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World Journal of Biology and Medical Sciences

Published by Society for Advancement of Science®

ISSN 2349-0063 (Online/Electronic)

Volume 3, Issue- 3, 106-121, July to September, 2016

Journal Impact Factor: 4.197



WJBMS 3/03/20/2016

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A Double Blind Peer Reviewed Journal / Refereed Journal

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RESEARCH PAPER

Received: 12/07/2016

Revised: 21/07/2016

Accepted: 22/07/2016

***Paenibacillus pabuli* strain ATSKP, an Unusual Aerobic Bacterium with Xylanase and Nitrogenase Activities Isolated from Soil Feeding Termites**

Pooja K. P. Jayaram and Chandra T. S.

Biochemistry Laboratory, Department of Chemistry, Indian Institute of Technology Madras, Chennai, India-600 036.

ABSTRACT

Seven bacterial strains were obtained in pure culture from soil feeding termites through enrichments with xylan as a carbon source in a nitrogen-free growth medium under aerobic conditions. One of the strains, designated ATSKP showed 98% 16S rRNA gene homology with *Paenibacillus pabuli*. The cells were Gram positive long rods with subterminal spores surrounded by a large slimy capsule of exopolysaccharide (EPS). Colonies appeared on nutrient agar but not on xylan agar. A variety of carbon sources like arabinose, galactose, xylose, carboxy methyl cellulose; starch, glycerol etc were utilized. Xylanase activity of 4 $\mu\text{mol}/\text{min}/\text{mg}$ protein and nitrogenase activity of 16 $\text{nmol}/\text{hr}/\text{mg}$ cell protein were observed under optimized assay conditions. ATSKP appears to be the only aerobic *Bacillus* that can fix nitrogen by using xylan as a sole carbon source and is thus of scientific interest. Phylogenetic and biochemical characterization indicates it to be a new strain if not a new species of *Paenibacillus*.

Key words: *Paenibacillus pabuli* strain ATSKP, nitrogen fixing, Xylanolytic and Exopolysaccharide (EPS).

INTRODUCTION

For a long while termite guts were recognized simply as anoxic fermentors. Recent studies have revealed that they are in fact axially and radially structured environments with physicochemically distinct microhabitats. The most important metabolic activities attributed to the gut microbiota are hydrolysis of cellulose and hemicelluloses, fermentation of the depolymerization products to short-chain fatty acids which are then reabsorbed by the host, and intestinal nitrogen cycling and dinitrogen fixation [Brune et al 2000, Fall et al. 2007].

Termites feed on wood and soil which lack combined nitrogen [Abe et al. 2000]. Therefore, there is a possibility of the presence of nitrogen fixers in termite gut for the maintenance of C/N ratio. Various nitrogen fixing bacteria have been isolated from the gut of earthworms and termites [Karsten et al. 1994]. Noda et al. [Noda et al. 2002] were successful in amplifying a nitrogen fixation gene, *nifH*, from mRNA of the gut microbial community of the termite *Coptotermes formosanus*. Presently molecular methods like gene expression studies, microsatellite genotyping, 16S sequencing, and genetic engineering are being used to provide knowledge about the ecology of termite's symbiotic gut flora.

Very few free living diazotrophs in soils degrade cellulose/lignocellulose (the most abundant biopolymers on earth) as energy sources for N₂ fixation [Halsall et al. 1986]. They have potential to function as ecofriendly bioreactors for single step bio-processing like conversion of ligno-cellulosic agricultural waste products with a wide C/N ratio to nitrogen rich bio-fertilizers by supplementing them with nitrogen sources. In 2007 there was a report on cellulase activities in nitrogen fixing *Paenibacillus* isolated from soil in nitrogen-free media [Emtiazi et al. 2007]. *Teredinibacter turnerae* gen. nov. sp. nov. [Distel et al. 2002], *Azospirillum* sp. [Halsall et al. 1986] and *Clostridium hungatei* sp. nov. [Leschine et al. 1988, Monserrate et al. 2001] were reported to be able to grow on xylan in the absence of a combined nitrogen source. The obligately anaerobic cellulolytic *Clostridium papyrosolvans* [Madden et al. 1982], *C. papyrosolvans* strain C7 [Leschine et al. 1983] and strain JW-2 [Warshaw et al. 1985] exhibited ammonium-repressible nitrogenase action. They fixed nitrogen only under microaerophilic or anaerobic conditions. However, except for *C. hungatei*, unequivocal proof for xylan utilization and xylanase activity is not available for the others.

Efforts were made here to enrich and isolate aerobic bacteria which could perform the dual function of nitrogen fixation and xylan degradation from soil feeding termites collected from mounds in IIT Madras campus. The biochemical and phylogenetic characterization and enzyme activities of *Paenibacillus pabuli* strain ATSKP obtained from these enrichments is described here.

MATERIALS AND METHODS

Chemicals

Birch wood xylan was obtained from Sigma, USA and other chemicals were of analytical grade.

Sources of inoculum

Soil feeding termites were collected from termite mounds in IIT Madras campus, washed, surface sterilized with 70 % ethanol, ground in 3 ml of autoclaved double distilled water using pestle and mortar, and used as inoculum.

Enrichment of the cultures

Fifty mg of this homogenized paste was inoculated into 10 ml mineral base culture broth [Murthy et al. 1989] containing birchwood xylan as the sole carbon source. The nitrogen-

free mineral base medium consisted of (in g/L): KH_2PO_4 : 0.45; K_2HPO_4 : 0.45; NaCl : 0.9; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.18; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.12; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.1; MnSO_4 : 0.5; CoCl_2 : 0.1; ZnSO_4 : 0.1, CuSO_4 : 0.01; $\text{AlK}(\text{SO}_4)_2$: 0.01; H_3BO_3 : 0.01; Na_2MoO_4 : 0.01. The pH of the medium after sterilization was 7.0. The culture broth was incubated on a shaker at 180 rpm and 30 °C.

Isolation and purification of the cultures

About 15 transfers of the enrichments were performed in xylan broth using 10^7 and 10^8 dilutions of inoculum each time. Since microscopic examination showed some bacilli to appear first, a rapid transfer at every 6 hrs of growth was done to separate these bacilli from being overgrown by other members. After two weeks the cultures were plated on nutrient, xylose, xylan or glucose agar (1, 1.2, 1.5, or 2 % agar). Single colonies appeared only on nutrient agar and were picked several times to bring the culture to purity.

