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Paraburkholderia tropica PKI7 and *Kosakonia arachidis* PKI8: Two newly reported tannase producing bacteria isolated from forest soil and study of their tannase producing potentiality

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Abstract

Tannins are the polyphenolic secondary metabolites of plants and having antimicrobial properties due to their protein precipitation abilities and other toxic effects towards microbes. However, with the help of an inducible enzyme called tannase, a variety of microbes including bacteria can resist tannin toxicity. Tannase catalyses the dissociation of ester bonds in gallotannins like tannic acid, resulting in release of gallic acid and glucose. Because of its vast industrial applications and ease of manipulation, interest in identifying and applying bacteria as tannase producers has grown in recent years. The bacterial strains reported in the present work were isolated from soil and identified as *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8 through morphological, biochemical, and 16s rDNA molecular approach. Simultaneous tannase and gallic acid production by these two bacterial strains were observed through submerged fermentation. This is the first report of *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8 strains as tannase producing bacteria. The initial tannase production were 0.75 U/ml and 1.49 U/ml respectively for *P. tropica* PKI7 and *K. arachidis* PKI8 after 24 h of submerged fermentation while gallic acid release was 8.4 µg/ml and 10.74 µg/ml respectively. There is a potential scope in higher tannase and gallic acid biosynthesis by the two strains.

Keywords: 16S rDNA; bacterial strains; gallic acid; submerged fermentation; tannase; tannin

Introduction

Tannins are water-soluble plant secondary metabolites that belong to the polyphenolic family. After cellulose, hemicelluloses, and lignin, they are estimated to be the fourth most prevalent element in plants. Based on their structures, they are divided into two groups: hydrolysable and condensed. Tannins are important in plant defence mechanisms because they protect plants from microbial attack by rendering viruses and bacteria inactive (Jana *et al.*, 2013). Despite their benefits, tannins are considered as antinutritional and antimicrobial compounds because they can precipitate proteins, block enzymatic reactions, and interfere with other critical microbial processes (Jiménez *et al.*, 2014). In nature, however, a variety of bacteria can withstand tannin toxicity

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by digesting tannin with the help of an enzyme called tannase (Kumar *et al*., 1999; Govindarajan *et al*., 2019; Lekshmi *et al*., 2020).

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an industrially important intra- or extracellular enzyme that belongs to the "hydrolase" enzyme class and is mostly found in bacteria, fungi, and yeast (Biswas *et al*., 2022). They are induced by tannin and hydrolyse the ester and depside linkages of a variety of compounds, including gallotannins, ellagitannins, gallic acid esters, epicatechin gallate, and generate gallic acid and glucose. Tannase has wide applications in a variety of industrial disciplines, including food, feed, drinks, pharmaceuticals, leather, and dye production (Biswas *et al*., 2020). It is predominantly utilised in the production of gallic acid, acron wine, instant tea, and fruit juice and beer clarification. It demonstrates its practical applicability in the treatment of tannin-containing waste water and leather industry effluent treatment (Das Mohapatra *et al*., 2020). The by-product gallic acid of tannin breakdown is a potential antioxidant and having anticancerous properties (Mitra *et al*., 2021). In comparison to the entire bacterial population, there are few reports in the literature on the bacterial origin of tannase (Biswas *et al*., 2020). As a result, looking for new and potential tannase producers is a never-ending effort, because large-scale enzyme synthesis necessitates maintaining a bacterial strain in a continuous culture. Furthermore, because tannase is an inducible enzyme, optimising culture conditions for maximum enzyme output is quite difficult.

Paraburkholderia tropica is a plant growth promoting bacteria (PGPB) and persists in nonleguminous plants and has been found in the rhizosphere, rhizoplane, internal tissues of sugarcane stem from Brazil and South Africa, as well as maize plants from Mexico (García *et al*., 2020). *P. tropica* demonstrates a variety of in vitro skills that are engaged in promoting plant growth, including the fixation of nitrogen, the formation of organic acids that solubilize phosphate, and the generation of antifungal compounds. *P. tropica* sp. has boosted maize productivity in terms of *in vivo* plant-growth promotion; in addition, wheat inoculation with *P. tropica* MTo-293 led to an increase in production under field conditions, however there was no discernible difference (García *et al*., 2020).

