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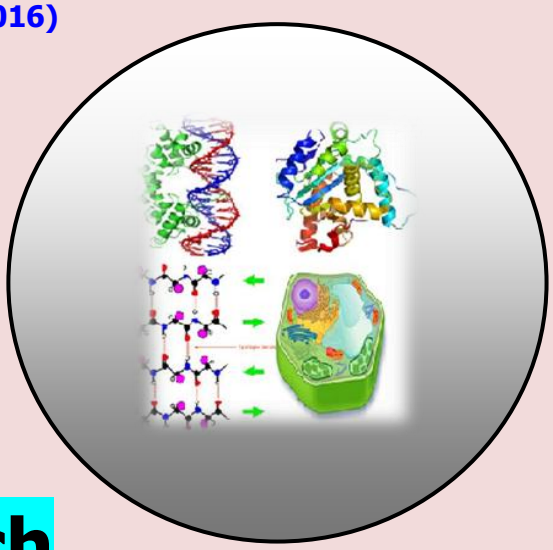

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Isolation and Characterization of Hyper-xylanase Producing Bacteria from Faecal of Indian Flying Fox, *Pteropus giganteus* (Chiroptera: Pteropodidae)

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ABSTRACT

A number of bacterial xylanases, including their production technology have been patented for various applications, and have been marketed for numerous commercial applications worldwide. Considering this, the present study was investigated to isolate and characterize new strain of hyper-xylanase producing bacteria from faecal of Indian flying fox, Pteropus giganteus. Initially, bacteria were isolated using xylan agar medium and selected based on high zone of hydrolysis. The potent bacterium was subjected to morphological, biochemical, and molecular characterization tools using standard methodologies. The isolate was identified as Pseudomonas nitroreducens strain LITH28 after 16S rRNA sequencing. Strain LITH28 showed maximum xylanase activity of 1040.32 ± 19.6 U/mL after 48 h of incubation period. In conclusion, the hyper-xylanse producing potentiality of strain LITH28 makes this bacterium ideal candidate for varied industrial applications. To the best of our knowledge, the present study evidenced the first report on the isolation of xylanolytic P. nitroreducens from faecal of P. giganteus.

Keywords: Bacterial xylanases, Hyper-xylanase, Pseudomonas nitroreducens and P. giganteus

INTRODUCTION

Xylan is a major skeletal component of the plant cell wall in primary and secondary cells. It is an abundant organic compound found on storage cells and photosynthesizing cells. Hydrolysis of xylan to monomers requires several enzymatic activities, among which xylanase plays a key role (Tomme et al., 1995; Anand et al., 2012). Xylans are biopolymers of glucose and xylose linked by β-1, 4 glycosidic linkages and β-1, 4 xylanopyranose linkages respectively (Warren, 1996). Xylans are twisted ribbons and are variously substituted with acetyl, arabinofuranoside and glucuronosyl residues (Tomme et al., 1995). The digestion of cellulose and xylan is only possible by breaking the β -1, 4 glycosidic bonds (Lynd et al., 2002). Xylanases are of increasing commercial interest because they allow the utilization of xylan, abundant in wastes from agriculture, and the food industry.

Although animals that commonly consume cellulose and xylan, most species do not produce cellulase and xylanase by themselves. They live in symbiotic relationship with cellulolytic and xylanolytic microorganisms

(Anand et al., 2012). In flying animals, food needs to be processed quickly to reduce the energy demand caused by increased flight mass. Bats feed on a variety of plant parts, mainly fruits (usually ripe), shoots, buds, flowers and/or flower products such as pollen and nectar (Bhat, 1994). Among bats, genus *Pteropus* has a high mass-specific energy demand because of its size and ability to fly (Speakman and Thomas, 2003).

Pteropus giganteus (Indian flying fox; Chiroptera: Pteropodidae) are widely distributed in the Indian sub-continent (Anand and Sripathi, 2004). Several studies focus on bacterial communities associated with the bat intestinal tract (Daniel et al., 2013; Galicia et al., 2014). It has led to the identification of different bacteria such as *Salmonella* (Islam et al., 2013), *Shigella, Enterobacter* (Mühldorfer et al., 2011), *Yersinia* (Mühldorfer et al., 2010) and many other enteric pathogens (Adesiyun et al., 2009) from the bat digestive tract (Muhldorfer, 2013). In view of the vast uses of xylanase in varied industries, in the present context, an attempt was undertaken to isolate and characterize potent hyper-xylanase producing bacteria from faecal of *P. giganteus.*

MATERIALS AND METHODS

Sample Collection

A total of five bat roosts were selected for faecal sample collection from Tirunelveli (TIR), Toothukudi (TUK), and Kanyakumari (KKU) districts of South India (Dhivahar and Isaac, 2018). The faecal sampling collection was made in the sampling sites between 6-7 A.M. by a non-invasive method in which three sterilized pieces (36×45 inches) of cotton material were spread under the roosting trees (Table 1). Fresh faecal samples were collected with sterile swab sticks and brought to the laboratory for immediate processing and processed for subsequent culture. Some portion of faecal was also preserved in the laboratory at 4°C for further utilization.