Characterization of the cultures

Microscopic studies: The cultures were examined under phase contrast in a Leica DM LB microscope for motility, Gram's and spore stain.

For Scanning electron microscopy cells were pelleted, washed and subjected to gradual dehydration with ethanol (20 %, 50 %, 70 % and absolute ethanol), fixed in 8 % (v/v) gluteraldehyde, spread on a cover slip and then subjected to sputter coating with gold particles for observation under JEOL ASM 3500 SEM.

Transmission electron micrographs were obtained at 80 KV on a JEOL JEM 100SX TEM. Ultra thin sections (less than 100 nm) of the specimen were obtained by using Leica Ultracut R ultramicrotome and then stained with uranyl acetate and lead citrate.

Colony characteristics and phenotypic characterization: Manual of Methods for General Bacteriology [Gerhardt et al. 1981] was used for phenotypic characterization. Modified Hungate technique was used for growing under strict anaerobic conditions.

Phylogenetic analysis based on the 16S rRNA gene: The 16S rRNA was amplified and sequenced separately at University of Florida, USA and Agharkar Research Institute, India. In both the places MoBio Power Soil DNA extraction kit, USA was used for DNA isolation. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described by Rawlings [Rawlings et al. 1995].

Primer FDD2 5'GGATCCGTCGACAGAGTTTGATCITGGCTCAG3'34-mer.

Primer RPP2 5'CCAAGCTTCTAGACGGITACCTTGTTACCACTT3'33-mer.

The phylogenetic tree was constructed using top blast hits from NCBI alongwith top hits from EZTaxon and RDP (excluding uncultured hits). All the sequences were aligned using clustalx version 1.83. The alignment was manually edited using DAMBE software. The phylogenetic tree was constructed with neighbor-joining method using kimura 2-parameter as a model of nucleotide substitution with MEGA4 software. Bootstrap values are shown at each node. Scale bar represents nucleotide substitution per site.

Measurement of growth: The total cell protein was estimated by Lowry method [Lowry et al, 1951] using bovine serum albumin as standard.

Estimation of residual xylan: Residual xylan in the growth medium was estimated at regular intervals of growth by the phenol-sulfuric acid method [Dubois et al. 1956].

Xylanase: Xylanase activity was determined by measuring released reducing sugars using the dinitrosalicylic acid (DNS) method [Hutcheon et al, 2005]. The enzyme assay parameters were optimized with respect to pH (5.5 to 9.0), protein content in the cell free supernatant (0.1 to 0.6 ml), temperature (20-55 °C) and incubation time (5 to 50 min). Acetate (pH 5.5),

phosphate (pH 6.0, 6.5, 7.0, 7.5), tris (pH 8.0, 8.5) and glycine-NaOH (pH 9.0) buffers were used. All the experiments were done in duplicates and each sample analyzed thrice.

Nitrogenase: Nitrogenase assay was done by acetylene reduction test using gas chromatography [Postgate 1972]. An AIMIL NUCON 5764 GC (FID, Poropak N column) was used for the assay. 3, 5 or 10 ml of the culture broth was retrieved from the aerobic culture broth and transferred into 25 ml serum vials and closed with butyl rubber stoppers and crimped with aluminium seals. 5 ml commercially available pure acetylene gas was injected into the vial after removing the same amount of air from the head space. In another set of experiments the cultures were shaken at 180 rpm during incubation with acetylene. 1 ml of gas sample was retrieved from the bottles after incubation for 1, 12, 24 or 48 hrs using a gas tight syringe and injected into the GC column. The oven, injection port, detector temperatures were 60 °C, 90 °C and 110 °C respectively. Ethylene gas was prepared in the lab by adding 40 % 2-chloroethyl phosphonic acid into 5M KOH solution under air tight conditions and was used as standard. This method yields ethylene of 99 % purity.

Time course of nitrogenase activity of *Paenibacillus pabuli* strain ATSKP² was investigated with 0.5 % of glucose, carboxymethyl cellulose (CMC), xylose or birchwood xylan (BW xylan) in nitrogen-free medium in shake flasks.

The nitrogenase activity was compared with that of *Azotobacter*, *Azospirillum* and *Acetobacter diazotrophicus* grown in nitrogen-free mannitol broth. The influence of a combined nitrogen source on nitrogenase activity of the strain was determined by supplying the medium with 0.5 % (w/v) yeast extract, ammonium sulphate and sodium nitrate in separate setups.

RESULTS

A distinctive feature of all the cultures enriched from soil feeding termites was the secretion of viscous sticky EPS around the cells. Under all growth conditions, the enrichments produced sticky polymer secretions which held on strongly to the glass walls of the growth container. This polymer exhibited a very sticky consistency that made it difficult even to tweeze it with needles. The polymer secretion diminished as the enrichments underwent further transfers. On continuous microscopic observation it was found that darting capsulated bacilli appeared first. Within an interval of 8-12 hrs various other organisms were observed. Interestingly this secondary population included symmetrical green structures resembling diatoms and even filamentous fungi. However it was difficult to isolate all of them.

Tetracycline (100µg/ml) was initially added to the growth media with the intension to eliminate prokaryotes and enrich fungi. However, contrary to expectations the bacterial population continued to survive in the mixed enrichment cultures through all the transfers containing tetracycline. The purified bacillus culture however was sensitive to tetracycline, penicillin G, polymixin B and erythromycin and resistant to cephaloridine and gentamycin.

Seven bacterial isolates similar to each other in growth and morphology were obtained. The bacilli isolate which showed fastest growth and higher xylan utilization was chosen for further studies and was designated as strain ATSKP. The biochemical characteristics and enzyme activities of the strain were studied.

ATSKP cells were motile, long rods with sub terminal spores when observed under light microscope. Capsule formation is a constitutive property of strain ATSKP [25]. The true morphology of the cells was masked by the presence of this exopolysaccharide (EPS) capsule. SEM analysis showed cocco-bacillary shaped cells prior to removal of the capsule. However

on removal of the capsule long rod shaped bacteria of about 0.75 μm width and 2 μm length could be distinguished clearly (Fig. 1 a). Some of the cells appeared slightly curved. TEM micrographs showed free mature spores with seven apparent "spikes" (Fig. 1 b, c).