Kosakonia arachidis is a plant-growth-promoting bacterium with activities such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, IAA production, and nitrogenase. Groundnut (*Arachis hypogaea* L. 'ALR-20) rhizospheric soil was previously isolated from the Tamil Nadu Agricultural University experimental plot in Coimbatore, India, containing *K. arachidis*. The root length of Indian mustard, tomato, and rice plants was considerably increased by seeds inoculated with *K. arachidis* (cyg seed germination pouch, Mega International). Methanol dehydrogenase is produced by *K. arachidis* (Madhaiyan *et al*., 2010).

These above two bacteria were mainly documented as plant-growth-promoting bacterium in literature (Madhaiyan *et al*., 2010; García *et al*., 2020). Although there are reports on other species of *Paraburkholderia* as tannase producers (Biswas *et al*., 2020) however no report was found for *P. tropica* PKI7 and *K. arachidis* PKI8 strains. In the present study this is the first report of these two newly isolated bacteria *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8 as potential tannase producers.

Materials and Methods

Isolation and screening of potent tannase producer

Soil sample was taken from the Kulik forest floor in Raiganj, Uttar Dinajpur, West Bengal, India (Figure 1). Using a selective tannic acid agar media, a number of tannase-producing bacterial strains were identified using a serial dilution approach (Mondal and Pati, 2000). The serially diluted soil samples were then inoculated in the specific tannin medium and incubated for 72 hours at 35 °C. Tannase producers were chosen based on the appearance of a distinct halo zone encircling the bacterial colony. Two bacterial strains with the highest

clear zone to colony size ratio (cz/cs) were selected and designated as PKI7 and PKI8. The strains were kept at 4 °C and subcultured on tannic acid agar slant at regular intervals.