Table 1. Descriptions of the faecal sample collection sites of *P. giganteus.*

Site code	Location in South India	Bat population	Roost tree spp.	Latitude / Longitude
TIR1	Sivagiri	740	*Bassia latifolia*	9˚.34''N 77˚.42''E
TIR2	Kadayam	420	*Ficus benghalensis*	8˚.82''N 77˚.37''E
TUK1	Paramankurichi	930	*Ficus benghalensis* *Azadirachta indica* *Polyalthia longifolia*	8˚.48''N 78˚.04''E
TUK2	Srivaikundam	8600	*Terminalia arjuna*	8˚.62''N 77˚.91''E
KKU1	Boothapandi	1200	*Bassia latifolia* *Termarindus indica*	8˚.26''N 77˚.44''E

Isolation and screening of hyper-xylanolytic producing bacteria

Xylan agar media (in g/L: polypeptone-10.0; yeast extract -5.0; KH_2PO_4 - 1.0; $MgSO_4$. $7H_2O$ - 0.2; Na_2CO_3 - 10.0; xylan birchwood - 5.0; NaCl - 3.0; agar - 20.0; and pH - 7.0) were prepared and transferred to the sterile petriplates aseptically. The swab stick with faecal sample was inserted into a sterile test tube containing 9 mL of sterile distilled water, swirled briefly to discharge the content in to the test tube, and serially diluted (Akobi *et al*., 2012). Hundred microtiters of suspension were spread aseptically on sterilized agar plates. The plates were incubated overnight at 37°C for the appearance of xylanolytic bacterial colonies. After overnight incubation, xylanase producing bacterial colonies were selected by staining the plates with Congo red (1% w/v) for 5 min followed by destaining with 1M NaCl for 10-15 min. The selected cultures were purified and maintained in glycerol stock at -80˚C for further assay. Isolated pure cultures were screened on the basis of their extracellular xylanase production efficiency. Isolates were further assayed for estimating extracellular xylanase production.

Extracellular xylanase production

Five hundred microliters of overnight grown bacterial inoculum were inoculated into 50 mL of fermentation medium. The fermentation medium consisted of (g/L): polypeptone - 5.0; yeast extract - 5.0; KH_2PO_4 - 1.0; $MgSO_4 \cdot 7H_2O$ - 0.2; Na_2CO_3 - 10.0; xylan birchwood - 10.0; NaCl -3.0; and pH - 7.0. The flask was kept for incubation in rotatory shaker at 37°C. After overnight incubation, the broth culture was centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected and the qualitative assay for xylanase production was performed as per the methodology of Khusro et al. (2016).

Estimation of xylanase activity

Xylanase activity was measured according to Bailey et al., (1992) with some modifications. The supernatant obtained from overnight bacterial broth culture was used as crude enzyme. One milliliter of enzyme solution was added to the test tube containing 1 mL of 1% (w/v) solubilised birchwood xylan solution. The reaction mixture was incubated at 50°C in water bath for 10 min. The reaction was stopped by adding 1 mL of dinitrosalicylic acid (DNS) reagent into it. The tubes were incubated in boiling water bath for 5 min. After cooling, the solution was centrifuged at 8000 rpm for 5 min at 4°C, and the supernatant was analyzed for absorbance at 540 nm using a UV-Visible spectrophotometer. A reagent blank and an enzymatic blank were prepared under the same condition. D-xylose (100–1000 µg/mL) was used as the standard. One unit (IU) of xylanase activity was defined as the amount of enzyme that liberates 1µmol of reducing sugars equivalent to xylose per minute under the assay conditions described.

Morphological and biochemical tests

Purified isolate was characterized by biochemical methods using indole test, methyl red test, voges proskauer test, citrate utilization test, catalase test, urease test, oxidase test, and amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining and motility test were performed under morphological test.

Molecular characterization and identification

Genomic DNA of bacteria was isolated and amplification of the 16S ribosomal RNA was done using the thermal cycler (Eppendorf Gradient) with Taq DNA polymerase and primers. The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min, and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by gel doc. The amplicon was purified and the same was sequenced using an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The sequence was compared for similarity search with the reference species of bacteria contained in genomic database banks, using NCBI BLAST.

Phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

RESULTS

Screening of hyper-xylanolytic bacterial strain

Out of 27 isolates, 9 showed the production of xylanase as indicated by the appearance of yellow clearing zone around the colonies after congo red staining at 37°C (Figure not shown). Isolate TIR2 J was reported to show high production of xylanase. Maximum zone of xylan hydrolysis was observed for TIR2 J. In contrary to this, other isolates revealed comparatively lower yield of xylanase (Table 2).

Table 2. Screening and selection of hyper-xylanolytic bacterial strains.

S. No.	Isolates	Zone of hydrolysis
1	TIR1 F	+
2	TIR2 D	+
3	TIR2 J	+++
4	TUR1 A	+
5	TUR1 J	+
6	TUR2 G	++
7	TUR2 J	++
8	KKU1 D	++
9	KKU1 J	+

Note: High zone of hydrolyse - (+++); Moderate zone of hydrolyse - (++); Low zone of hydrolyse - (+)

Xylanase activity (Quantitative estimation)

Xylanase producing isolates were further used for the quantification of extracellular xylanase activity at distinct incubation periods (24-72 h).

Among selected 9 xylanolytic isolates, maximum xylanase activity (1040.32 ± 19.6 U/mL) was estimated for isolate TIR2 J after 48 h of incubation period (Fig. 1). These hyper-xylanase producing isolates were further selected for morphological, biochemical, and molecular characterization.

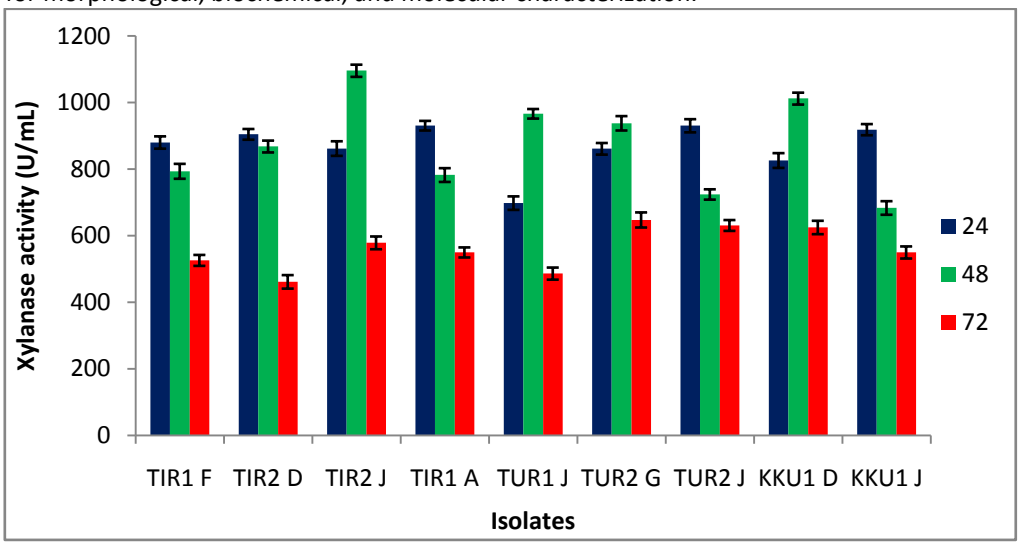

Figure 1. Xylanase activity of nine bacterial isolates up to 72 h of incubation, (Values represent mean ± SD).

Morphological and biochemical properties.

The potent hyper-xylanase producing bacterium (TIR2 J) was observed to be Gram-negative and motile. The isolates exhibited negative results towards indole, methyl red, voges proskauer, urease, and oxidase tests. Isolates exhibited positive results towards citrate utilization, catalase, and amylase tests (Table 3). The selected isolate was categorized under genus *Pseudomonas* based on the taxonomical characteristics.

Table 3. Morphological and biochemical tests report of selected isolate.

Tests	Result
Morphology	Rod shaped
Gram staining	Positive
Motility	Positive
Indole	Negative
Methyl Red	Negative
Voges Proskauer	Negative
Citrate utilization	Positive
Urease	Negative
Catalase	Positive
Amylase	Positive
Oxidase	Negative

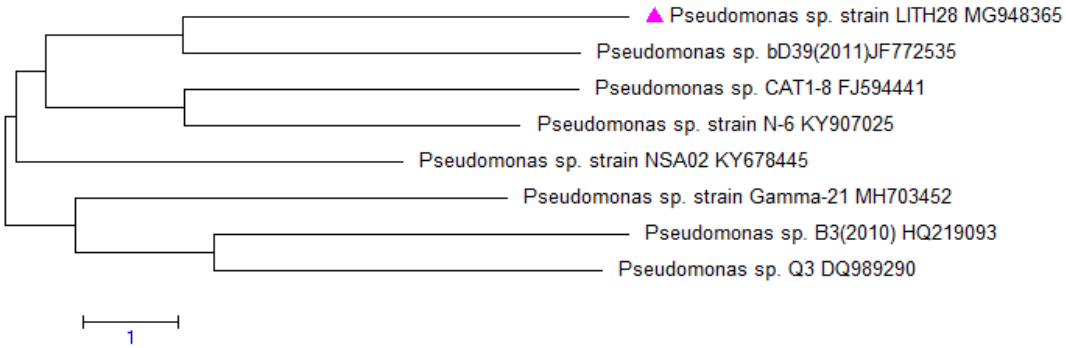

Figure 2. Phylogenetic tree construction of strain LITH28. The pink bullet indicates the isolate of the current study.