Inability to form colonies on xylan, xylose and glucose agar was a distinct trait of the strain, although it could grow in the broth with these sugars. However colonies of two sizes (small colonies of 1 mm diameter and bigger colonies of 3-4 mm diameter) appeared on nutrient agar plates after 24 hrs incubation at 30 °C. Nutrient agar was therefore used for picking the isolated colonies of strain ATSKP which were flat, cream coloured and glistening. They had undulate margin on thin agar (1.5 %) but entire edges on 2 % nutrient agar.

Based on the 16S rRNA gene sequence analysis, strain ATSKP was identified as belonging to genus *Paenibacillus* with 98 % 16S rRNA gene sequence similarity with *Paenibacillus pabuli* strain SW12. Fig. 2 shows the comparative sequence analysis of 16S rRNA gene from strain ATSKP and representative strains of the genus *Paenibacillus* from GenBank, using the neighbour-joining method. Table 1 gives the distinctive phenotypic characteristics of species phylogenetically related to *Paenibacillus pabuli* strain ATSKP.

Growth was supported only up to 2 % NaCl concentration. Many carbon sources like birchwood and oat spelt xylan, arabinose, galactose, glucose, xylose, carboxymethyl cellulose (CMC), starch and glycerol supported growth in the presence and absence of combined nitrogen source (Table 2). Mannitol and sucrose failed to support growth even in the presence of a combined nitrogen source.

Growth increased substantially when 1.0 % xylan was provided in place of 0.5% with yeast extract as nitrogen source. The cells reached stationary phase of growth by 120 hr with doubling time of 14 hrs and substrate utilization 72 % on 1.0% birchwood xylan medium (Fig. 3a). pH did not drop below 6.5 during growth. Increase in extracellular protein paralleled growth (Fig. 3b).

Xylanase activity was 4 $\mu\text{mol}/\text{min}/\text{mg}$ extracellular protein when grown in N- free medium with 0.5 % w/v birchwood xylan (Fig. 3b). The optimum conditions for xylanase assay were pH 6.0, incubation time 20 min and temperature 50 °C. Xylanase activity was induced only when grown on arabinose, galactose, glucose, xylan and xylose (Table 2). The activities were similar in the presence or absence of combined nitrogen source (0.5 % w/v yeast extract) (Table 2). The culture supernatant also showed endoglucanase, protease and amylase activity on different carbon sources in the absence of any combined nitrogen source (Table 2).

Nitrogenase activity was observed when the organism was grown on various carbon sources like glucose, xylan and xylose and a relatively low activity on CMC (0.5 % w/v) (Fig. 4). The highest activity was recorded at 72 hrs (Fig. 4). Nitrogenase activity of 16 nmol/hr/mg cell protein was obtained under optimized conditions of assay viz. 72 hrs of growth, incubation with 16.67 % v/v acetylene for 24 hrs under shaking at 180 rpm when grown on 0.5 % birchwood xylan (Fig.5a). The activity of nitrogenase of ATSKP was very low when the cultures were not shaken during incubation with acetylene (Fig. 5b). Nitrogenase activity was less than that of other standard nitrogen fixing organisms like *Azotobacter*, *Azospirillum* and *Acetobacter* grown on N-free mannitol medium (Fig 5 a,b).

The nitrogenase activity decreased on removal of the exopolysaccharide (Fig.4a). Nitrogenase was completely repressed in the presence of combined nitrogen sources like yeast extract, sodium nitrate and ammonium sulphate.

DISCUSSION

Even after a series of transfers, a highly diverse population was getting enriched every time probably due to cross feeding and nutritional synergism between the organisms adhering to the polymer. Several rapid transfers (every 6 hrs) in broth (after microscopic examination) had to be performed in order to finally obtain pure culture of the bacilli that appears at the early stage. This was a very tedious and exhaustive process. The susceptibility to many antibiotics on purification of the cultures could be attributed to the reduction in the amount of polymer around the cells.

ATSKP was deposited at two culture collection centres namely Microbial Type Culture Collection, Institute of Microbial Technology (MTCC), India and National collection of Industrial Micro-organisms (NCIM), National Chemical Laboratory, India with the accession numbers MTCC 8531 and NCIM 5291 respectively. The 16S rRNA gene sequence was submitted to GenBank with the accession number EU721728. Further chemotaxonomic studies have to be performed to confirm the exact phylogeny of ATSKP and to know if it is a new species of *Paenibacillus* or a new strain of *P. pabuli*.

Inability of the strain to grow under higher NaCl concentrations indicates that the strain is not halo-tolerant.

A possible explanation for the reduced nitrogenase activity when the cultures were not shaken could be that acetylene could not penetrate across the capsule in to the cells in the absence of shaking. Diazotrophs are known to secrete exopolymers for maintaining the anaerobic environment required for nitrogenase action [Sabra et al. 2006]. The reduction in the nitrogenase activity on removal of the exopolymer can be linked to this explanation. The fact that nitrogenase was not expressed when grown in the presence of a combined nitrogen source proves that strain ATSKP has an ammonium repressible nitrogenase activity.

The ability to grow with xylan as a sole carbon source by fixing nitrogen is a rare combination which makes strain ATSKP interesting and unusual. There are many *Paenibacilli* reported to fix nitrogen but these are not reported to utilize xylan [Elo et al 2001]. Strain ATSKP appears to be the only *Paenibacillus* with the dual capacity to degrade xylan and fix atmospheric nitrogen.

Among the genera other than *Paenibacilli*, the capacity to degrade hemicelluloses (major component xylan) degradation and fix atmospheric nitrogen has been reported in three other bacteria; *Azospirillum spp.* [Halsall et al. 1986], *Teredinibacter turnerae gen.nov.sp. nov.* [Distel et al.2002] and *Clostridium hungatei* [Monserate et al. 2001]. However these bacteria are reported to be either microaerophilic or strictly anaerobic and did not exhibit nitrogen fixing capabilities when grown with continuous shaking. ATSKP is not a strict anaerobe but is microaerophilic and fixes dinitrogen better under aerobic continuous shaking conditions.