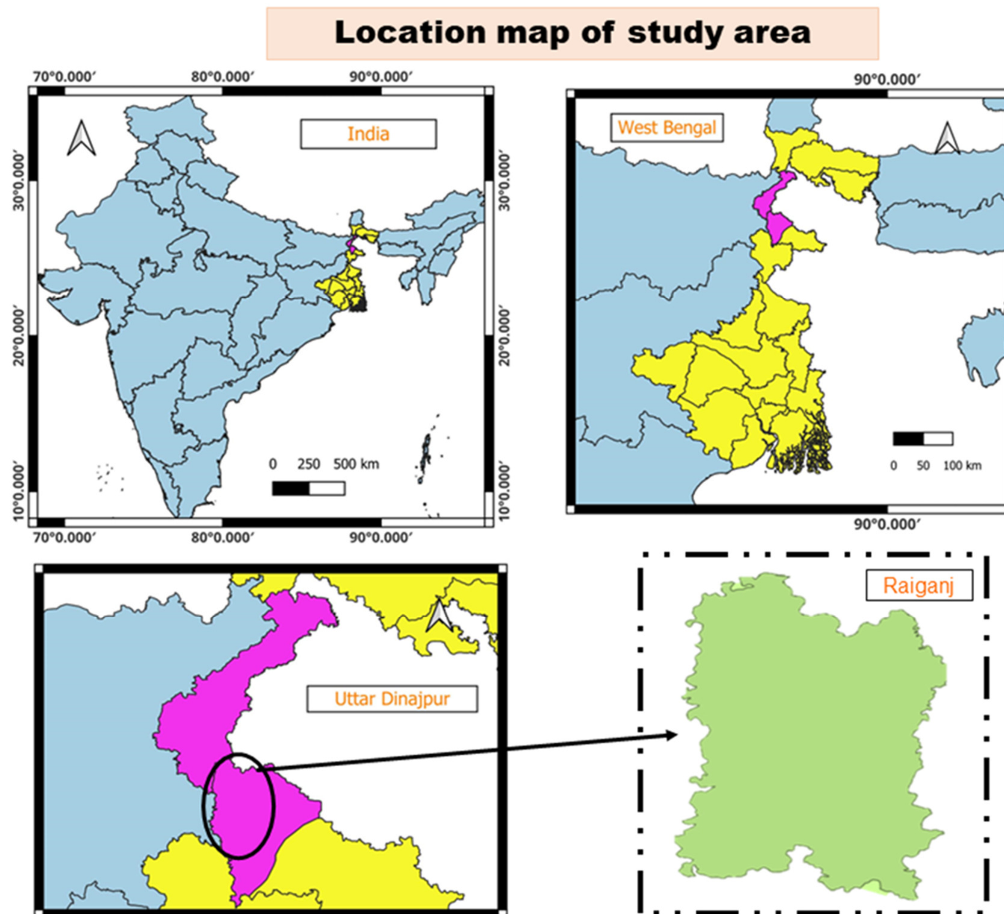

Figure 1. Study area map of Kulik forest, Raiganj, Uttar Dinajpur, West Bengal, India

Identification of selected bacterial strain

The selected bacterial strains PKI7 and PKI8 were identified based on their morphological, biochemical, and 16S rDNA sequencing properties (Biswas *et al*., 2021). Gram staining, colony morphology, and pigmentation were used to characterise bacteria morphologically, whereas biochemical characterization for the isolates included carbohydrate utilisation, catalase production, oxidase production, hydrolysis of starch and casein, citrate utilisation, nitrate reduction, and gelatine liquefaction. Carbohydrate fermentation studies were carried out using phenol red as an indicator and a basal medium supplied with appropriate carbohydrate such as glucose, fructose, lactose, sucrose, mannose, and mannitol. The colony morphology such as form/shape, colour, margin, elevation, size, appearance, and optical quality of the bacterial isolates were also studied.

Using a DNA isolation kit (ZymoTM, USA), the genomic DNA of each isolate was extracted. About 10 mL of a culture that had been cultivated for 24 hours (nutrient broth) was centrifuged at 8000 rpm for 15 minutes, and DNA was extracted from the cell pellet in accordance with the kit's instructions. Using a UV spectrophotometer (Nanodrop, Thermo Scientific), the quality and amount of DNA were assessed. Gene segment of 16S rDNA of the bacteria was amplified by PCR up to 30 cycles using 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) primers

(Suzuki and Giovannoni, 1996) and purified samples of PCR were sequenced through Sanger sequencing. The obtained forward and reverse reading sequences were assembled into contigs using the CAP3 assembly programme (Huang and Madan, 1999), and DECIPHER v2.0 (Wright, 2016) was used to screen for the presence of chimaeras. The sequencing was then submitted to the NCBI (https:/ submit.ncbi.nlm.gov/subs/genbank/) and received the accession number.

BLAST was used to match the 16S rDNA sequences of the bacterial isolates to the nucleotide database of NCBI genbank. Ten sequences were chosen based on their maximum identity score, and the sequences were aligned using the multiple sequence alignment software application ClustalW. MEGAX software was used to create a phylogenetic tree based on the Tamura-Nei model, which included 1000 bootstrap replications and a standard pattern of partially deleted nucleotide changes (Tamura and Nei, 1993; Kumar *et al*., 2016).

Preparation of inoculum and production of tannase through submerged fermentation

Inoculum was prepared by growing a loop full amount of stock culture of the bacterium in 50 ml sterile tannic acid medium (pH 5.0) as described above at 35 °C for 20h. The inoculation medium and the fermentation medium were identical. The bacterial isolates PKI7 and PKI8 were grown for 24 hours on a rotary shaker (120 rpm) at 35 °C with 50 ml of sterilised media in a 250 ml Erlenmeyer flask. The culture broth was centrifuged at 5000 g for 15 min to check for the production of tannase and gallic acid in the supernatant.

