig. 3 Hydrolytic enzyme activity under submerged fermentation (SmF) of *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18)

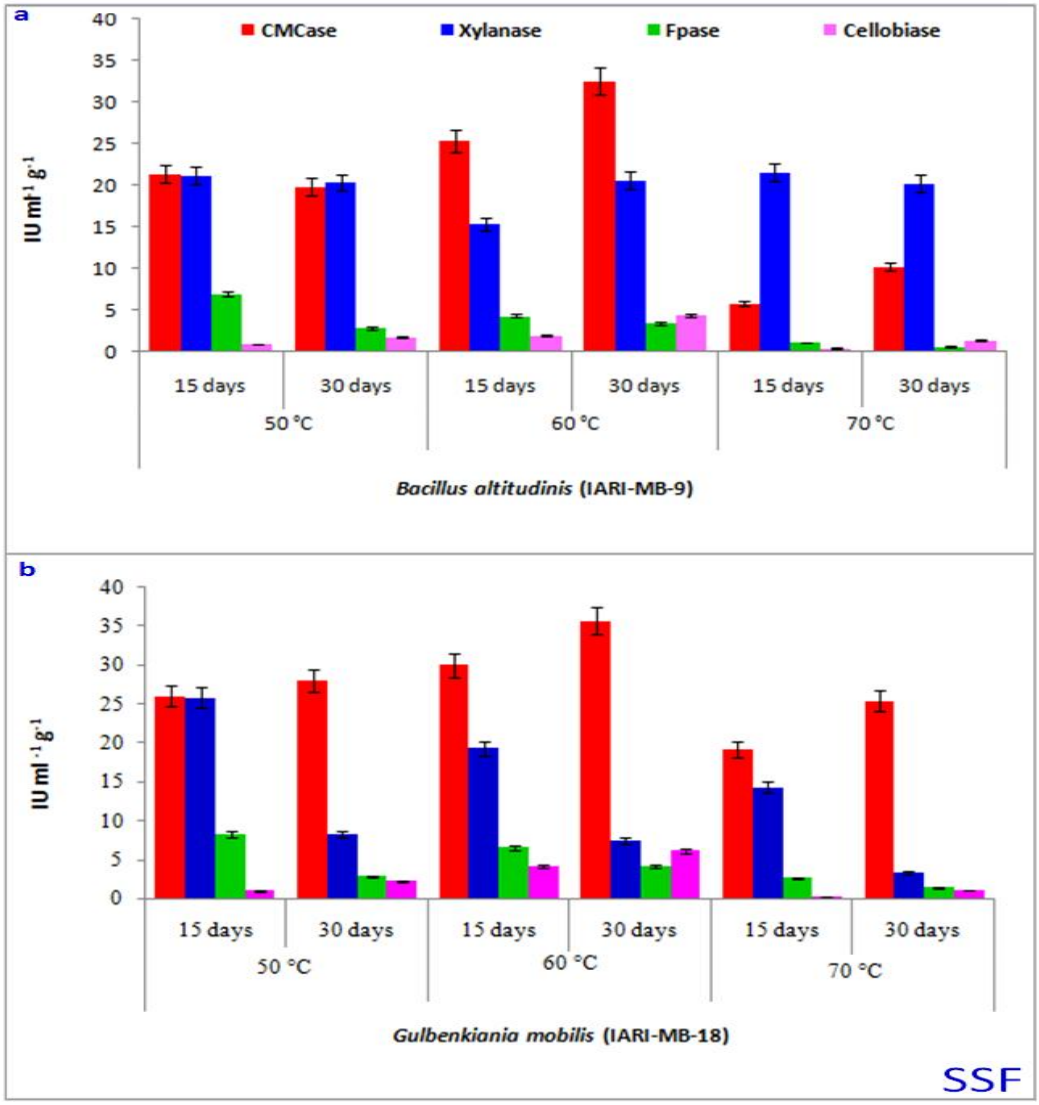

Fig. 4 Hydrolytic enzyme activity under solid state fermentation (SSF) of *Bacillus altitudinis* (IARI-MB-9) and *Gulbenkiania mobilis* (IARI-MB-18)

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Conflict of interest statement

There are no conflicts of interest.

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