Teredinibacter turnerae gen.nov.sp. nov. has been reported to grow on xylan but xylanase activity has not been reported. Same is the case with *Azospirillum spp.* *Clostridium hungatei* is reported to have xylanase activity of 14 U/mg protein. It appears that xylanolytic ability has not been unequivocally proved in the above three organisms viz *Teredinibacter*, *Azospirillum* and *C.hungatei* by substrate utilization measurements and xylanase assays. Commercially available xylan such as birch wood, oat spelt, barley etc are natural extracts from plant fibres and generally contain a fair amount of monosaccharides which can support the growth of a non-xylanolytic organism for a while. Therefore a direct measurement of the residual xylan and xylanase activity is required to confirm xylan utilization. Apart from *Clostridium hungatei* wherein xylanase activity of 14 U/mg protein was reported, a complete

study on the xylanase activity is lacking in *Teredinibacter* and *Azospirillum*. In *Azospirillum* spp. substrate utilization has been inferred only by showing the increase in CO₂ emission with growth [Halsall et al. 1986]. *Clostridium hungatei* differs from strain ATSKP by the fact that it is an obligate anaerobe and is cellulolytic. ATSKP on the other hand is not anaerobic and non-cellulolytic. In the present study a direct measurement of the residual substrate xylan has been employed to substantiate and prove that the ATSKP degrades xylan.

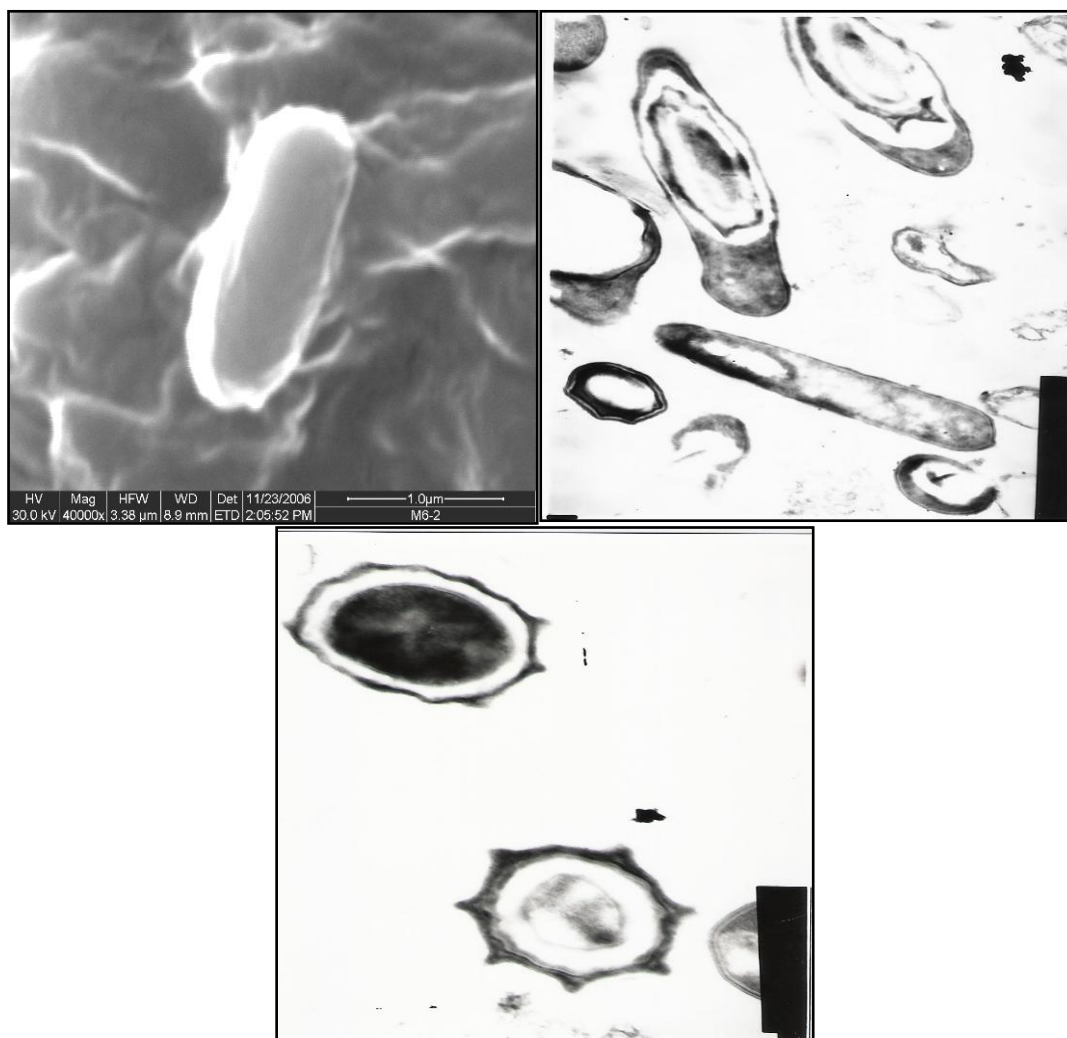


Figure 1. a) SEM image of *Paenibacillus pabuli* strain ATSKP after removing the extracellular polymer (Magnification 40,000x). b, c) TEM images of ultra thin sections of *Paenibacillus pabuli* strain ATSKP showing (b) different stages of sporulation. The exosporangium surrounding the maturing spore is visible (arrow). The developing spikes have started to become visible (arrow) (c) free mature spore with seven apparent “spikes” (arrow).

Teredinibacter turnerae gen.nov, grows on cellulose in the absence of combined nitrogen source. However, nitrogenase activity during growth on xylan is not specified. *Azospirillum* spp. is reported to show a nitrogenase activity of 2.4 nmols/hr/mg cell protein during growth on xylan. *Clostridium hungatei* shows very high nitrogenase activity (700 nmols/hr/mg cell protein) when compared to ATSKP (16 nmols/hr/mg cell protein).

Teredinibacter turnerae gen.nov.sp. nov. could not fix nitrogen when the culture was aerated [Distel et al. 2002]. We attribute the ability of *Paenibacillus pabuli* strain ATSKP [2] to fix nitrogen under continuous shaking to the presence of capsular exopolysaccharide which probably provides protection against oxygen.