Study of bacterial growth

The cell concentration was evaluated using turbidimetry at 620 nm to measure the growth by UV-VIS spectrophotometer (Das Mohapatra *et al*., 2020).

Enzyme assay

Tannase activity in the fermented medium was determined according to the colorimetric method of Mondal *et al*. (2001). The specific extinction coefficient of tannic acid at 530 nm was 0.577. One unit (U) of tannase was defined as the amount of enzyme, which can hydrolyse 1 µmole of the ester linkage of tannic acid per minute.

Estimation of gallic acid

Gallic acid content of the culture filtrate was estimated by the method of Bajpai and Patil (1996). The following equation was used to determine the concentration of gallic acid using a specified extinction coefficient:

Concentration of gallic acid (mg/ml) = 21.77 ($A_{254.6}$) - 17.17 ($A_{293.8}$)

Results and Discussion

Isolation and screening of potent tannase producer

On tannic acid agar plates, microorganisms isolated from soil samples were screened. After 72 hours of incubation on tannic acid – agar plates, a diverse population of bacteria and fungi was discovered. PKI7 and PKI8 were chosen as a powerful tanninolytic bacteria based on higher clear zone to colony size (cz/cs) ratio of 1.36 and 1.78 respectivelywith clear zone around the colonies of 15 mm and 16 mm respectively on tannic acid agar plate (Figure 2A and 2C). After 72 hours of incubation, the strain PKI8 changes pigmentation and appears brownish in colour in tannic acid agar medium (Figure 3). The bacterial isolates were cultured in liquid medium with tannic acid (1% w/v) as the sole carbon source, and after 24 hours of incubation, the tannin degradation potentiality of PKI7 and PKI8 were found to be 7.6% and 15.67% respectively.

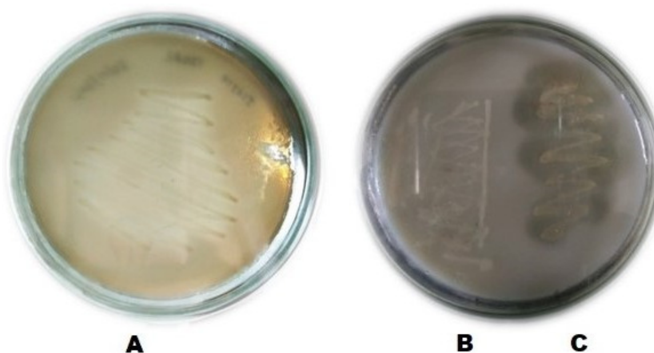

Figure 2. Growth of PKI7 (A) and PKI8 (C) on tannic Acid agar medium showing zone of tannin hydrolysis after 24 h of incubation; while B showing tannin tolerant bacteria

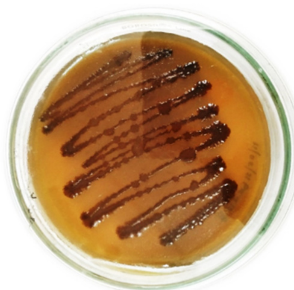

Figure 3. Growth of PKI8 on tannic acid agar medium showing pigmentation after 72 h of incubation

Identification of selected bacterial strain

The selected bacterial isolates PKI7 and PKI8 were characterized and identified based on the morphological, biochemical, and molecular characteristics. Colonies of PKI7 were tiny, circular, off white in colour, opaque, smooth in texture, flat with entire margin while PKI8 colonies had a diameter of 3-4 mm, were off white in colour, smooth, erose, opaque, and had an entire margin. Table 1 shows the colony features of PKI7 and PKI8 in further detail. The cells of both the strains were aerobic, Gram-negative rods (Figure 4A and 4B). Strain PKI7 was found to produce spore but PKI8 did not sporulate. Strain PKI7 were found to be aerobic, Gram-negative rods and were positive for catalase and nitrate reduction while negative to gelatin utilization which indicates that it belongs to the family Burkholderiaceae while the main characteristics of PKI8 were acid and gas production from D-glucose and other carbohydrates, catalase positivity while oxidase negativity, and inability to convert nitrate to nitrite. This strain has also been discovered to grow on Mac Conkey's medium, with red colonies. These traits led to the conclusion that the isolate PKI8 belongs to the Enterobacteriaceae family.