Molecular characterization and phylogenetic tree construction

Genomic DNA and amplicon of the isolate (TIR2 J) was visualized under UV- transilluminator (Figure not shown). In the present study, 16S rRNA gene sequencing of the isolate was further investigated. The isolate was identified as *Pseudomonas nitroreducens* strain LITH28 by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. Sequences were submitted to GenBank and the accession numbers MG948365 was assigned for the isolate. Fig. 2 shows the phylogenetic tree of isolates inferring their evolutionary relationship with other *Pseudomonas* spp. based on accession numbers. The results demonstrate the close resemblance of strains with other *Pseudomonas* spp.

DISCUSSION

There were many organisms isolated from animal faecal with high cellulase and xylanase activity (Sadhu et al., 2013; Thite and Nerurkar, 2015; Vijayaraghavan et al., 2016; Kalim and Ali, 2016; Aarti et al., 2018). However, xylanolytic *Pseudomonas* sp. from such environmental sample were not reported. In the present study, among various xylanolytic strains isolated from bat fecal, *P. nitroreducens* strain LITH28 was identified to be hyper-xylanase producing bacteria, thereby revealing first report on the isolation of xylanolytic *P. nitroreducens* from the faecal of *P. giganteus.*

Strain LITH28 hydrolyzes xylan into glucose units (Anand and Sripathi, 2004). This glucose can be easily absorbed in the intestine, supplying energy for *P. giganteus*. Bat diet is the major determinant of the intestine and faecal microbial flora (Moschen *et al*., 2012), therefore, microbial community structure may help in understanding the diet of an organism. Previously, Schwab et al. (2011) investigated that faecal microbiota composition of grizzly bears (*Ursus arctos*) and the presence of microorganisms are related to the food resources consumed by grizzly bears. The gut microorganisms have the ability to adapt themselves to changes in host diet, by induction of enzymes or by population changes in the microbial community (Santo Domingo et al., 1998). It was shown in the adult pigs that dietary fibres influenced xylanolytic and cellulolytic bacteria and an increase in fibre-degrading bacteria is directly proportional to extent of fibre digestion (Varel et al., 1987).

Fruit bats were considered consistent for their frugivory dietary habits and also found to occasionally feed on insects as well (Herrera et al., 2001). *P. giganteus* extensively forages on leaves of *Tamarindus indica*, *Erythrina indica* and *Ficus religiosa* (Bhat, 1994) and also on *F. bengalensis* (Ezilvendan, 2003). Likewise, Ryukyu fruit bats (*P. dasymallus*) feed on leaves of nine species, making up a large part of their diet for much of the year (Funakoshi et al., 1993). *P. voeltzkowi* consumes a high proportion of leaves, considered to be the third major diet of these bats (Entwistle and Corp, 1997). The Egyptian fruit bat (*Rousettus aegyptiacus*) forages on leaves of *F. religiosa* (Korine et al., 1999). The grey headed flying fox (*P. poliocephalus*) and the great fruit-eating bat (*Artibeus lituratus*) have folivorous diets as well (Parry-Jones and Augee, 1991; Zortea and Mendes, 1993)

The presence of *P. nitroreducens* in *P. giganteus* may be due to water or food source contamination, or possibly through transmission from other bats within the roost colony (Daniel et al., 2013). The possibility of transmission between individuals in the harem or large groups of bats maybe a threat to the survival of several threatened species such as *P. vampyrus* and *Penthetor lucasi* as these species are known to live together in large groups (Mohd-Azlan et al., 2001). *Pseudomonas* sp. was reported to be present in *Cynopterus brachyotis brachyotis* (Daniel et al., 2013).

CONCLUSION

In brief, hyper-xylanase producing bacterium was isolated from the faecal of *P. giganteus* and identified as *P. nitroreducens* strain LITH28 after morphological, biochemical, and molecular characterization tools. The strain showed maximum xylanase activity of 1040.32±19.6 U/mL after 48 h of incubation period. The hyper-xylanse producing ability of strain LITH28 makes the bacterium ideal candidate for varied industrial applications. Further study is required to enhance the production of xylanase using various optimization tools.

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