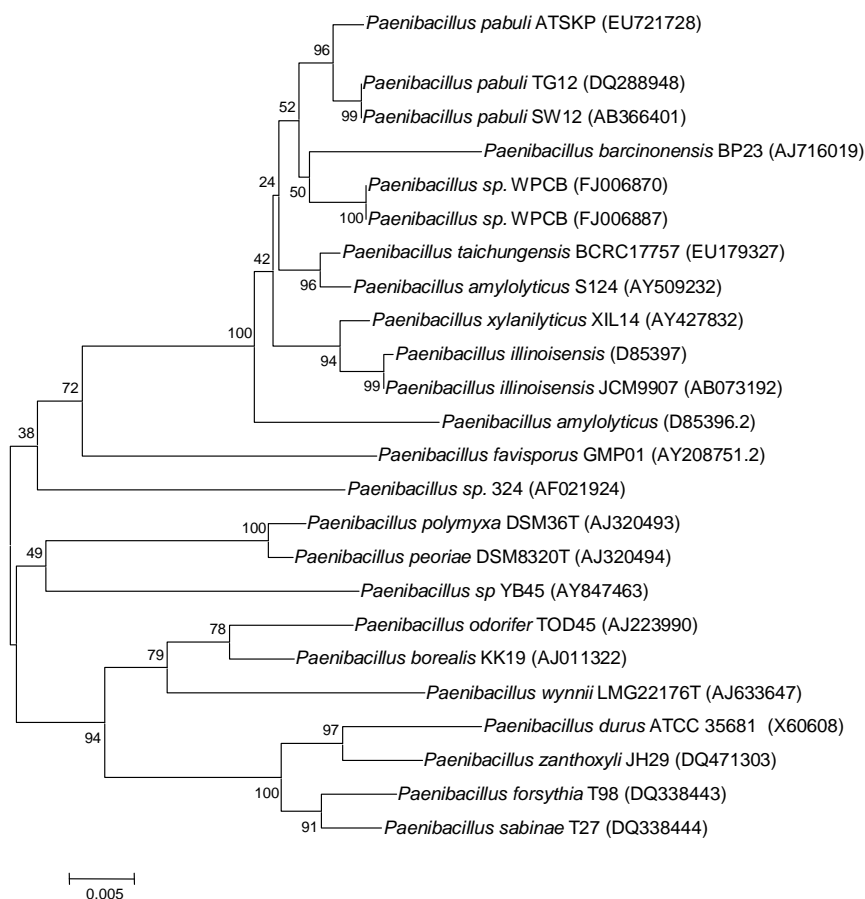


Figure 2. Phylogenetic relationship of the isolated strain ATSKP with top hits from NCBI, RDP and EZTaxon databases. The tree was constructed using Neighbor-Joining method with kimura 2-parameter as a model of nucleotide substitution. Sequences in this study are shown in bold. Names are those of the species followed by strain names. Genbank accession numbers for each species are shown in bracket after each species name. Scale bar represents nucleotide substitutions per site.

The above bacteria required the presence of vitamins and growth factors. ATSKP could be grown and fixed nitrogen aerobically on a synthetic medium with xylan as the sole carbon source in complete absence of a combined nitrogen source or growth factors.

To our knowledge, the termite gut isolate *Paenibacillus pabuli* strain ATSKP is the only Gram positive *Paenibacilli* reported to date that can fix atmospheric nitrogen and grow with xylan as a sole carbon source in the complete absence of any growth factor, and the only bacterium that combines these capabilities to grow under strict aerobic conditions. Nitrogen fixation reaction being expensive in terms of energy equivalents, the organism's metabolism and energetics in simultaneously performing these two functions provides an interesting area for further research. *Paenibacillus* strain ATSKP with its dual ability to fix atmospheric

nitrogen and degrade hemicelluloses can find potential application in the recycling of recalcitrant organic wastes with high C/N ratio. The organism is therefore of considerable interest for both basic research and applications in aerobic composting.