Table 1. Colony characteristics of the bacterial isolates

Isolates	Characteristics							
	Size (mm)	Shape	Colour	Margin	Elevation	Opacity	Texture /surface	Appearance
Paraburkholderia tropica PKI7	Tiny	Circular	Off white	Entire	Flat	Opaque	Smooth	Dull
Kosakonia arachidis PKI8	Moderate (3- 4)	Circular	Off white (24h)/ brown (72 h)	Lobate	Raised	Opaque	Smooth	Dull

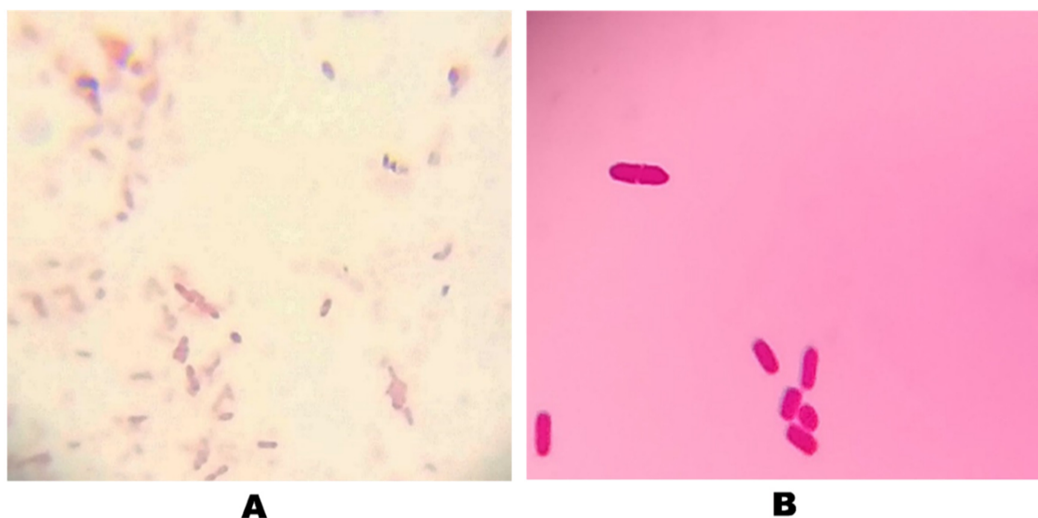

Figure 4. Gram staining view of PKI7 (A) and PKI8 (B)

Table 2 summarises the morphological and biochemical properties of the isolates.

Table 2. Morphological and biochemical characterization of bacterial isolates

Characteristics studied	Result	
	(*Paraburkholderia tropica* PKI7)	(*Kosakonia arachidis* PKI8)
Gram's staining	-	-
Shape	Rod	Rod
Colour	Off white	Off white
Motility	+	+
Oxygen tolerance	+	+
Spore	+	-
Citrate utilization	+	+
Starch hydrolysis	-	-
Casein hydrolysis	+	-
Gelatin hydrolysis	-	-
Indole Production	+	+
MR/VP test	-	+
Catalase	+	+
Oxidase	+	-
Nitrate reduction	+	-
Carbohydrate Fermentation Test: (acid production)		
Glucose	-	+
Fructose	-	+
Sucrose	-	+
Lactose	-	-
Mannitol	+	+
Mannose	+	+
Maltose	-	+

(+) positive, (-) negative

The Voges-Proskauer test revealed positive result for the strain PKI8 but negative for PKI7 while for gelatinase both are negative. Nitrate cannot be converted to nitrite by PKI8 but PKI7 is potent in this task. Fermentation of glucose, fructose, sucrose, mannitol, mannose, and maltose by PKI8 produces acid and gas but not through PKI7, however lactose fermentation produces neither by them (Table 2). Citrate utilisation was positive for both, whereas casein and starch hydrolysis were negative for PKI8 but PKI7 showed positive response for casein hydrolysis. These characteristics revealed that the bacterial strain PKI7 belongs to the genus *Paraburkholderia* and PKI8 to the genus *Kosakonia*. The growth profile of the bacterial isolates in nutrient broth medium has been shown in Figure 5.

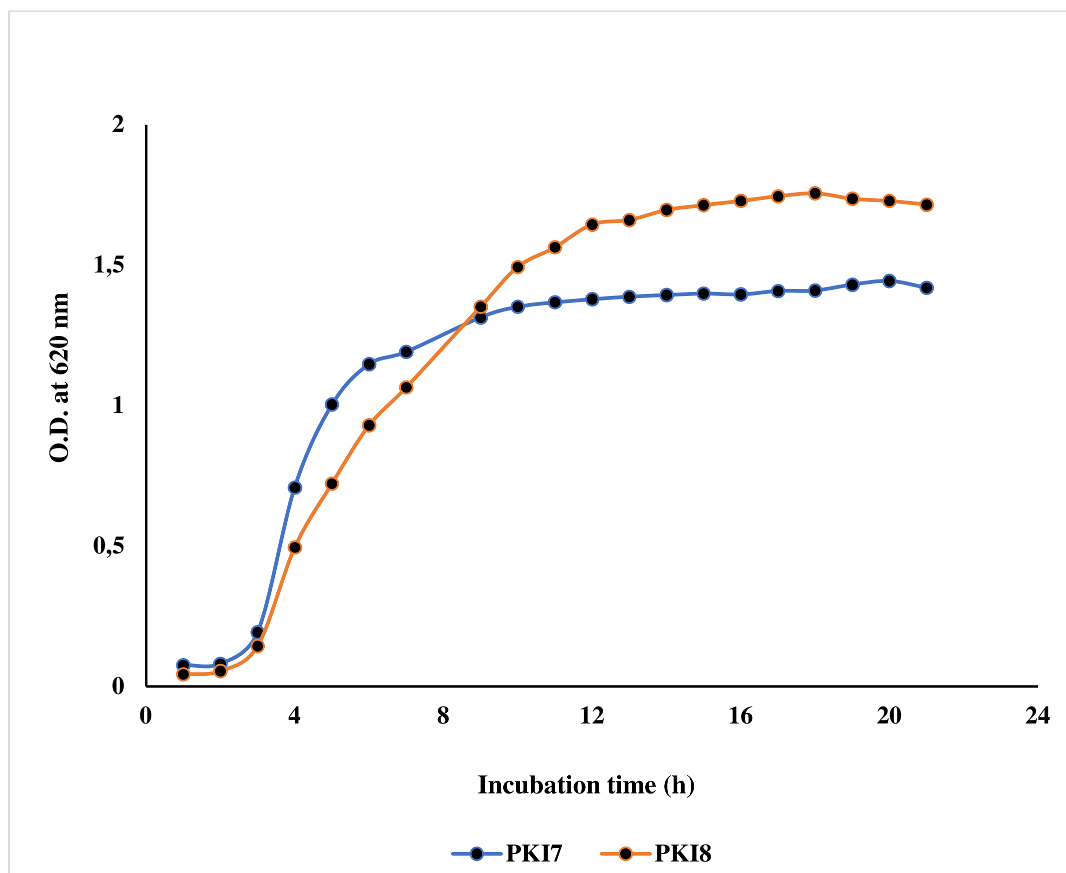

Figure 5. Growth curve of PKI7 (A) and PKI8 (B)