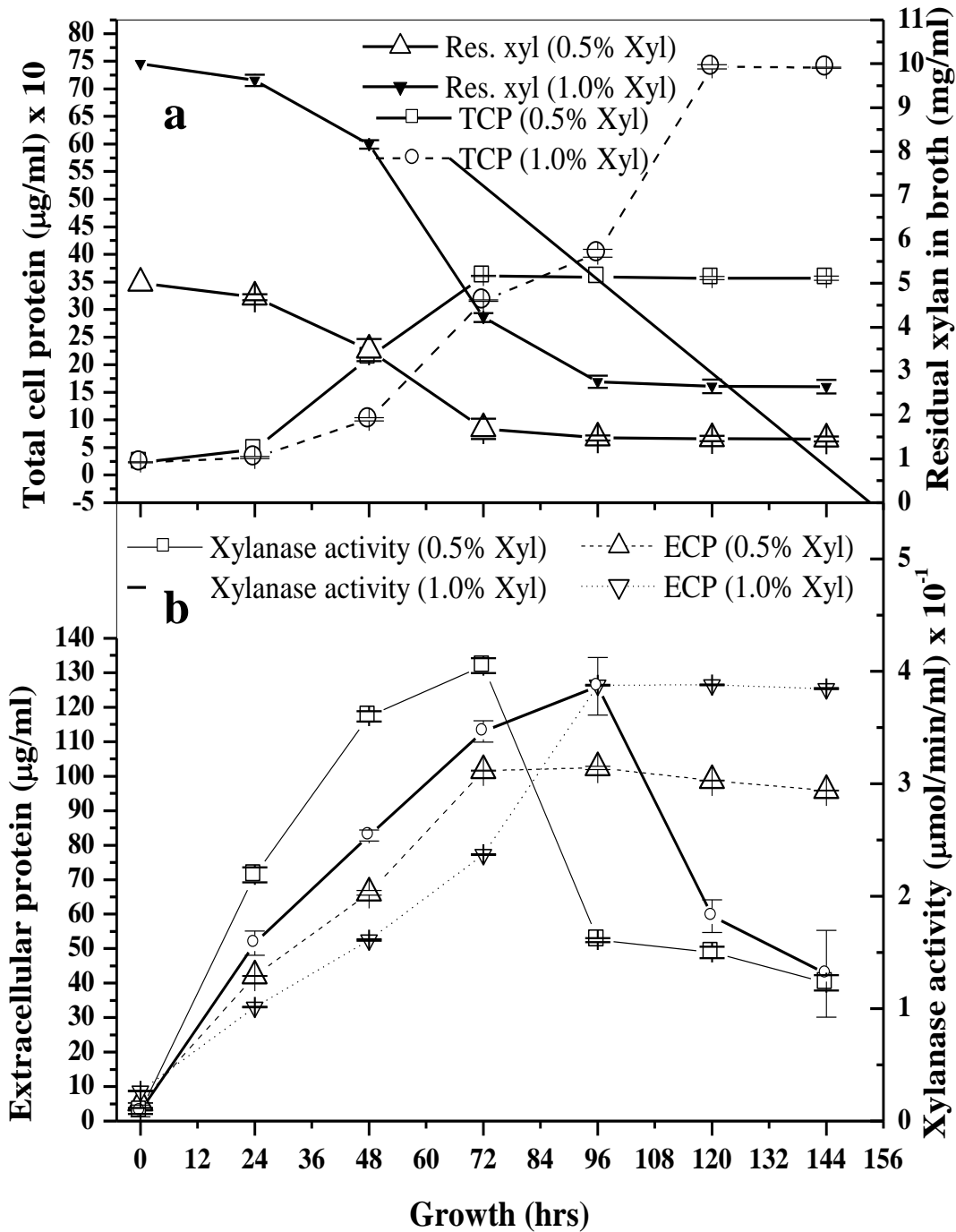


Figure 3. Growth and xylanase activity of *Paenibacillus* strain ATSKP under optimized conditions. (a) Total cell protein (TCP) and birchwood xylan (Xyl) utilization (b) xylanase activity and extracellular protein (ECP) in N-free medium.

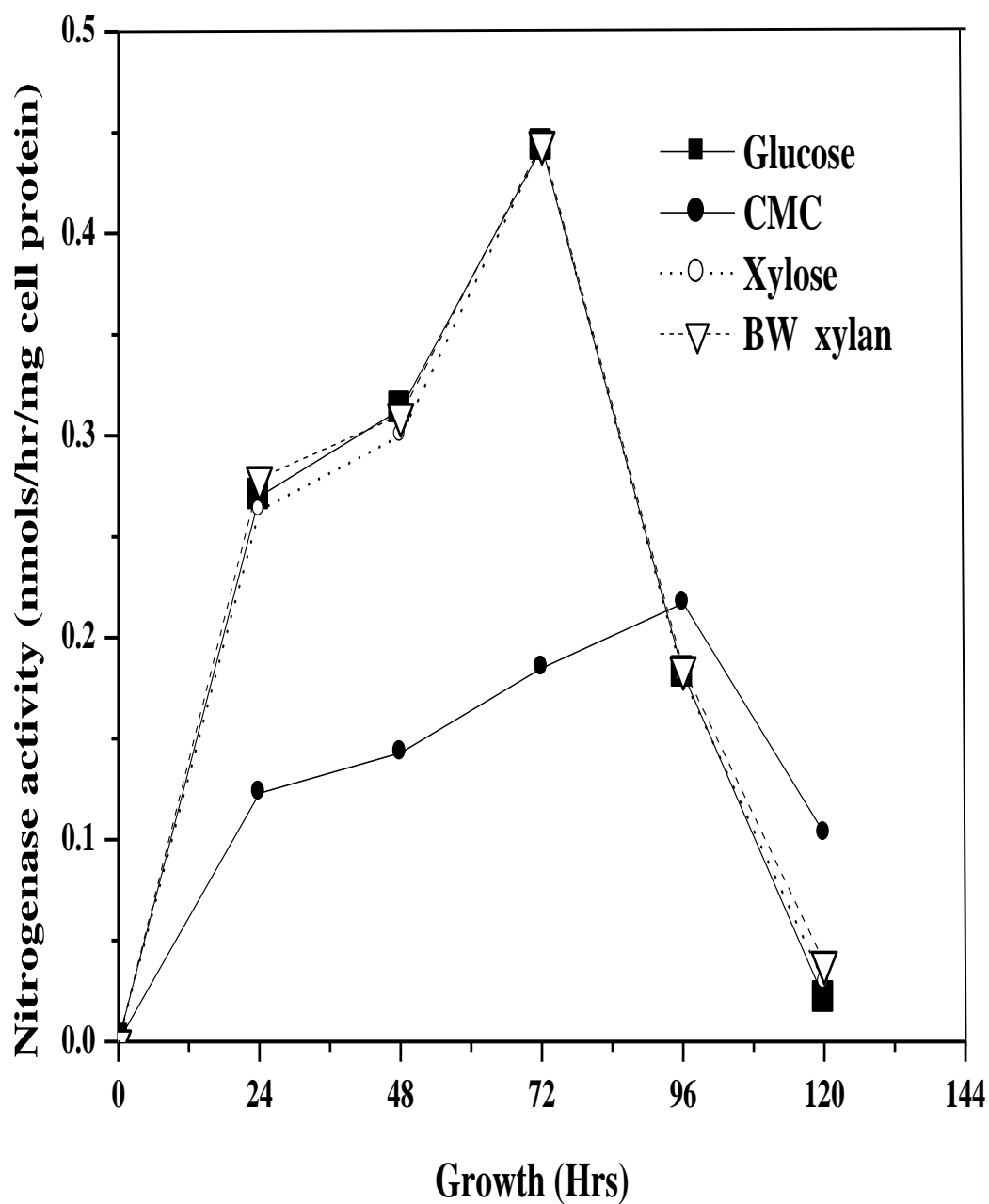


Figure 4. Time course of nitrogenase activity when grown on different carbon sources in N-free medium. Carbon sources were 0.5 % glucose, carboxymethyl cellulose (CMC), xylose or birchwood xylan (BW xylan).