16S rDNA profiling and a BLAST study were used to confirm the identity of isolate. The 16S rDNA nucleotide sequence of strain PKI7 was found to be 99.03 identical to that of *Paraburkholderia tropica* strain 202 (Genbank accession as MK389460.1) while PKI8 was 98.95 percent identical to that of *Kosakonia arachidis* strain Ah-143 (Genbank accession as NR 116403.1). Strain PKI7 was found to be clustered with *Paraburkholderia tropica* strain 202 on the phylogenetic tree and therefore the bacterial strain was designated as *Paraburkholderia tropica* strain PKI7 (Genbank accession number MW784720.1) (Figure 6), on the other hand, PKI8 was found to be clustered with *Kosakonia arachidis* strain Ah-143 on the phylogenetic tree, therefore, the bacterial strain was named as *Kosakonia arachidis* strain PKI8 (Genbank accession number MZ127794.1) (Figure 7). This is the first report on tannin fermentation and tannase production by *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8, two newly isolated tannase producers.

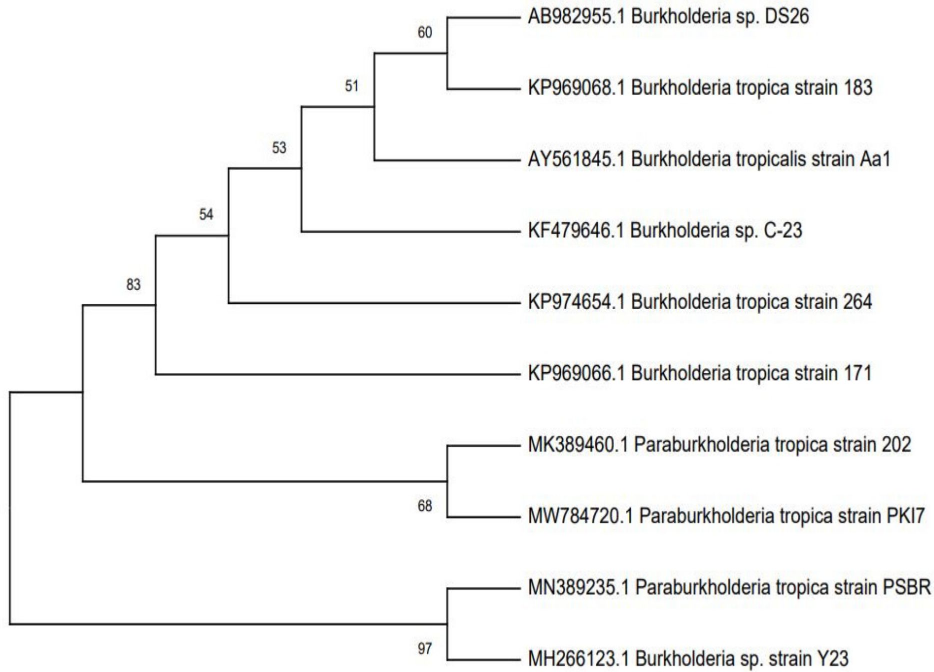

Figure 6. Phylogenetic tree (Neighbor-Joining consensus tree) on the basis of 16S rRNA sequence of gene showing the distance between strain PKI7 with other strain of *Paraburkholderia* species. The numbers over branches represent bootstrap confidence values (%) based on 1000 replicates. The Genbank accessions are shown in parenthesis