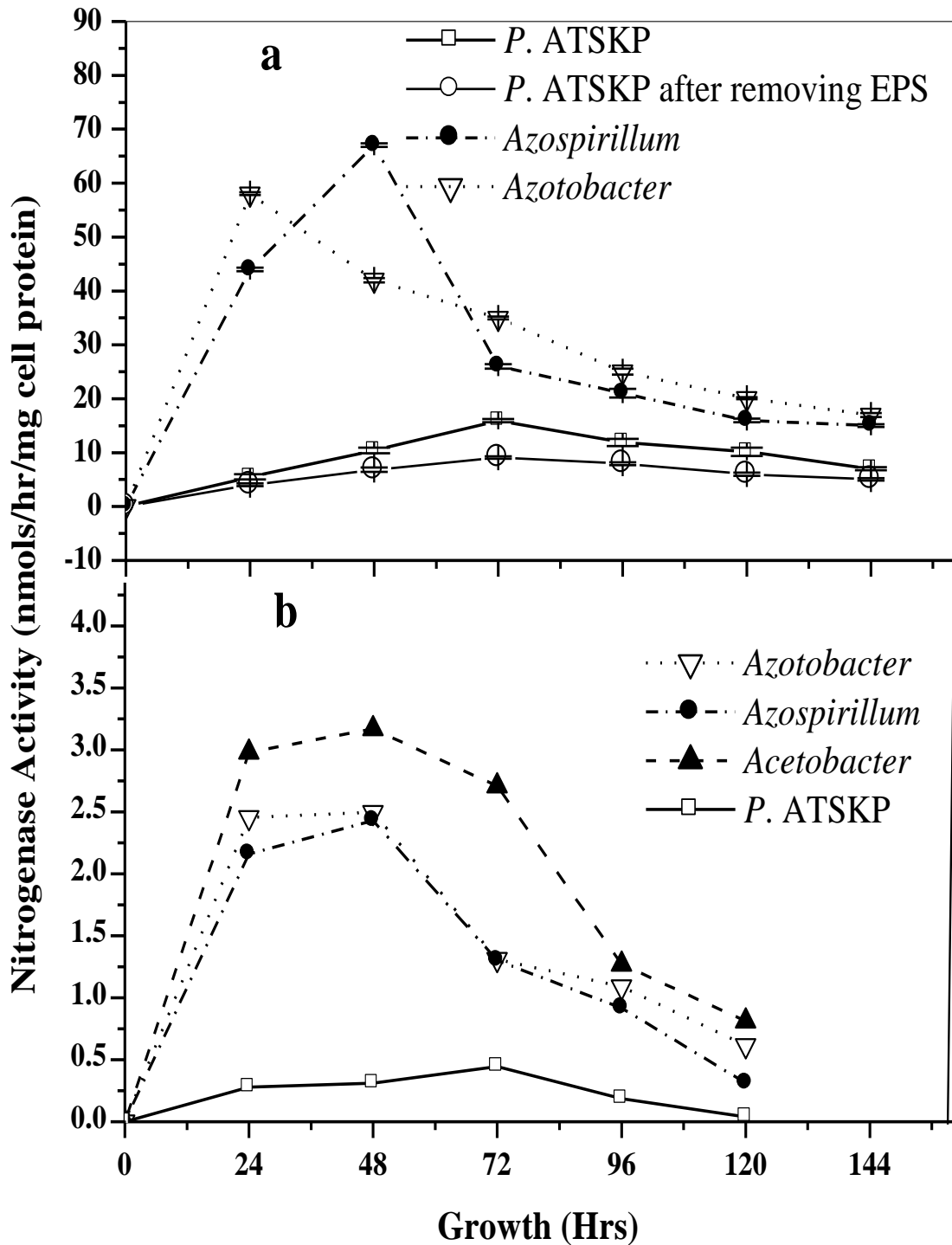


Figure 5. Nitrogenase activity of *Paenibacillus* strain ATSKP in comparison with the activities of other nitrogen fixing bacteria. (a) Activity when the cultures were shaken at 180 rpm during incubation with acetylene, (b) Activity when the cultures were not shaken (Strain ATSKP₂ was grown on N-free mineral base medium with 0.5 % birchwood xylan as carbon source whereas the other cultures were grown on N-free mannitol broth).

Table 1. Distinctive phenotypic characteristics of species phylogenetically related to *Paenibacillus pabuli* strain .

TEST	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15a	16b	17b	18c	19d	20e	21e	22f	23g	24h	25i	26j	27k	28l	
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Optimum growth temperature (°C)	30	30-37	30	30	28-30	28-30	20	28-30	40	37	37	37	NR	37	37-42	10-35	5-35	28	30	30	30-37	30-32	37	37	20	28	30	30	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	NR	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	NR	-	-	NR	-	-	+	-	-	+	-	-	-	-	-	-	-	-	NR	+	-	-	+	-	+	
Urease	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	NR	-	NR	NR	+	-	-	-	-	+	
H ₂ S	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	NR	NR	NR	+	NR	NR	NR	-	NR	NR	NR	NR	-	-	-	-	-	-	
Nitrate reduction	+	-	+	+	+	+	-	-	NR	-	-	+	V	-	-	+	+	-	+	+	+	+	+	-	+	+	-	+	
Production of:																													
Acetyl methyl carbinol	+	+	+	-	-	-	-	-	NR	-	-	-	NR	-	+	NR	NR	-	+	NR	NR	+	-	+	-	+	-	W	
Indole	-	-	-	-	-	-	-	-	-	+	NR	-	NR	-	-	NR	NR	-	NR	NR	NR	NR	-	-	NR	-	NR	W	
Dihydroxyacetone	+	-	-	-	NR	NR	-	NR	NR	-	NR	-	NR	-	NR	NR	NR	-	NR	NR	NR	-	-	-	NR	NR	NR	+	
pH in V-P broth#	4.5-6.8	4.5-5.1	5.5-6.6	4.5-5.0	5.5-5.5	5.5-5.5	5.6	5.5-5.5	NR	5.6	5.0-5.2	4.6-4.7	NR	4.6-4.7	NR	NR	NR	NR	5.3-5.9	NR	NR	NR	NR	5.0	4.4	-	5.3	4.7	4.5-5.1
Tyrosinase	-	-	-	-	-	-	-	-	NR	-	-	-	NR	-	NR	NR	NR	NR	NR	-	-	NR	-	-	NR	NR	NR	NR	
Caseinase	+	-	+	-	-	-	-	V	+	+	+	-	V	-	NR	-	NR	+	+	+	+	+	-	-	-	-	-	+	
Amylases	+	-	+	+	+	+	+	+	+	+	+	+	NR	+	NR	+	+	-	+	+	+	+	+	+	NR	+	NR	+	
Citrate	-	-	+	V	-	-	-	-	NR	-	-	-	V	-	-	-	NR	-	-	W	W	+	-	-	NR	NR	+	-	
Growth at/in:																													
pH 5-6	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	NR	NR	+	+	+	+	+	+	+	-	-	NR	+	
50 °C	-	-	-	+	-	-	-	-	-	-	+	+	NR	+	-	-	-	-	NR	-	-	-	-	-	+	-	-	-	
5% NaCl	-	-	-	-	+	-	-	V	+	+	-	-	+	-	+	NR	NR	-	-	-	-	-	W	+	-	-	+	-	
Utilization of:																													
L-Arabinose	+	-	+	+	+	+	-	+	+	-	+	+	V	-	+	+	+	+	+	+	-	-	-	+	-	NR	+	+	
Mannitol	+	+	+	+	+	+	+	+	-	-	+	+	NR	+	-	+	-	V	+	+	+	+	+	+	NR	+	+	+	
D-Xylose	+	-	+	+	+	+	+	+	+	-	+	+	NR	+	+	+	+	+	+	+	-	-	+	+	NR	-	+	+	
Acid production:																													
Glucose	NR	NR	+	+	+	NR	+	+	NR	NR	NR	+	+	NR	NR	NR	NR	+	+	NR	NR	+	NR	+	NR	NR	+	+	
Lactose	+	-	+	+	+	NR	+	+	NR	NR	NR	+	+	NR	NR	+	+	+	+	NR	NR	NR	NR	NR	NR	+	NR	-	
Sucrose	NR	NR	+	+	+	NR	+	+	NR	NR	NR	+	+	NR	NR	NR	NR	+	+	NR	NR	+	NR	NR	+	NR	NR	-	
Mannitol	NR	NR	+	+	+	NR	+	+	NR	NR	NR	+	+	NR	NR	NR	NR	+	+	NR	NR	NR	NR	+	+	NR	+	+	
Gas production:																													
Glucose	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	+	NR	-	-	-	+	W
Lactose	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	-	-	+	-
Sucrose	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	-	-	+	-
Xylanolytic	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	NR	-	NR	NR	+	+	NR	+	+	
Nitrogen fixing	+	+	+	+	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	+	+	NR	-	NR	+	-	-	+	-	+	
% homology of 16S rRNA gene with strain ATSKP	90	88	93	91	92	97	92	98	93	92	96	91	89	93	91	91	92	91	93	92	93	92	94	96	92	91	96	100	