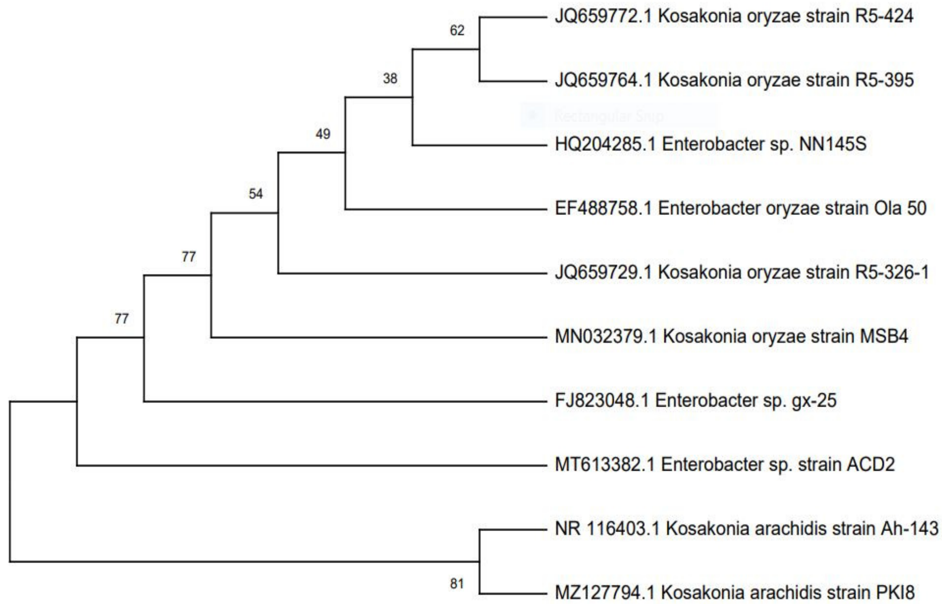

Figure 7. Phylogenetic tree (Neighbor-Joining consensus tree) on the basis of 16S rRNA sequence of gene showing the distance between strain PKI8 with other strain of *Kosakonia* species. The numbers over branches represent bootstrap confidence values (%) based on 1000 replicates. The Genbank accessions are shown in parenthesis

Estimation of tannase activity and gallic acid

The estimated initial enzyme yield by *P. tropica* PKI7 and *K. arachidis* PKI8 was found to be 0.75 and 1.49 U/ml respectively and gallic acid production was 8.4 and 10.74 µg/ml respectively at 35 °C with medium pH 5.0 (Table 3).

Table 3. Initial enzyme activity and gallic acid production by the bacterial isolates

Bacterial strains	pH	Temperature	Tannase activity (U/ml)	Gallic acid production (µg/ml)
Paraburkholderia tropica PKI7	5.0	35 °C	0.75	8.4
Kosakonia arachidis PKI8			1.49	10.74

Raghuwanshi *et al.* (2011) identified a potent tannase-producing bacteria *Bacillus sphaericus* from soil and found that it had the greatest enzyme activity (11.2 IU/ml) under optimal conditions. Similarly, Jana *et al.* (2013) found that under optimal conditions *Bacillus subtilis* PAB2 produced more tannase (10.69 U/ml) and detected the maximum quantity of gallic acid accumulation (6.45 mg/ml) after 36 hours of fermentation. Another report observed that for *B. subtilis* AM1 and *L. plantarum* CIR1, the maximum tannase production levels were 1400 and 1239 U/L after 32 and 36 hours of fermentation, respectively while highest gallic acid release for *B. subtilis* AM1 was 24.16 g/L and for *L. plantarum* CIR was 23.73 g/L (Aguilar-Zárate *et al*., 2015). Borah *et al.* (2023) documented highest tannase activity and gallic acid yield of 0.0497 U/mL and 225 µg/mL respectively by *Bacillus Gottheilii* M2S2.

Conclusions

The bacterial strains *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8 were tested for tannase activity and gallic acid production. Under submerged fermentation conditions, both the isolates can produce extracellular tannase. Further, the strains were able to produce gallic acid. More research is being done to improve the culture conditions for maximal enzyme production. This is the first report of *Paraburkholderia tropica* PKI7 and *Kosakonia arachidis* PKI8 as potential tannase producers.

Authors' Contributions

Conceptualization and design of experiments (IB and PKDM); Methodology (IB); data collection, analysis, and interpretation (IB); Supervision (PKDM); Writing - original draft (IB); Writing – review, and editing (PKDM). All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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