ATSKP: 1, *Paenibacillus polymyxa* ATCC 842^T; 2, *Paenibacillus azotofixans* ATCC 35681^T; 3, *Paenibacillus peoriae* LMG 14832^T; 4, *Paenibacillus macerans* ATCC 8244^T; 5, *Paenibacillus lautus* NRRL NRS-666^T; 6, *Paenibacillus amylolyticus* NRRL NRS-290^T; 7, *Paenibacillus macquariensis* ATCC 23464^T; 8, *Paenibacillus pabuli* NCIMB 12781^T; 9, *Paenibacillus campinasensis* KCTC 0364BP^T; 10, *Paenibacillus dendritiformis* T168^T; 11, *Paenibacillus illinoisensis* NRRL NRS-1356^T; 12, *Paenibacillus chibensis* NRRL B-142^T; 13, *Paenibacillus glucanolyticus* DSM 5162^T; 14, *P. azoreducens* DSM 13822^T; 15, *Paenibacillus turicensis* MOL722^T; 16, *Paenibacillus graminis* RSA19^T; 17, *Paenibacillus odorifer* TOD45^T; 18, *Paenibacillus borealis* KK19^T; 19, *Paenibacillus jamilae* B.3^T; 20, *Paenibacillus terrae* AM141^T; 21, *Paenibacillus kribbensis* AM49^T (*Paenibacillus taejonensis*); 22, *Paenibacillus brasiliensis* PB172^T; 23, *Paenibacillus favisporus* GMP01^T; 24, *Paenibacillus xylanilyticus* XIL14; 25, *Paenibacillus wynnii*; 26, *Paenibacillus phyllosphaerae*; 27, *Paenibacillus barcinonensis*; 28, *Paenibacillus pabuli* strain ATSKP. Data are from Meehan *et al.* (2001), Elo *et al.* (2001) and von der Weid *et al.* (2002) except where indicated. +, Positive; -, negative; V, variable; W, weak; NR, not recorded.

a Bosshard *et al.* (2002), **b** Berge *et al.* (2002), **c** Elo *et al.* (2001), **d** Aguilera *et al.* (2001), **e** Yoon *et al.* (2003), **f** von der Weid *et al.* (2002), **g** Velazquez *et al.* (2004), **h** Rivas *et al.* (2005), **i** Rodriguez-Diaz *et al.* (2005), **j** Rivas *et al.* (2005), **k** Sanchez *et al.* (2005), **l** Data obtained in this study. #V–P broth, Voges-Proskauer broth.

Table 2. Extracellular enzyme activities of *Paenibacillus* strain ATSKP during growth on different carbon sources in N-free and N-supplemented medium.

Growth substrate (0.5%w/v)	TCP (µg/ml) in N-free medium	*Xylanase (µmol/min/ml) in N-free medium	*#Xylanase (µmol/min/ml) in N-supplemented medium	@ Endoglucanase (µmol/min/ml) in N-free medium	@ Amylase (µmol/min/ml) in N-free medium	@ Protease (µmol/min/ml) in N-free medium
Arabinose	61	0.281	0.298	-	-	-
Galactose	67	0.193	0.197	-	-	-
Glucose	340	0.283	0.298	-	-	-
Birchwood xylan	360	0.501	0.491	0.432	0.214	0.404
Oatspelt xylan	354	0.489	0.479	-	-	-
Xylose	360	0.325	0.331	-	-	-
Mannitol	NG	ND	ND	-	-	-
CM cellulose	216	ND	ND	0.493	0.074	0.257
Starch	67	ND	ND	1.530	1.110	0.413
Sucrose	NG	ND	ND	-	-	-
Glycerol	104	ND	ND	-	-	-

TCP=Total cell protein on 5th day of growth .The initial TCP on 0th day was 23 µg/ml.

*Enzyme activity on 4th day of growth in nitrogen-free medium;

Yeast extract 0.5% was used as nitrogen supplement;

@ Enzyme activity on 5th day of growth in nitrogen-free medium;

ND: Not detectable; NG =no growth.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. Andrew Ogram, University of Florida, USA and Dr. Prashanth Dhakephalkar, Agharkar Research Institute, Pune, India for performing the 16S rRNA gene sequencing. Dr. Yogesh Souche, National Centre for Cell Science, India and Dr. Sharmila Anishetty, Anna University, India are thanked for helping in the construction of the phylogenetic tree.

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Corresponding author: Dr. Pooja K. P. Jayaram, Biochemistry Laboratory, Department of Chemistry, Indian Institute of Technology Madras, Chennai, India-600 036.

E-mail: pooja.poojakp@gmail.com pj_kp@yahoo.co.in